

Signalling via vascular endothelial growth factor receptor complexes

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by

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Signalling via vascular endothelial growth factor receptor complexes

Abstract

Selene K. Roberts

Vascular endothelial growth factor (VEGF) is an essential pro-angiogenic protein. Blood vessels are lined with endothelial cells that express VEGF receptors -1 and -2, and the multitude of adapter proteins, signalling enzymes and structural proteins required for VEGF signalling. Many proteins are involved in the integration of signalling pathways that induce angiogenesis, a process crucial for the formation of the vasculature in embryos, and for several physiological, and pathological, processes in adults. This study sought to investigate the role of various components of VEGF receptor signalling complexes using fluorescence microscopy to complement conventional biochemical techniques.

Using DsRedII-Grp1 to visualise the product of the reaction catalysed by PI3K, activation of PI3K downstream of VEGF receptor-2 is shown. There was no obvious relocalisation of p85 to phosphorylated receptors however a small amount of GFP-p85 associates with, and is phosphorylated in response to, activated VEGF receptor-2.

The Shc-related adaptor ShcB/Sck was localised to the plasma membrane in stimulated endothelial cells and associated with activated VEGF receptor-2. PTB and SH2 protein domains, contained within Sck, facilitated these events. Key tyrosine amino acids found within Grb2 binding motifs of ShcA and Sck were phosphorylated in response to VEGF. Tyrosine residues 315 and 316 of Sck were phosphorylated to a greater extent when compared to the corresponding residues of ShcA. Cells expressing Sck proteins lacking tyrosine phosphorylation sites showed a reduced amount of phospho-ERK and DNA synthesis.

VEGF receptor-2 was internalised and degraded in response to VEGF. This occurred, at least in part, through the conventional endocytic pathway. VEGF receptor-2 is localised in caveolin-1 and EEA-1 containing vesicles, which is suggestive of internalisation via caveolae and/or early endosomes.

Nedd4 co-localises with VEGF receptor-1 at regions of the plasma membrane and this association was confirmed by co-immunoprecipitation. The over-expression of Nedd4 enhanced the degradation of VEGF receptor-1.

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Addenda

Chapter 4 – Signalling via PLC γ 1 and PI3K

The addenda consists of 1 compact disc (CD) containing QuickTime movies (Figures 4.5 and 4.11)

Abbreviations

aFGF	acidic fibroblast growth factor
AIP4/Itch	atrophin-1-interacting protein 4
Akt/PKB	protein kinase B
Amp	ampicillin
Ang	angiopoietin
APS	ammonium persulphate
Arf	ADP-ribosylation factor
Asn	asparagine
ATP	adenosine triphosphate
BAE	bovine aortic endothelial
Bcl-2	B-cell lymphoma/leukaemia 2
bFGF	basic fibroblast growth factor
bp	base pair
BrdU	5-bromo-2'-deoxy-uridine
BSA	bovine serum albumin
Cbl	casitas B lineage lymphoma
cdc42	cell division cycle 42
cDNA	complementary DNA
c-fms	colony stimulating factor-1 receptor
CIN85	c-Cbl interacting protein
Cou	coumermycin A1
CSF	colony stimulating factor
Csk	C-terminal Src kinase
C-terminal	carboxy-terminal
DAG	diacylglycerol
DEP	density enhanced phosphatase
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphates
DTT	dithiothreitol

ECGS	endothelial cell growth supplement
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid
eNOS	endothelial nitric oxide synthase
Eph	ephrin receptor
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
EtBr	ethidium bromide
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
GCSF	granulocyte colony stimulating factor
Grb	growth factor receptor bound
GTP	guanine triphosphate
GyrB	B subunit of bacterial DNA gyrase
HECT	homologous to the E6-AP carboxyl terminus
HEK	human embryonic kidney
HGF/SF	hepatocyte growth factor/scatter factor
HGFR	hepatocyte growth factor receptor
HIF	hypoxia inducible factor
hr	hour
HRE	hypoxia response element
HRP	horseradish peroxidase
HSP	heat shock protein
HSPG	heparan sulphate proteoglycans
HUVE	human umbilical vein endothelial

Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IGFR	insulin-like growth factor-1 receptor
IKK	inhibitor of kappa-B kinase
IL	interleukin
Ins (3,4,5) P ₃	inositol (3,4,5) trisphosphate
IP	immunoprecipitation
Jnk	c-jun N-terminal kinase
Kan	kanamycin
Kb	kilobase
kDa	kilodalton
KDR	kinase insert domain containing receptor
LB	Luria Bertani
LMW-PTP	low molecular weight protein tyrosine phosphatase
MAP	mitogen activated protein
MAPKAP	mitogen activated protein kinase activated protein
MEK	MAP/ERK kinase
min	minute
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT1-MMP	membrane type-1 matrix metalloproteinase
Nedd4	neuronal precursor cell expressed developmentally down regulated
NGF	nerve growth factor
NO	nitric oxide
NOS	nitric oxide synthase
NRP-1	neuropilin-1
N-Shc	neuronal Shc
OD	optical density
PI3K	phosphatidylinositol 3 – kinase
PAE	porcine aortic endothelial
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PH	pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PIGF	placental growth factor
PMA	phorbol 12-myristate 13-acetate
Pro	proline
PTB	phosphotyrosine binding
PtdIns (3,4,5) P ₃	phosphatidylinositol (3,4,5) trisphosphate
PtdIns (4,5) P ₂	phosphatidylinositol (4,5) bisphosphate
PTP	protein tyrosine phosphatase
pY	phosphotyrosine
RING	really interesting novel gene.
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
SAPK	stress activated protein kinase
Sck	shc-like protein
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH2	src homology-2
Shc	src homology collagen
SHP	SH2-containing phosphatase
Sos	Son of sevenless
SRE	serum response element
SV	simian virus
sVEGFR-1	soluble vascular endothelial growth factor-1
TBS	tris-buffered saline
TBST	tris-buffered saline with tween
TEMED	N,N,N',N'-tetramethylethylene diamine

TGFβ,	transforming growth factor β
TNF	tumour necrosis factor
TPA	12-O-tetradecanoyl phorbol 13-acetate
uPA	urokinase plasminogen activator
UTR	untranslated region
V	volts
VE-Cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VE-PTP	vascular endothelial protein tyrosine phosphatase
VRAP	VEGF receptor associated protein
wt	wild-type
XIAP	X-linked inhibitor of apoptosis

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1 - Development of the vascular system

VEGF (vascular endothelial growth factor) is an essential signalling molecule required for the de novo generation of blood vessels (vasculogenesis) (Carmeliet and Collen, 1999; Risau and Flamme, 1995) and for initiating the formation of new blood vessels from pre-existing ones (angiogenesis) (Korpelainen and Alitalo, 1998; Risau, 1997). The entire vasculature system, a series of hollow tubes through which blood flows, is lined by a monolayer of vascular endothelial cells surrounded by mural cells and rests on a basement membrane. Mural cells, smooth muscle cells, pericytes and cardiomyocytes, regulate the contraction and dilatation of blood vessels and tighten the endothelial cells to provide stability. New vessel growth and maturation is a highly complex and coordinated process that involves many growth factors, cytokines and angiopoietins (reviewed in Risau, 1997). Signalling pathways initiated by VEGF however often represent a critical rate-limiting step in physiological angiogenesis.

1.1.1 Vasculogenesis

Vasculogenesis only takes place during embryonic development. The processes of angiogenesis and vascular remodelling are also required however for the migration and proliferation of endothelial cells and for the formation a support network made up of mural cells. The vascular system is one of the first systems to form in the embryo. It is initiated by mesoderm induction and embryonic stem cell differentiation in the yolk sac, forming haemangioblasts and precursors for smooth muscle cells. Haemangioblasts clump together in blood islands, the outer cells of which develop into primitive endothelial cells, angioblasts, and the inner cells develop into haematopoietic cells. These two cell types co-operatively form a primitive blood vessel. At this stage of embryonic blood vessel development, embryonic day 7 in the mouse, expression of VEGF-A (Dumont et al., 1995) and one of its receptors, VEGF receptor-2 (Kaipainen et al., 1993; Yamaguchi et al., 1993), have been detected in blood island cells. VEGF receptor-1 is expressed in these primitive endothelial cells (Peters et al., 1993) at day 8 in the mouse and is involved in the formation of tubes from the proliferating and differentiating angioblasts and haematopoietic precursor cells. Angiopoietins and ephrins, additional pro-angiogenic molecules, and their receptors Tie-2 (Schnurch and Risau, 1993) and EphB2 and EphB3 respectively are also expressed around this time. These molecules, as well as many others, act with VEGF in the remodelling, i.e.

branching, sprouting and pruning, and in the maturation, i.e. arterio-venous formation and stabilisation of vascular smooth muscle cells within the vasculature (Adams et al., 1999).

1.1.2 *Angiogenesis*

Angiogenesis, the formation of new capillaries by sprouting or by splitting from their vessel of origin, requires the loosening of support cells and the ability of the newly exposed endothelial cells to multiply (Figure 1.1). VEGF is a major pro-angiogenic factor and initiates angiogenesis by stimulating endothelial cells to proliferate and migrate towards this stimulus. Angiogenesis is an invasive process and begins with the degradation of the basement membrane surrounding the pre-existing vessel to allow for the migration of new endothelial cells. Fibroblast growth factor (FGF), another pro-angiogenic protein, acts with VEGF to stimulate endothelial cells to secrete several proteolytic enzymes that are involved in the degradation of the extracellular matrix (ECM). These proteases include the urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 (reviewed in Pepper, 2001). The action of these proteases on ECM proteins exposes new epitopes and changes the structure of proteins, which results in an induction of endothelial cell and smooth muscle cell migration. Migrating endothelial cells adapt their shape and become elongated (Ichijima et al., 1993). This generates a larger surface area for the endothelial cell surface expressed ECM receptors, integrins, to promote the attachment, release and re-attachment to the ECM that is required during cell migration. In addition proteases release matrix-bound angiogenic factors such as bFGF, VEGF and IGF-I that enhance endothelial cell sprouting (Bergers et al., 2000; Conway et al., 2001; Sato and Rifkin, 1988). bFGF and VEGF are mitogenic growth factors that also act to promote endothelial cell proliferation. This is essential to provide the lining for the newly formed vessels. When the endothelial cells have formed solid vessels, the joining together of two sprouts, a process called anastomosis, generates a vascular loop. Mural cells are established from the maturation of PDGF receptor- β -positive cells that surround the new loop and a new basement membrane is deposited. Pericytes, one type of mural cell, contain long processes that embrace and hence stabilise the endothelial cells. In addition they secrete TGF β to prevent further proliferation of the endothelial cells. Angiopoietins are also key molecules that mediate the stabilisation of newly formed vessels. Angiopoietin-1 (Ang-1) is a local derived vessel-stabilising factor and acts on Tie-2 expressed on endothelial cells. Angiopoietin-2 also binds to Tie-2, is an antagonist to Ang-1 and together they control the tightness of the vessels (Maisonpierre et al., 1997; Thurston et al., 2000). The distinction between arteries and veins is established in the

final maturation stage and this process involves the Eph-ephrin system (Adams et al., 1999; Gale et al., 2001). The complete process of angiogenesis involves interactions between endothelial cells, stabilising cells and components of the ECM and signalling through many receptor tyrosine kinases to co-ordinate the complex process of endothelial cell migration, proliferation and blood vessel formation.

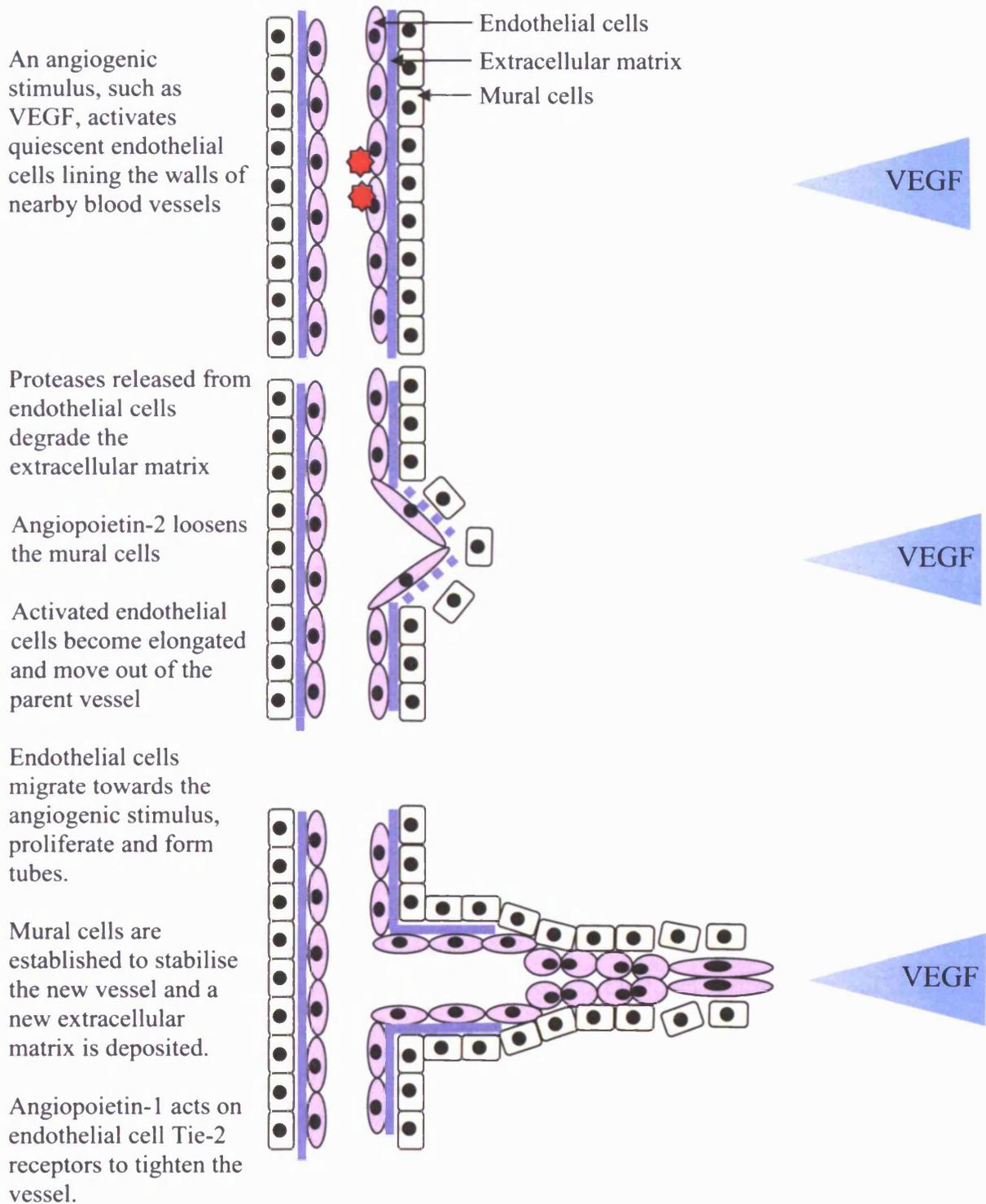


Figure 1.1 – The cascade of events during angiogenesis.

1.2 - Angiogenesis in pathological conditions

Angiogenesis is controlled by a net balance between pro- and anti-angiogenic factors, some examples of which are given in Table 1, and is initiated when the pro-angiogenic factors trigger the “angiogenic switch”(Hanahan and Folkman, 1996). The vasculature is remarkably quiescent in the adult however angiogenesis does occur physiologically during the female reproductive cycle (Ferrara et al., 1998; Phillips et al., 1990; Shweiki et al., 1993) and wound healing (Brown et al., 1992; Nissen et al., 1998; Peters et al., 1993) and pathologically in conditions such as diabetic retinopathy (Funatsu et al., 2001), rheumatoid arthritis (Koch, 2003), psoriasis (Creamer et al., 2002) and tumour growth (Folkman, 1990). Solid tumours require a blood supply to be able to expand to a size greater than 1 mm³; hence active proliferation of endothelial cell is particularly striking during tumour-induced angiogenesis. Hypoxia, a low level of oxygen, is often a feature in the centre of solid tumours and as a result of this VEGF expression is enhanced and stimulates an excess growth of vessels into the tumour. A functional vascular network is required to provide all cells, including cancerous cells, with oxygen and nutrients and to remove toxic waste products generated by cellular metabolism. Tumours therefore adopt enhanced angiogenesis for the sustained growth and survival of cancerous cells. The promotion of angiogenesis in tumours is aided, in part, by the expression of pro-angiogenic growth factors, cytokines and MMPs by tumour cells, the loss of pericytes that suppress the turnover of endothelial cells, an increase in microvascular permeability, the up regulation of $\alpha v\beta 3$ integrin, which promotes cell survival and the activation of oncogenes and/or inactivation of tumour suppressor genes to potentiate deregulation cell growth (reviewed in Ribatti, 2005). VEGF and its receptors are however essential for the growth of solid tumours. A study by Kim et al., in 1993, provided the first “proof of principle” that interfering with VEGF signalling can inhibit tumour growth. In this study a monoclonal antibody to VEGF inhibited tumour growth *in vivo* and reduced the density of blood vessels (Kim et al., 1993). Furthermore, Millauer et al. showed that a dominant negative VEGF receptor mutant could inhibit tumour growth in mice (Millauer et al., 1996) and Lin et al showed that expression of soluble VEGF receptor-2 inhibits tumour growth and reduces the vascular density within tumours (Lin et al., 1998). A greater understanding of VEGF receptor signalling is likely to identify other pro-angiogenic proteins that when targeted may inhibit angiogenesis and hence the growth of solid tumours. To date several clinical trials have used, or are currently using, small molecule inhibitors to block

VEGF receptor signalling (Blakey et al., 2002; D'Amato et al., 1994; Davis et al., 2002; Kenyon et al., 1997; Siemann and Shi, 2004), small molecule vascular-disrupting agents (Hori et al., 1999; Horsman et al., 1998; Lash et al., 1998; Murata et al., 2001; Tozer et al., 1999) and anti-VEGF neutralising antibodies (Willett et al., 2004; Yang et al., 2003) to rectify aberrant angiogenesis. Bevacizumab (Avastin), is a specific example of a monoclonal antibody against VEGF that has been tested in a phase III clinical trial for metastatic colorectal cancer (Hurwitz et al., 2004), likewise Pegaptanib (Macugen) is a specific example of a drug targeting angiogenesis (Gragoudas et al., 2004) that has been tested in a phase III clinical trial for neovascular age-related macular degeneration. In the near future, gene therapy (reviewed in Baker, 2004) and adenoviruses (reviewed in Kanerva and Hemminki, 2004) are also likely to be effective in delivering anti-angiogenic proteins to treat human cancers.

In contrast, insufficient angiogenesis occurs when pro-angiogenic factors are absent or anti-angiogenic molecules are prevalent. The resulting lack of vessel growth and abnormal vessel regression, which occurs normally when the new vasculature consists of too many vessels, can cause myocardial ischaemia, stroke, pre-eclampsia, delayed wound healing, respiratory distress, osteoporosis and many other diseases (Carmeliet, 2003). Pro-angiogenic growth factors, such as VEGF, FGF and granulocyte colony stimulating factor (G-CSF), are being used in clinical trials to promote neovascularisation.

Pro-Angiogenic Factors	Anti-Angiogenic Factors
Growth Factors Acidic and basic Fibroblast growth factors Vascular endothelial growth factor Platelet-derived growth factor-BB Placental growth factor Transforming growth factors α and β Hepatocyte growth factor Granulocyte colony stimulating factor Angiopoietin-1 Cytokines Tumour necrosis factor α Interleukin-8 Enzymes Platelet derived endothelial cell growth factor Prostaglandins Prostaglandin E1 and E2	Protein fragments Angiostatin Endostatin Vasostatin Fibronectin fragment Prolactin fragment Metalloproteinase inhibitors Plasminogen activator inhibitor Heparinases Cytokines Interleukin-12 Interferon $\alpha/\beta/\gamma$ Growth Factors Transforming growth factor β Angioarrestin

Table 1 – Summary of pro- and anti-angiogenic factors.

1.3 - Vascular Endothelial Growth Factors (VEGFs)

The mammalian VEGF family of proteins include VEGF-A (the original VEGF), VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PlGF). A viral open reading frame codes for VEGF-E. Several VEGF-A protein isoforms are produced by the alternative splicing of pre-mRNA from a single VEGF gene containing 8 exons separated by 7 introns (Figure 1.2) (Robinson and Stringer, 2001). These isoforms contain between 121 and 206 amino acids, but can be modified further through proteolytic cleavage by uPA and plasmin (Houck et al., 1992; Plouet et al., 1997), and differ in their ability to bind to heparin sulphate (Houck et al., 1992; Park et al., 1993). VEGF₁₂₁ is a frequently expressed isoform but due to the splicing of exons 6 and 7 from the VEGF gene it is unable to bind heparin and is therefore freely diffusible. The remaining isoforms all bind heparin, to some degree, which is important for their mitogenic activity (Neufeld et al., 1996). The larger isoforms, VEGF₁₈₉ and VEGF₂₀₆, contain a region rich in charged basic amino acids in their C-terminal domains and bind to heparin with high affinity (Park et al., 1993). They are almost completely sequestered by heparan sulphate proteoglycans on the cell surface and by the extracellular matrix. VEGF₁₆₅ is the most abundant VEGF isoform and has properties that are intermediate between soluble VEGF₁₂₁ and cell surface-bound VEGF₁₈₉ and VEGF₂₀₆. The properties of this isoform allow for cell secretion but a significant fraction remains bound to the extracellular surface. VEGF₁₆₅ is also able to interact with a co-receptor, neuropilin-1 (NRP-1) (Soker et al., 1998), via a basic, NRP-1 binding region, encoded by exon 7 of the VEGF gene. VEGF₁₆₅ associated with NRP-1 has an increased affinity for one of the VEGF receptors resulting in VEGF₁₆₅ having the greatest mitogenic potency among the VEGF subtypes. VEGF₁₆₅ has the optimal characteristics of bioavailability and biological potency and has been used extensively in studies of VEGF signalling. VEGF-A₁₆₅ will now be referred to as VEGF.

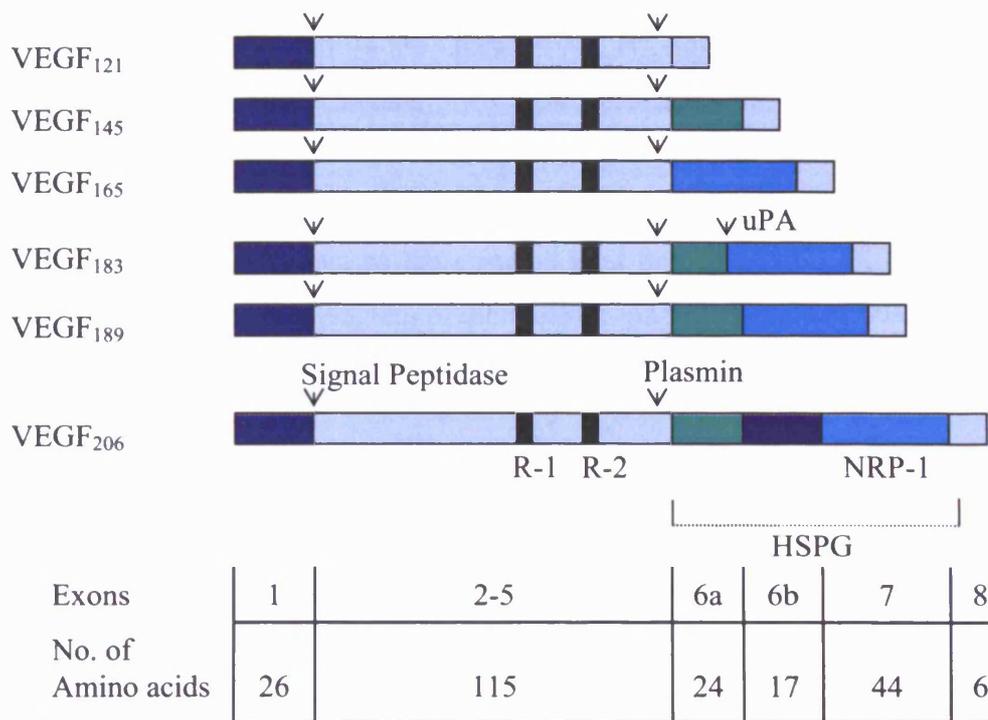
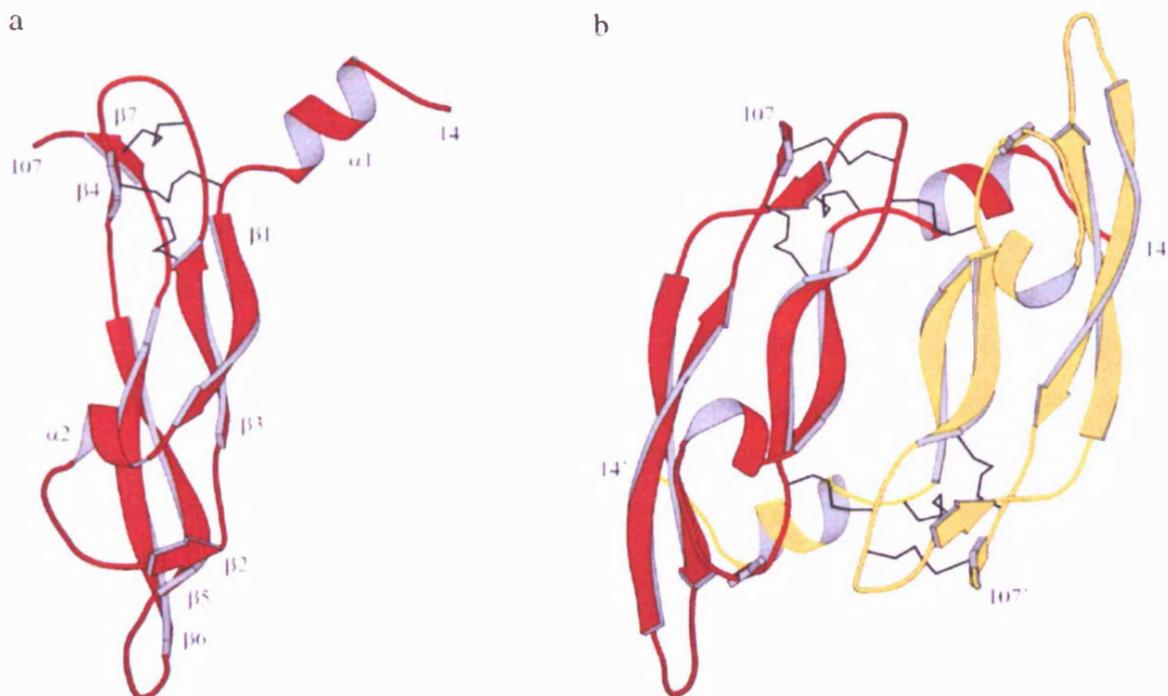


Figure 1.2 – The splice variants of human VEGF-A

This diagrammatic representation of the VEGF-A isoforms shows that exons 6 and 7 are subject to alternative splicing. The size of the protein domains and the exons from which they derive are shown at the bottom of the figure. The first 26 amino acids of VEGF-A represent a signal peptide and this is cleaved during secretion. Plasmin cleavage sites are shown. uPA is able to cleave VEGF₁₈₃ however the exact site is not known. Sites of interaction with VEGF receptor-1 (R-1), VEGF receptor-2 (R-2), neuropilin-1 (NRP-1) and heparan sulphate proteoglycans (HSPG) are shown.

Members of the VEGF family are characterised by eight characteristically spaced cysteine residues in their protein structure. Two cysteine residues are used to form intermolecular cross-linking disulphide bonds between anti-parallel VEGF homodimers or VEGF family member heterodimers. The other six cysteine residues form three intramolecular bonds to make loop structures known as the cysteine knot homology domain (Muller et al., 1997). Figure 1.3 shows a ribbon diagram of the receptor-binding domain of VEGF in its monomeric and dimeric forms.



**Muller, Yves A. et al. (1997) Proc. Natl. Acad. Sci. USA 94, 7192-7197
Vascular endothelial growth factor: Crystal structure and functional
mapping of the kinase domain receptor binding site**

Figure 1.3 – A ribbon diagram representing the receptor-binding domain of VEGF. The monomeric VEGF structure is shown (a) and the structure of a VEGF dimer is also shown (b). This diagram was created by Muller et al using the programme Molscript. The black lines represent the disulphide bonds in the cysteine knot motif.

1.3.1 *The role of the VEGFs*

A major role of VEGF is in the development of embryonic blood vessels and consequently the homozygous knockout of VEGF, or the loss of just one VEGF allele, results in embryonic lethality in the mouse (Carmeliet et al., 1996; Ferrara et al., 1996). The development of the cardiovascular system is dependent upon precise VEGF concentration gradients and a decrease in the amount of VEGF results in abnormal endothelial cell development and reduced angiogenesis with fatal consequences. In adults, normal endothelial cells are quiescent, except in the reproductive organs of fertile women or during wound healing, however VEGF is still an important survival factor to prevent endothelial cell apoptosis.

1.3.2 *VEGF expression in response to hypoxia*

VEGF-A expression is regulated by hypoxia and by other growth factors. Under hypoxic conditions the hypoxia-inducible factors, HIF1 α and HIF2 α are stabilised and translocated to the nucleus. In nuclei they cooperate with HIF β to activate genes that contain a hypoxia response element (HRE) (Guillemin and Krasnow, 1997). VEGF expression is increased during hypoxia and this is regulated by HREs contained within the 5' and 3' ends of the gene (Ladoux and Frelin, 1993; Minchenko et al., 1994). Several growth factors including epidermal growth factor (EGF), transforming growth factors (TGF α and TGF β), keratinocyte growth factor, Insulin-like growth factor-I (IGF-I) and platelet-derived growth factor (PDGF) also up-regulate VEGF mRNA expression. It is thought that paracrine or autocrine release of these growth factors cooperate with local hypoxia to regulate VEGF release (Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999). In addition VEGF is regulated post-transcriptionally by changes in mRNA stability. Sequences in the 5' and 3' untranslated region of VEGF mRNA mediate this regulation (Coles et al., 2004; Ikeda et al., 1995; Levy et al., 1996).

1.4 - The VEGF receptors

Active forms of VEGF family members are synthesised as disulphide linked dimers. VEGF-A, and other family members, initiate angiogenic signals by binding to receptor tyrosine kinases. VEGF receptors are structurally and functionally similar to the PDGF receptor family (Klagsbrun and A. D'Amore, 1996) and are expressed predominantly in endothelial cells (de Vries C et al., 1992; Terman BI et al., 1992), but also in neurons (Nico et al., 2002; Nico et al., 2004), pancreatic duct cells (Oberg et al., 1994), retinal progenitor cells (Yang and Cepko, 1996), megakaryocytes and hematopoietic cells (Katoh et al., 1995), normal human testicular tissue (Ergun et al., 1997) and the myometrium (Brown et al., 1997; reviewed in Matsumoto and Claesson-Welsh, 2001). In addition increased expression of VEGF receptor-2 mRNA has been detected in many types of tumour cell (Boocock et al., 1995; Brown et al., 1993; Hatva et al., 1995; Plate et al., 1993; Wizigmann-Voos et al., 1995; Yoshiji et al., 1996; reviewed in Matsumoto and Claesson-Welsh, 2001). The three classes of receptor are VEGF receptor-1 (or Flt-1, Fms-like tyrosine kinase, as it was previously known (Shibuya et al., 1990)) VEGF receptor-2 (KDR, kinase insert domain containing receptor, or Flk-1, fetal liver kinase-1, (Terman et al., 1991)) and VEGF receptor-3 (Flt4 (Aprelikova et al., 1992; Galland et al., 1992)) and each receptor interacts with one or more of the VEGF family members (Figure 1.4). VEGF-A binds to VEGF receptors 1 and 2 however different residues within VEGF-A bind to each receptor. Asparagine 63, Glutamine 64 and Glutamine 65 are required for VEGF-A binding to VEGF receptor-1 and Arginine 82, Lysine 84 and Histidine 85 are critical for binding to VEGF receptor-2. VEGF-B and PlGF only bind to VEGF receptor-1. VEGF-C and -D bind primarily to VEGF receptor-3 but also to VEGF receptor-2 and VEGF-E binds exclusively to VEGF receptor-2.

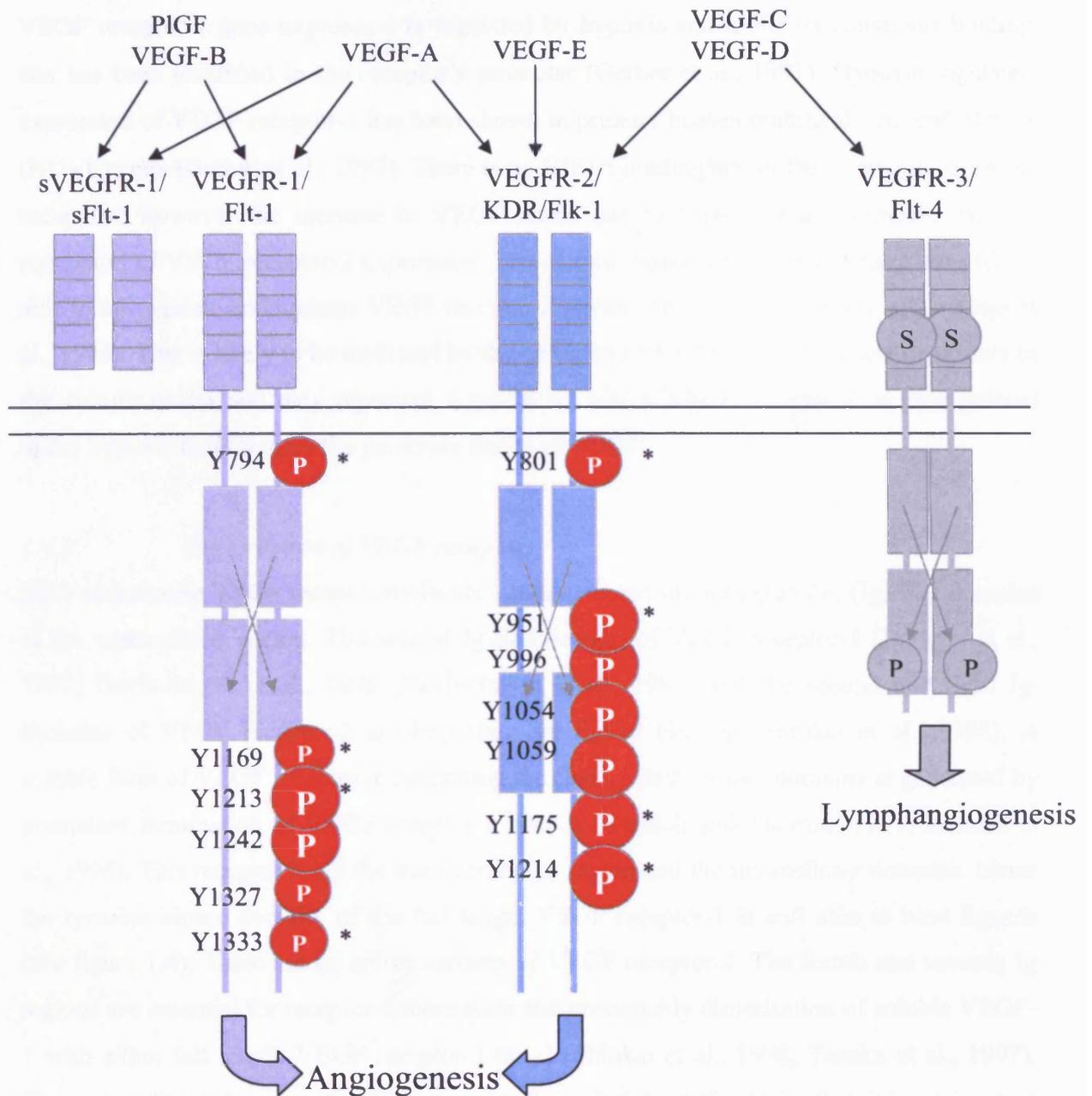


Figure 1.4 - A schematic diagram of the activation of VEGF receptors in response to the family of VEGF ligands.

VEGF receptors are activated by PlGF and different isoforms of VEGF. The major and minor tyrosine phosphorylation sites are shown for VEGF receptor-1 and -2, by large and small circles respectively, and those shown to bind to downstream signalling proteins are indicated by an asterisk. VEGF receptor-1 and -2 signal to induce angiogenesis and VEGF receptor-3 initiates lymphangiogenesis.

1.4.1 *VEGF receptor expression in response to hypoxia*

VEGF receptor-1 gene expression is regulated by hypoxia and a HIF1 α -consensus binding site has been identified in the receptor's promoter (Gerber et al., 1997). Hypoxia-regulated expression of VEGF receptor-1 has been shown in primary human umbilical vein endothelial (HUVE) cells (Gerber et al., 1997). There is no HIF1 α binding site in the promoter of VEGF receptor-2 however the increase in VEGF levels due to hypoxia may stimulate the up-regulation of VEGF receptor-2 expression. The culture media from hypoxic myoblast cells is able to up-regulate endogenous VEGF receptor-2 protein by 13-fold in HUVE cells (Brogi et al., 1996). This is likely to be mediated by the secretion of VEGF by the hypoxic myoblasts in the culture media and may represent a model by which VEGF receptor-2 is up-regulated under hypoxic condition by the paracrine action of VEGF.

1.4.2 *The structure of VEGF receptors*

Each monomeric VEGF receptor molecule contains seven immunoglobulin (Ig)-like domains in the extracellular region. The second Ig-like domain of VEGF receptor-1 (Barleon et al., 1997; Davis-Smyth et al., 1996; Davis-Smyth et al., 1998) and the second and third Ig-domains of VEGF receptor-2 are important for ligand binding (Shinkai et al., 1998). A soluble form of VEGF receptor-1 containing the first to sixth Ig-like domains is generated by premature termination of VEGF receptor-1 mRNA (Kendall and Thomas, 1993; Kendall et al., 1996). This receptor lacks the transmembrane region and the intracellular domains, hence the tyrosine kinase domain, of the full length VEGF receptor-1 is still able to bind ligands (see figure 1.4). There are no splice variants of VEGF receptor-2. The fourth and seventh Ig regions are essential for receptor dimerisation and presumably dimerisation of soluble VEGF-1 with either full length VEGF receptor-1 or -2 (Shinkai et al., 1998; Tanaka et al., 1997). The extracellular domain of VEGF receptor-3 is slightly different in that it contains two extracellular peptides linked by a disulphide bridge.

All VEGF receptor monomers contain a single hydrophobic transmembrane domain and a catalytic domain in the intracellular region. The tyrosine kinase domain is split by a non-catalytic, kinase insert sequence of approximately 70 amino acids, and is flanked by a juxtamembrane region and a C-terminal tail. The PDGF family of receptors also contain a kinase insert sequence of similar length and Tyr-X-X-Met motifs found in this region are important for the activation of phosphatidylinositol-3-kinase (PI3K) in response to receptor

stimulation. These motifs are not found in the insert region of the VEGF receptors indicating that they use different motifs or mechanisms to those in the PDGF receptors to activate signal transduction (Shibuya et al., 1990). VEGF receptors 1 and 2 share ~44% amino acid sequence identity and all three receptors show the greatest amount of similarity in their kinase domains (80%) and the least amount in their C-terminal tails. All three receptors are vital for the development of the vasculature during embryogenesis.

1.4.3 *VEGF receptor-1*

The homozygous knockout of VEGF receptor-1 in mice is embryonically lethal (Fong et al., 1995). Disruption of the VEGF receptor-1 gene severely impairs the development of endothelial cells into organised, functional blood vessels. Vessel-like structures are formed but they are not obvious and are obstructed by the overgrowth of endothelial cells. There is an increased number of haemangioblasts in these embryos (Fong et al., 1999), which indicates that changes in the fate of early endothelial cells may be responsible for these vascular defects. The overgrowth of endothelial cells suggests that VEGF receptor-1 has a negative regulatory role during normal vascular development. In mice lacking only the tyrosine kinase domain of VEGF receptor-1 however embryos develop a vascular system that shows no obvious defects and is comparable with that found in normal mice (Hiratsuka et al., 1998). Macrophages derived from these mice did show impaired migration in response to VEGF and PlGF. This is only a slight defect in comparison to the lethality caused by a complete VEGF receptor-1 deficiency, however it does suggest that tyrosine activity of this receptor is required for a secondary physiological function, the induction of monocyte/macrophage migration.

VEGF receptor-1 has a high affinity for VEGF-A however tyrosine kinase activity is only increased two-fold in response to ligand binding. With this information and the phenotypic profiles of the VEGF receptor-1-null mice it has been proposed that this receptor may not function primarily to transmit a mitogenic signal but instead act as a decoy receptor (Park et al., 1994). The soluble form of VEGF receptor-1 is expressed at considerable levels in placenta during pregnancy (Clark et al., 1998a) and may cooperate with the full length VEGF receptor-1 to regulate angiogenesis in normal embryos. The high binding affinity for VEGF could enable VEGF receptor-1, in both forms, to negatively regulate the activity of VEGF on the vascular endothelium by preventing mitogenic signalling via VEGF receptor-2.

Additionally soluble VEGF receptor-1 may create dominant negative receptor dimers by pairing with one class of full-length receptor.

PlGF is one member of the VEGF family that specifically binds to VEGF receptor-1. The study of PlGF-null mice has therefore led to suggestions regarding the potential roles for VEGF receptor-1 in embryogenesis and pathological angiogenesis. Embryonic angiogenesis is not affected in mice deficient for PlGF and these animals are born at mendelian frequencies, are healthy and also fertile (Carmeliet et al., 2001). PlGF is normally expressed at embryonic day 10.5 in mice however embryos engineered to lack PlGF may compensate for this deficiency by up regulating VEGF.

The cDNA for PlGF was identified from a human placental cDNA library and was investigated as a ligand for VEGF receptors because it encodes for a protein that has 53% identity with the platelet-derived growth factor-like region of VEGF (Park et al., 1994). PlGF was found to bind to VEGF receptor-1 with high affinity but, like VEGF, showed no mitogenic or permeability-enhancing activity. As mentioned above VEGF receptor-1 binds VEGF with high affinity and can therefore limit the bioactivity of VEGF. PlGF however is proposed to displace the proportion of VEGF that binds to VEGF receptor-1, thereby increasing its availability to VEGF receptor-2. Although this role for PlGF is well accepted recent studies have suggested that PlGF, either alone or in combination with VEGF, may stimulate angiogenesis by transmitting signals through VEGF receptor-1 (Luttun et al., 2002c).

PlGF does not appear to have a significant role in embryogenesis. In the adult however it stimulates angiogenesis by synergising with VEGF and amplifying endothelial cell responses to VEGF in pathological conditions (Carmeliet et al., 2001). These conclusions are supported by the localisation of VEGF receptor-1. In the embryo, VEGF receptor-1 is predominantly in its soluble form, which is compatible with its role as a “VEGF sink”. In pathological conditions the membrane localisation of VEGF receptor-1 is increased indicating that PlGF could activate this receptor to transmit signals.

Studies involving PlGF-null mice were prompted by observations regarding changes in pathological angiogenesis (Luttun et al., 2002a; Luttun et al., 2002c). Loss of PlGF impairs endothelial cell responses to VEGF and therefore also impairs angiogenesis, plasma

extravasation and collateral growth during ischaemia, inflammation, wound healing and cancer. PlGF, acting with VEGF, may also affect vascular permeability in response to conditions such as skin wounding and stimulate arteriogenesis in ischaemic hearts and limbs.

Evidence that PlGF modulates the endothelial response to VEGF is illustrated in a study that examined the capillary outgrowth in intact aortic rings from PlGF-deficient mice (Carmeliet et al., 2001). Treatment with PlGF or VEGF alone resulted in minimal capillary outgrowth however treatment with a combination of both growth factors strongly stimulated this response. The use of antibodies specific for VEGF receptor-1 completely blocked outgrowth in response to PlGF and VEGF, indicating that activation of this receptor, possibly by PlGF, is required for angiogenesis. Furthermore, these antibodies provide a protective effect in inflammatory disorders driven by angiogenesis, such as arthritis and atherosclerosis. Studies by Lutun et al. show that anti-VEGF receptor-1 suppresses VEGF-driven neovascularisation in ischaemic retina (Lutun et al., 2002c). In addition these antibodies block angiogenesis and tumour growth in nude mice in a dose-dependent manner by reducing vascularisation and proliferation and increasing necrosis and apoptosis. These observations support the evidence for active signalling via this receptor in pathological angiogenesis.

VEGF receptor-1 is also expressed in haematopoietic stem cells, macrophages and monocytes and in these cells the tyrosine kinase activity may be required for cellular migration towards PlGF or VEGF. VEGF receptor-1 is an important factor in the function of the haematopoietic system and the accumulation of haematopoietic cells in tumour tissue promotes tumour vascularisation. The growth of PlGF-expressing tumours is impaired in mice that lack the intracellular domain of VEGF receptor-1 and the use of neutralising antibodies to inhibit VEGF receptor-1 function decreases tumour growth (Hiratsuka et al., 2001; Lutun et al., 2002b). These observations suggest that the intracellular domain of VEGF receptor-1 might regulate tumour angiogenesis.

1.4.3(i) Activation of VEGF receptor-1

The level of phosphorylation of VEGF receptor-1 is an indication of its activation but this has only been convincingly detected using cells engineered to over-express the receptor (Ito et al., 1998; Sawano et al., 1997). In these systems phosphorylation sites have been mapped in the cytoplasmic domain of the receptor. The phosphotyrosine residues identified to date however are not contained within p85-, Shc- or Grb2-binding consensus sequences. In addition, a

repressor sequence in the juxtamembrane region of VEGF receptor-1 may influence protein folding (Gille et al., 2000). It has been suggested that this could either inhibit the accessibility of the kinase domain active site or regulate the interactions of phosphatases. This region has been shown to constitutively inhibit the activation of PI3K and endothelial cell migration in response to VEGF.

Several phosphorylation sites have been mapped in the VEGF receptor-1, which include tyrosine residues 794, 1169, 1213, 1242, 1309, 1327 and 1333 and all but one of these (794) are found in the C-terminal tail. Some of these may serve as binding sites for SH2-binding proteins as increased tyrosine phosphorylation of PLC γ 1, Crk, SHP-2 and ERK1/2 has been shown in porcine aortic endothelial (PAE) cells expressing VEGF receptor-1 and stimulated with VEGF (Ito et al., 2001). The identification of signalling roles for these residues has not yet been possible as mutations result in deregulated kinase activity. Studies investigating the effects of VEGF signalling in endothelial cells often conclude that VEGF receptor-2 is critical for migration, differentiation and proliferation but VEGF receptor-1 is not. In contrast however VEGF receptor-1 has been implicated in the migration of HUVE cells and monocytes towards VEGF (Barleon et al., 1996; Hiratsuka et al., 1998). In addition, under some circumstances, VEGF receptor-1 is able to generate a mitogenic response (Meyer et al., 2004a). The natural ligands for wild type VEGF receptor-1 include PlGF and VEGF however the tyrosine phosphorylation profiles of the receptor differ depending on the ligand. PlGF stimulates the specific phosphorylation of tyrosine residue 1309 within VEGF receptor-1 whereas VEGF phosphorylates tyrosine 1213 and, to a lesser extent, tyrosines 1242 and 1333 (Autiero et al., 2003). Microarray data, from RNA of cells in which VEGF receptor-1 signalling can be studied independently of that of VEGF receptor-2, show that VEGF results in minimal VEGF receptor-1 signalling (no effect on expression of target genes) however activation by PlGF resulted in the change in expression levels of over 50 genes involved in proliferation, apoptosis and angiogenesis (Autiero et al., 2003).

1.4.4 *VEGF receptor-2*

VEGF receptor-2 is expressed in most, if not all, embryonic tissues. More specifically it is expressed in vascular endothelial and lymphatic endothelial cells, megakaryocytes and haematopoietic stem cells. Embryos lacking the VEGF receptor-2 gene show defects in the development of endothelial and haematopoietic precursors required for yolk-sac blood island formation and this, together with the consequential failure of vasculogenesis, results in death

in-utero (Shalaby et al., 1995). The insufficient differentiation and proliferation of endothelial and haematopoietic cells demonstrates the essential requirement of VEGF receptor-2 in these processes.

VEGF receptor-2 is the primary receptor that mediates the growth and permeability of endothelial cells. It is a high affinity receptor for VEGF-A, VEGF-C, VEGF-D and VEGF-E. VEGF receptor-2 has a lower binding affinity for VEGF-A (K_d = about 200 pM) than that of VEGF receptor-1 (K_d = 1-10 pM) (Seetharam et al., 1995; Shibuya et al., 1999). The increase in kinase activity however is much greater than that observed for VEGF receptor-1 and phosphorylation can easily be detected in primary endothelial cells such as HUVE cells. VEGF receptor-2 depends on its intrinsic kinase activity for autophosphorylation of tyrosine residues. Mutation of the cDNA encoding for a chimeric VEGF receptor-2, so that the lysine 866 in the ATP binding pocket of the VEGF receptor-2 protein was substituted for an arginine, showed that the tyrosine kinase activity of the receptor is required for *in vitro* and *in vivo* tyrosine phosphorylation (Rahimi et al., 2000). Several autophosphorylation sites have been identified in VEGF receptor-2, which include tyrosine residues 801, 951, 996, 1054, 1059, 1175 and 1214 located in the juxtamembrane, kinase, kinase insert and C-terminal domains of the receptor (Dougher and Terman, 1999; Takahashi et al., 2001). Yeast two-hybrid screens have identified signalling proteins that bind to these phosphotyrosine residues and single amino acid mutations has identified their importance in downstream signalling pathways. Briefly, tyrosines 1054 and 1059 in the activation loop of the kinase domain and tyrosine 1214 in the C-terminal tail of the receptor are required for maximum activation (Dougher and Terman, 1999), tyrosine 951 is essential for VEGF –induced HUVE cell migration (Zeng et al., 2001) and is the binding site for VRAP (VEGF receptor-associated protein) (Wu et al., 2000b), tyrosine 1175 mediates PLC γ 1 binding and activation leading to cell proliferation via PKC (protein kinase C) and ERK (Guo et al., 1995; Takahashi et al., 1999b; Takahashi et al., 2001), Sck also binds to tyrosine 1175 providing a potential link to the Grb2-Sos-MAP kinase cascade (Ratcliffe et al., 2002; Warner et al., 2000) and Grb2 may bind to tyrosine 1214 creating yet another possible link to the MAP kinase pathway. As VEGF receptor-2 is a major component necessary for many signalling pathways in endothelial cells details of its signalling capacity are illustrated in Figure 1.5 and detailed in subsequent sections. VEGF receptor-2 signalling is enhanced by co-receptors and the adhesion of endothelial cells to the extracellular matrix therefore proteins that are important for efficient signal transduction are also described.

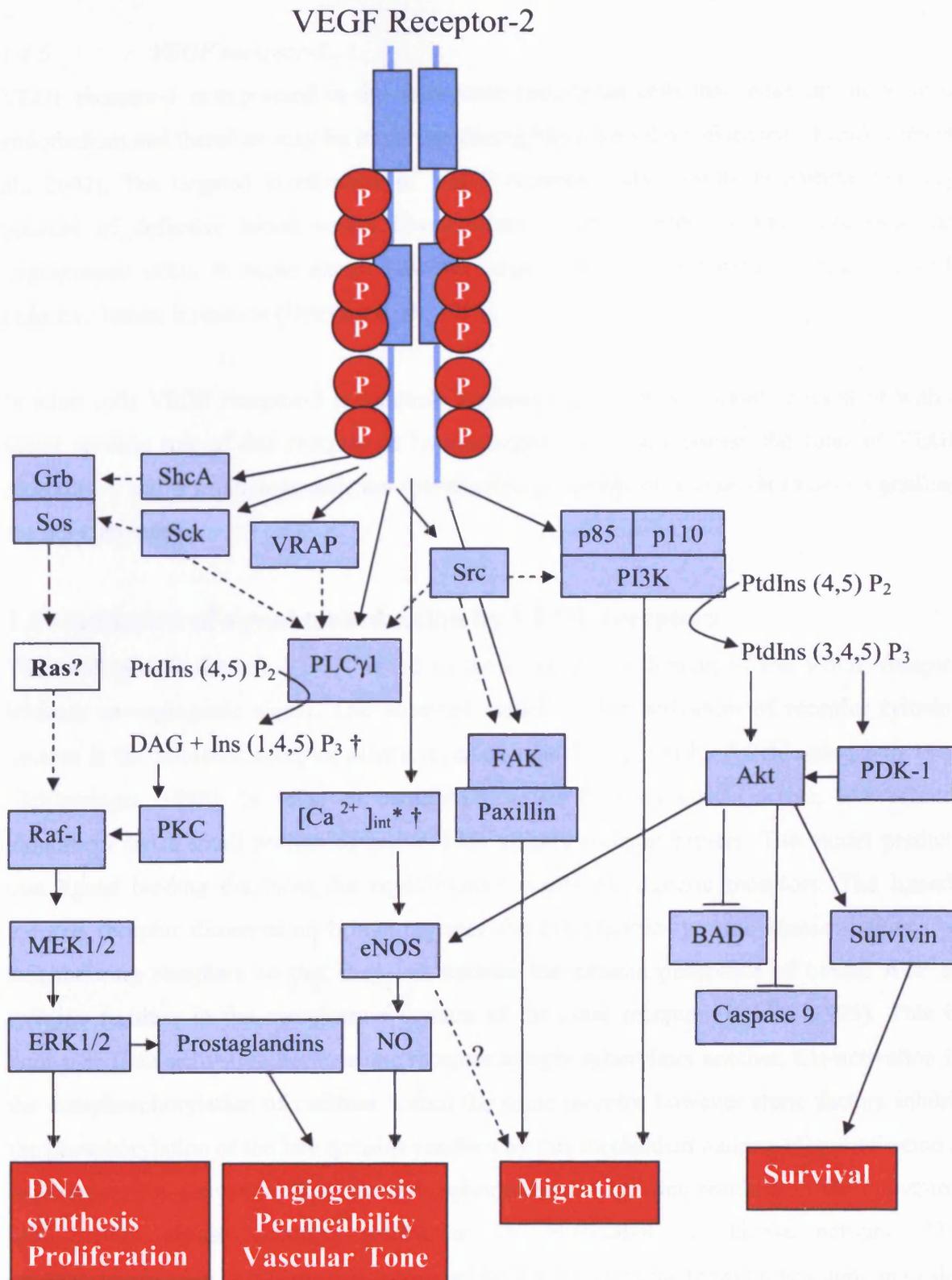


Figure 1.5 – A summary of the pathways downstream of VEGF receptor-2. Full arrows show pathways that are well characterised. Dotted arrows and the question mark indicate pathways and outcomes still under investigation. Caspase-9 and BAD are phosphorylated by Akt, hence their activities are inhibited. * (Bates and Curry, 1997; Chandra and Angle, 2005; Pocock et al., 2000) † (Brock et al., 1991)

1.4.5 *VEGF receptor-3*

VEGF receptor-3 is expressed in the embryonic endothelial cells that make up the venous endothelium and therefore may be important during blood vessel development (Karkkainen et al., 2002). The targeted inactivation of VEGF receptor-3 also results in embryo lethality because of defective blood vessel development. In these embryos vasculogenesis and angiogenesis occur to some extent however large vessels are abnormally organised with defective lumen formation (Dumont et al., 1998).

In adult cells VEGF receptor-3 is uniquely expressed in lymphatic vessels consistent with a major specific role of this receptor in lymphanogenesis. In this project the roles of VEGF receptors 1 and 2 in angiogenesis are investigated so details of VEGF receptor-3 signalling are not elaborated.

1.5 - Initiation of signal transduction by VEGF receptors

The binding of a dimeric VEGF ligand to the extracellular domain of the VEGF receptor initiates an angiogenic signal. The accepted model for the activation of receptor tyrosine kinases is the allosteric receptor oligomerisation model proposed by J Schlessinger in 1988 (Schlessinger, 1988). In cells, an equilibrium exists between kinase active, low affinity monomers and a small portion of active, high affinity receptor dimers. The model predicts that ligand binding displaces the equilibrium towards the dimeric receptors. The ligand-induced receptor dimerisation brings together the cytoplasmic tyrosine kinase domains of neighbouring receptors so that they can transfer the gamma phosphate of bound ATP to tyrosine residues in the cytoplasmic domain of the other receptor (Heldin, 1995). This is known as trans-activation because one receptor autophosphorylates another. Cis-activation is the autophosphorylation of residues within the same receptor however steric factors inhibit the phosphorylation of the key tyrosine residues by this mechanism making oligomerisation a requirement for activation. Trans-autophosphorylation of tyrosine residues in the activation loop within kinase domains results in the stimulation of kinase activity. The autophosphorylation of tyrosines in the juxtamembrane region, the kinase insert domain or the C-terminal tails of receptors generate docking sites for modular domains that recognise phospho-tyrosine within a specific sequence. These phosphorylation events could occur by either a trans or cis mechanism.

The crystal structure of inactive VEGF receptor-2 has provided a molecular understanding of the tight controls imposed upon its catalytic activity. In unstimulated VEGF receptor-2 the activation loop is positioned so that it prevents substrate tyrosines from binding to the active site (McTigue et al., 1999). Ligand binding to the extracellular domain of the receptor repositions the activation loop, and possibly other inhibitory sequences in the juxtamembrane and C-terminal tail, opening up the active site and allowing substrate access. An inhibitory juxtamembrane sequence has been identified in VEGF receptor-1 (Gille et al., 2000). In some cases however dimerisation is not always sufficient for kinase activation and ligand-induced conformation changes are also required (Jiang and Hunter, 1999). This is evident for ErbB2 receptors that have been engineered to contain cross-linking cysteine residues in their transmembrane helix. This generates a permanent receptor dimer however only a subset of these have transforming activity (Burke et al., 1997; Burke and Stern, 1998). This indicates that activation is dependent on the relative orientation of the two receptors. In addition this study suggests that upon ligand binding, conformational switches must occur, to ensure the catalytic domains are juxtaposed in the correct configuration, to enable autophosphorylation in trans between the receptor subunits. Other examples in which dimerisation is insufficient include the ephrin receptors that require tetramerisation to elicit their full range of biological responses (Stein et al., 1998) and the Epo receptor that exhibits a number of different dimeric conformations with different activities, which are dependent on the ligand bound (Livnah et al., 1998).

1.6 - SH2- and PTB domain-containing adapter proteins

Adapter proteins are able to associate with activated receptors by means of specific protein modular domains that recognise phosphorylated tyrosine residues. Src-homology-2 domain (SH2-domain) and phosphotyrosine-binding domain (PTB-domain) are non-catalytic regions of approximately 100 amino acids that bind specifically to phosphorylated tyrosine residues (Forman-Kay and Pawson, 1999; Pawson et al., 2001; Pawson and Nash, 2000; Pawson et al., 2002; Yan et al., 2002). The affinity and specificity of the SH2 and PTB domains for a specific protein is dictated by the identification of amino acids that flank the phosphotyrosine residue. SH2 domains recognise between 3-6 residues C-terminal to the phosphotyrosine but for the PTB domain residues that are amino-terminal to the phosphotyrosine determine the target sequence. SH2 domain-containing proteins often contain other protein interaction domains such as SH3 domains that stably bind to Pro-X-X-Pro motifs. These motifs do not

need to be post-translationally modified. Adapter proteins lack intrinsic catalytic activity and couple a phosphotyrosine residue, recognised by its SH2 domain, to downstream targets with proline-rich motifs that bind to the SH3 domains. Some enzymes also contain one or more SH2 domains along with other protein and/or lipid-binding motifs. PLC γ 1 for example contains two SH2 domains, which increases its binding affinity and specificity, and SH3 and PH domains that regulate the activity of the linked catalytic domain and juxtapose the enzyme with its substrate, Phosphatidylinositol (4,5) bisphosphate (PtdIns (4,5) P₂).

Adapter proteins found in the cytoplasm of endothelial cells use phosphorylated residues as docking sites to associate with the receptors and other proteins at the cell surface. SH2 domain-containing proteins that have been shown to interact with activated VEGF receptors include adapters, docking proteins and enzymes such as, ShcA (Src-homology collagen protein) (Kroll and Waltenberger, 1997), ShcB/Sck (Shc-like protein) (Igarashi et al., 1998; Ratcliffe et al., 2002; Warner et al., 2000), Yes, Fyn, VRAP (VEGF receptor associated protein) (Wu et al., 2000b), Grb2 (Growth factor receptor bound) and Grb10 (Giorgetti-Peraldi et al., 2001), Crk, Nck (Stoletov et al., 2001) and PLC γ 1 (Takahashi and Shibuya, 1997). For most adapter proteins the SH2 domain(s) found within these proteins mediates its binding to the activated growth factor receptors.

1.6.1 *The Shc family of adapter proteins*

The Shc family of adapter proteins are unique because they contain both an amino-terminal PTB domain and a carboxy-terminal SH2 domain (Luzi et al., 2000). They are also known as docking proteins because they nucleate the formation of multiprotein complexes. The SH2 domain of ShcA has a particular preference for peptides with a leucine or isoleucine residue at the +3 position relative to the phosphotyrosine however it is the PTB domain of ShcA that associates with phosphotyrosine residues in activated receptors. The specific peptide binding-motif for the ShcA PTB domain is Asn-Pro-X-pTyr (Margolis, 1996) and this domain can also associate with acidic phospholipids such as PtdIns (4,5) P₂ and Phosphatidylinositol (3,4,5) trisphosphate (PtdIns (3,4,5) P₃) in cell membranes (Zhou et al., 1995b). This indicates that the interaction of Shc proteins with the cell membrane could occur independently of an interaction with tyrosine phosphorylated receptors. The production of PtdIns (3,4,5) P₃ as an intracellular messenger could however regulate the localisation and signalling of ShcA in response to phosphoinoside 3-kinase (PI3K) activation. In addition to ShcA, ShcB/Sck and ShcC/N-Shc proteins are also members of the Shc family. ShcB expression is mainly confined

to the nervous system but has been identified in endothelial cells, and ShcC appears to be exclusively expressed in neuronal cells (Kojima et al., 2001; Nakamura et al., 1998; Ratcliffe et al., 2002). These proteins have the same domain structure as ShcA (Pelicci et al., 1996) and are also likely to function as adapter proteins. Two groups independently showed the association of ShcB/Sck with VEGF receptor-2 in a yeast two-hybrid screen (Kojima et al., 2001; Warner et al., 2000). In addition a truncated protein co-immunoprecipitates with the receptor when both are co-transfected into HEK293 cells (Warner et al., 2000). This interaction is likely to be mediated by the SH2 domain of ShcB/Sck and not the PTB domain as reported for other ShcA-receptor interactions. In addition it has been proposed that ShcB/Sck is used in endothelial cells as a unique adapter to couple VEGF receptor-2 to the Ras/MAP kinase pathway. The expression of full length ShcB/Sck will enable comparative studies of ShcA and ShcB/Sck to investigate this further.

1.6.2 Grb10 as a VEGF receptor-2 adapter protein

Grb10 is another adapter protein that interacts with VEGF receptor-2 (Giorgetti-Peraldi et al., 2001). The exact biological role of Grb10 is not yet known however in response to VEGF signalling the protein is tyrosine phosphorylated and the levels of Grb10 mRNA are increased. Expression of Grb10 results in an increase in the amount of VEGF receptor-2 molecules, an increase of the tyrosine phosphorylation of VEGF receptor-2 and in HEK293 cells (Human Embryonic Kidney 293 cells) the activation of signalling molecules such as MAP kinase. This suggests that Grb10 could be involved in a positive feedback loop whereby VEGF signalling results in the expression of more VEGF receptor-2 molecules and facilitates receptor activation. Grb10 acts specifically on VEGF receptor-2 but elicits a different effect compared to that of Grb2 or Shc. These adapter proteins were unable to modify the amount or tyrosine phosphorylation of VEGF receptor-2 in response to VEGF.

1.7 - MAP kinase signalling pathways in endothelial cells

Receptor tyrosine kinase association with the Grb2-Sos complex has been shown to initiate the activation of a major proliferative pathway (Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993; Saucier et al., 2002). The protein tyrosine kinases involved in this pathway make up the highly conserved mitogen-activated kinase (MAP kinase) cascade. An important consequence of the activation of this cascade is the activation of one of the many extracellular signal-regulated kinases (ERKs). ERKs, under certain conditions, are able to

enter the nucleus and phosphorylate target substrates, initiating the transcription of key genes. These gene products then initiate progression through the cell cycle and hence cell proliferation. Grb2 is recruited to phosphotyrosine residues in activated receptors. As Grb2 is constitutively associated with Sos, through a Grb2-SH3 domain interaction, it also recruits the guanine nucleotide exchange protein Sos (Son of Sevenless), which activates Ras. Consequently the MAP kinase pathway is activated and Raf-1 (a MAP kinase kinase kinase) phosphorylates and activates MEK (a MAP kinase kinase), which phosphorylates and activates ERK (a MAP kinase).

VEGF is a strong activator of ERKs via VEGF receptor-2 and this is likely to contribute to VEGF-induced angiogenesis by inducing gene expression and proliferation of endothelial cells (Zachary, 2003). To initiate the MAP kinase pathway Grb2 may bind to phosphotyrosine 1214 in the C-terminal tail of VEGF receptor-2, as this residue is contained within the Grb2 SH2-binding motif. Alternatively VEGF has been shown to stimulate ShcA phosphorylation and promote the formation of a Shc-Grb2 complex (Kroll and Waltenberger, 1997) however there is no evidence that it is this association that leads to activation of Ras or the ERK pathway. Ras has shown to be activated in response to VEGF, by the increased binding of labelled GTP, and MAP kinase activation is dependent on the association of Ras with Raf-1 (Doanes et al., 1999). Expression of a dominant negative Ras protein, N17ras, which blocks MAP kinase activation by EGF does not affect levels of VEGF-stimulated phospho-MAP kinase. Further investigations demonstrate that VEGF can activate the Raf-MEK-ERK pathway through a mechanism that is Ras-independent and mediated by PKC (Takahashi et al., 1999b). PKC-specific inhibitors severely reduced VEGF-dependent phosphorylation of MEK, activation of MAP kinase and subsequent DNA synthesis.

Other member of the MAP kinase pathway, in addition to the extracellular signal-regulated kinases (ERKs) include stress activated protein kinase-1/c-Jun N-terminal kinase (Jnk) and Stress-activated protein kinase-2/p38 MAPK.

1.7.1 VEGF signalling via Jnk

Jnk is involved in a cross-talk mechanism with the extracellular signal-regulated kinases/ERKs (Pedram et al., 1998). VEGF signalling to Jnk has been demonstrated by Pedram et al and they show that activation of ERK is both necessary and sufficient for rapid Jnk activation. This group also show that the activation of ERK, mediated by a number of

different stimuli, can indirectly, but rapidly, activate Jnk and that both kinases mediate increased endothelial cell proliferation in response to VEGF. The authors conclude that Jnk action appears to be more directly responsible for the majority of the VEGF-induced proliferation indicating the importance of the cross-activation mechanism. The main role of ERK activation in response to VEGF is therefore to activate Jnk, a cross-talk system between MAP kinases that could contribute to the decision by the cell to divide or to terminally differentiate (reviewed in Zhang and Liu, 2002).

1.7.2 *VEGF signalling via p38 MAP kinase*

Stress-activated protein kinase-2 (SAPK-2)/p38 MAPK plays an essential role in VEGF-induced vascular permeability, and inhibition of p38 MAPK can induce a switch from this to pro-angiogenic activities. Inhibition of p38 MAPK enhances VEGF- and FGF- induced angiogenesis and p38 MAPK signalling may provide a general anti-angiogenic mechanism. Issbrucker et al report that inhibition of p38 MAPK activity, using SB202190 and SB203580, enhances VEGF-induced angiogenesis *in vitro* and *in vivo* accompanied by prolonged ERK activation, a reduction in apoptosis and reduced migration (Issbrucker et al., 2002). Also Matsumoto et al report that treatment with the p38 MAPK inhibitor SB202190 enhances FGF-2-induced tubular morphogenesis by decreasing apoptosis, increasing DNA synthesis and cell proliferation, and enhancing the kinetics of cell differentiation (Matsumoto et al., 2002).

Remodelling of the actin cytoskeleton is indicative of changes in vascular permeability and migration. The activation of p38 MAPK contributes positively to these processes in the formation of actin stress fibres. In HUVE cells VEGF induced profound cytoskeletal reorganisation characterised by the formation of cytoplasmic stress fibres (Rousseau et al., 1997). MAPKAP kinase 2/3 is a physiological target of p38 MAPK and can therefore be used as a measure of p38 MAPK activity. Neither PlGF nor VEGF stimulation of VEGF receptor-1 resulted in MAPKAP kinase 2/3 activation however VEGF stimulation of VEGF receptor-2 resulted in a 3-fold activation of p38 MAPK in PAE cells expressing VEGF receptor-2 (Rousseau et al., 1997). A VEGF receptor-2 neutralising antibody inhibited the activation of p38 MAPK and cell migration in HUVE cells (Rousseau et al., 2000). In addition VEGF receptor-2 mediated stress fibre formation is associated with a 30-40% increase in F-actin, which was inhibited by the potent p38 MAPK inhibitor, SB203580. Hence VEGF receptor-2 is responsible for the p38 MAPK-mediated actin reorganisation and cell migration. Activation

of FAK by VEGF receptor-2 however was independent of p38 MAPK and ERK activation (Rousseau et al., 2000).

1.8 - PLC γ 1 signalling downstream of VEGF receptors

The yeast two-hybrid system has been used to show the association of VEGF receptor-1 with PLC γ 1 (Cunningham et al., 1997). This work also shows that, single tyrosine mutations at positions 794 and 1169 are important for PLC γ 1 binding, and double tyrosine mutations are essential. VEGF stimulation of NIH3T3 cells expressing VEGF receptor-1 results in the phosphorylation of tyrosine residue 1169 in the C-terminal tail of VEGF receptor-1 (Sawano et al., 1997). This phosphotyrosine residue provides a binding site for PLC γ and association mediates PLC γ activation.

Wu et al show a strong direct association of full length PLC γ 1, and both SH2 domains of PLC γ 1, with VEGF receptor-2 in a yeast two hybrid screen using a HUVE cell cDNA library and also an *in-vivo* interaction between endogenous PLC γ 1 and VEGF receptor-2, expressed in HUVE cells, by immunoprecipitation (Wu et al., 2000a). In subsequent yeast two-hybrid experiments, using mutants of VEGF receptor-2, in which key tyrosine residues are mutated to phenylalanine residues, they show that tyrosine 951 of VEGF receptor-2 is essential for binding to PLC γ 1, tyrosine 996 is non-essential and mutation of tyrosine 1175 only weakens the interaction. Cunningham et al also used the yeast two-hybrid system to show that the N-terminal SH2 domain of PLC γ 1 associates with VEGF receptor-2 (Cunningham et al., 1997). This study however shows that mutation of tyrosines 801 or 1175 of VEGF receptor-2 abolishes the receptor-PLC γ 1 interaction.

Yeast two-hybrid mapping also identified VRAP as a protein that interacts with tyrosine 951 of VEGF receptor-2 (Wu et al., 2000b). In this study Wu et al show by co-immunoprecipitation a VEGF-stimulated association of VRAP with VEGF receptor-2 in HUVE cells and a constitutive association between VRAP and PLC γ 1 in HUVE cells. VRAP could therefore couple tyrosine 951 of VEGF receptor-2 to PLC γ 1.

Meyer et al use PAE cells expressing chimeric VEGF receptor-2, to show that tyrosine 1006 is the main binding site for the C-terminal SH2 domain of PLC γ 1 in an immunoprecipitation assay, and conclude that although the two domains act co-operatively, the C-terminal SH2

domain of PLC γ 1, but not the N-terminal SH2 domain, is able to associate with VEGF receptor-2 (Meyer et al., 2003). This study also shows that tyrosines 1006 and 1175 of the chimeric VEGF receptor-2 are responsible for the activation of PLC γ 1. In addition, PLC γ 1 is not activated in MSS31 and BAE endothelial cells expressing a Y1175F mutant VEGF receptor-2 (Takahashi et al., 2001).

The conclusions, as to the single phosphotyrosine residue, or the combination of phosphotyrosine residues in VEGF receptor-2 responsible for binding to PLC γ 1, are inconsistent however tyrosine 1175 has been implicated by several groups (Cunningham et al., 1997; Meyer et al., 2003; Takahashi et al., 2001; Wu et al., 2000a). The differences in the conclusions reached are likely to be due to the different methods used. In addition it is possible that an individual phosphotyrosine residue does not mediate the recruitment of PLC γ 1 and that some sites may be compensatory in their ability to bind to PLC γ 1.

PLC γ association with VEGF receptor-2, possibly via either Sck or VRAP adapter proteins, stimulates tyrosine phosphorylation and activation of PLC γ . Activated PLC γ then catalyses the hydrolysis of phosphatidylinositol (4,5) bisphosphate (PtdIns (4,5) P₂) to produce inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG). The subsequent activation of Protein Kinase C (PKC, particularly PKC α and PKC ζ isoforms (Xia et al., 1996)) by DAG results in signals that induce the proliferation of endothelial cells. As discussed above PKC mediates the activation of the MAP kinase pathway. In support of this PKC inhibitors (Glick et al., 2001; Takahashi et al., 1999b) and antisense oligonucleotides (Higaki et al., 1999; Wellner et al., 1999; Xia et al., 1996) have been shown to block VEGF induced activation of ERK and MEK and angiogenesis. Calcium mobilisation by IP₃ contributes to the production of nitric oxide by eNOS and prostacyclins via MAP kinase. In these studies Src activation by VEGF was shown to be required for the phosphorylation of PLC γ 1 (He et al., 1999). NO and prostaglandin I₂ promote angiogenesis, vascular hyperpermeability and vasodilation (Zachary, 2003).

1.9 - Phosphatidylinositol 3-kinase and Akt signalling

Phosphatidylinositol 3-kinase (PI3K) is a heterodimer consisting of a p85 regulatory domain and a p110 catalytic subunit. The p85 subunit contains two SH2 domains and a region between these domains that mediates the association with p110. p85 is phosphorylated in

response to VEGF and the enzymatic activity of PI3K is also increased in VEGF-stimulated endothelial cells (Thakker et al., 1999). PI3K signalling is required in a fundamental cell survival mechanism by which VEGF activates anti-apoptotic signals in endothelial cells.

PI3K phosphorylates the 3' position of inositol contained within lipids thereby generating a range of phospholipids that serve as substrates for protein modules. PtdIns (4,5) P₂ is used as a substrate for PI3K thereby creating a unique derivative, phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5) P₃). As PtdIns (3,4,5) P₃ is the primary product of activated PI3K, and can be subsequently dephosphorylated by a specific phosphatase to produce PtdIns (3,4) P₂, it has been suggested to function as a ubiquitous second messenger. The receptor-stimulated accumulation of PtdIns (3,4,5) P₃ can very quickly lead to the recruitment of Akt/PKB (Protein Kinase B). Akt is able to interact with PtdIns (3,4,5) P₃ via its pleckstrin homology (PH) domain (Franke et al., 1997), a structural motif first defined in the cytoskeletal protein pleckstrin, which anchors it to the membrane. PtdIns (3,4,5) P₃-activated protein kinase, PDK1, is also a PH domain containing protein that phosphorylates, and hence activates, Akt (Alessi et al., 1997; Andjelkovic et al., 1997).

Akt is a serine/threonine kinase that subsequently phosphorylates target proteins however the initiation of survival signals is also dependent on VE-cadherin (Carmeliet et al., 1999). Signalling events downstream of Akt include the enhanced synthesis of nitric oxide via endothelial nitric oxide synthase (eNOS), the inhibition of p38 MAPK and Jnk pathways, activation of the ERK pathway and phosphorylation, hence inhibition, of the pro-apoptotic proteins Bad and caspase-9. VEGF has also been shown to induce the expression of anti-apoptotic proteins such as Bcl-2, A1, survivin and XIAP (Gerber et al., 1998; Tran et al., 1999).

1.10 - Nitric oxide as a signalling molecule in endothelial cells

Nitric oxide (NO) release from endothelial cells is essential for VEGF-mediated increases in vascular permeability, angiogenesis and endothelial cell migration. The production of NO is inhibited by the down-regulation of VEGF receptor-2 following sustained VEGF stimulation. The pre-treatment of BAE cells with VEGF desensitises cells, prevents further activation of VEGF receptor-2, Akt and eNOS and hence inhibits NO production. The activation of eNOS to produce NO is mediated by PLC γ 1 and PI 3-kinase/Akt pathways, which increase

intracellular calcium levels, and the phosphorylation of eNOS by Akt (Dimmeler et al., 1999; Fulton et al., 1999).

1.11 - Signalling via Src family kinases

The Src family kinases, Src, Yes and Fyn are expressed in endothelial cells (Bull et al., 1994; Kiefer et al., 1994) and mice deficient in Src and Yes show impaired VEGF-induced vascular permeability (Eliceiri et al., 1999). The association of Src with VEGF receptor-2 has been shown in bovine aortic endothelial (BAE) cells and HUVE cells (Chou et al., 2002). Fyn and Yes however associate preferentially with VEGF receptor-1 (Chou et al., 2002). VEGF induces an increase in Src activity and Src has been implicated in the phosphorylation of VE-cadherin, PI3K, p38 MAPK and PLC γ 1.

1.12 - Other activators of angiogenesis

1.12.1 *Mechanical signals*

Fluid shear stress is caused by the dragging forces created by blood flow and is a mechanical stimulus that can be detected by cells. These haemodynamic forces are a major determinant of arterial tone, vascular remodelling and atherogenesis. The stimulation of cells by shear stress results in the activation of different types of receptors including integrins, VEGF receptor-2, G-protein-coupled receptors and ion channels, which in turn can activate diverse signalling pathways. Wang et al show that the association of VEGF receptor-2 with Cbl, an ubiquitin ligase enzyme implicated in the degradation of VEGF receptor-2, and Cbl phosphorylation is induced by shear stress (Wang et al., 2004). The activation and regulation of VEGF receptor-2 in response to shear stress is dependent on the activation of α v β 3 integrin (Wang et al., 2002). Fluid shear stress induces the formation of α v β 3 integrin clusters and, by the assembly of focal adhesion complexes that connect integrins and the ECM to the cytoskeleton, these proteins constitute a mechano-sensing and -transduction mechanism. The rearrangement of the cytoskeleton may bring together different molecules to create stable signalling complexes, that may include VEGF receptor-2 and Cbl for example. Focal adhesion complexes also contain other proteins including the adapter protein Grb2 and the non-receptor tyrosine kinase Src. In another study the inhibition of Src kinase, using the inhibitor PP2, abolished flow-induced VEGF receptor-2 tyrosine phosphorylation and downstream signalling (Davis et al., 2001). As Src is also required for the activation of EGFR by integrins Jin et al hypothesised that src kinases phosphorylate receptors to transduce signals from mechanosensors (Jin et al.,

2003). The activation of VEGF receptor-2 in response to shear stress results in the recruitment and activation of PI3K and the concomitant activation of Akt. These signalling proteins then phosphorylate eNOS promoting its activation and NO production in cultured cells. NO has an essential role in the regulation of vascular function and structure in response to blood flow. These roles include vessel relaxation, inhibition of apoptosis and vascular remodelling.

1.12.2 *Fibroblast Growth Factor Signalling*

There are many members of the fibroblast growth factor (FGF) family that bind to four FGF receptors. Heparan sulphate proteoglycans are co-receptors for the FGF receptors and FGF-2 binding to FGF receptor-1 is stabilised by heparin or heparan sulphates. Basic FGF, or FGF-2, was the first pro-angiogenic molecule identified (Shing et al., 1984) and binds to FGF receptor-1. The expression of a dominant negative FGF receptor-1 in mouse embryos shows that the wild type receptor is involved in the development and maintenance of the vasculature (Lee et al., 2000). The addition of recombinant FGF-2 or the up-regulation of endogenous FGF induces VEGF expression in cultured endothelial cells (Seghezzi et al., 1998). This represents an autocrine mechanism whereby FGF-2 signalling contributes to angiogenesis. The stimulation of endothelial cells with FGF results in cell survival, proliferation, migration and differentiation. In addition, FGF induces angiogenesis in the chicken chorioallantoic membrane assay. Angiogenesis observed in this assay was blocked following treatment with the MEK inhibitor PD98059 indicating that the Ras-MEK-MAP kinase pathway is important in FGF-mediated angiogenesis (Eliceiri and Cheresch, 1999). In cultured endothelial cells tube formation is an assay used to detect endothelial cell differentiation and sustained MAP kinase activity is implicated in this process. Inhibition of MEK and MAP kinase activity by the PD98059 inhibitor, or inhibition of PKC by bisindolylmaleimide, does not prevent tube formation in cultured endothelial cells however differentiation is abrogated by PP1-induced inhibition of Src (Klint et al., 1999). FGF-1 can also induce angiogenesis in the CAM assay and transfection of a gene encoding a secreted version of FGF-1 increases vascularisation. This is likely to be mediated by the Akt signalling pathway as FGF-1 induced upregulation of Akt mRNA and increased activity of Akt as shown by phosphorylation. In addition an Akt inhibitor reduced angiogenesis in response to FGF-1 (Forough et al., 2004).

1.12.3 *Angiopoietin and Tie Receptor Signalling*

The Tie family of receptor tyrosine kinases, Tie-1 and Tie-2, are also expressed in endothelial cells and bind to angiopoietins. Tie receptor function, at least in part is regulated by ligand

binding to the extracellular domain of the receptor. Angiopoietins 1-4 all bind agonistically or antagonistically to the Tie-2 receptor (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The ligands for Tie-1 receptor have not been identified yet however it may act as a co-receptor for Tie-2. The intracellular structure of the Tie receptors is similar to that for the VEGF and PDGF receptors however the extracellular portion is unique. Mice lacking either Tie-1 or -2 die due to loss of vascular integrity resulting in haemorrhaging or a decrease in endothelial cell numbers (Puri et al., 1995; Sato et al., 1995). Over-expression of Angiopoietin-1 results in increased vascularisation and induces chemotactic migration and endothelial cell sprouting. This is mediated by modifications to the actin cytoskeleton, via PI3K signalling and Rac GTPase activation, and coordinated interactions with ECM proteins (Suri et al., 1998). Signalling via Tie receptor-2 modulates cell-cell and cell-matrix interactions that are required for vascular remodelling and maturation. In addition, during the final stages of angiogenesis Ang-1 acts with Tie-2 to recruit pericytes, which tightens newly-formed blood vessels (Davis et al., 1996). Tie-1 and Tie-2 exist as heterodimers and this interaction promotes positive Tie-2 signalling. VEGF modulates this complex by the creation of truncated Tie-1. Cleavage of the extracellular domain of Tie-1, leaving a membrane bound fragment containing the transmembrane domain and the intracellular domain, results in a heterodimer composed of full length Tie-2 complexed to the Tie-1 ectodomain (Tsiamis et al., 2002). VEGF therefore modulates these receptors and this potentially affects the ability of these receptors to stabilise vascular vessels.

1.12.4 Ephrin Signalling

Ephrin A and B ligands are membrane-bound and associate with Ephrin tyrosine kinase receptors. Studies using Ephrin B2 ligand or Ephrin receptor knockouts show that these signalling molecules are required for vascular phenotypes (Adams, 2002; Adams et al., 2001; Cheng et al., 2002). Ephrin A1, together with the Ephrin A2 receptor, contributes to signalling during tumour angiogenesis and a dominant negative Ephrin A2 receptor blocked the formation of capillary endothelial tubes in VEGF-induced angiogenesis.

1.13 - VEGF receptor co-receptors

1.13.1 *Neuropilins*

Neuropilin-1, NRP-1, a transmembrane protein implicated in semaphorin-activated axonal guidance, is also a receptor for VEGF family members (Soker et al., 1998). The NRP-1 ligands semaphorin 3A and VEGF have opposing effects in the growth and survival of neurons and endothelial cells, with Sema-3 being inhibitory and VEGF stimulatory. Protein sequences encoded for by exon 7 of the VEGF-A gene are involved in NRP-1 binding as well as conveying binding to heparin. This is probably not a co-incidence as the interaction between VEGF-A₁₆₅ and NRP-1 is heparin-dependent. This interaction results in an increased affinity of VEGF receptor-2 for NRP-1 and may facilitate NRP-1 enhanced VEGF-A₁₆₅-mediated signalling. It has a short cytoplasmic tail with no intrinsic catalytic function however its cytoplasmic domain does interact with PDZ domain-containing molecules (Cai and Reed, 1999). It may function as a docking co-receptor, to present VEGF to receptors and form stable complexes through which signals can be transduced. This function of NRP-1 may be negatively regulated by VEGF receptor-1. The binding of NRP-1 to the extracellular domain of VEGF receptor-1 prevents NRP-1 from binding VEGF and consequently the biological effect of VEGF is diminished (Fuh et al., 2000). Similarly a naturally occurring soluble NRP-1 can act as a tumour antagonist, probably through the withdrawal of VEGF from its receptors (Gagnon et al., 2000).

Over-expression of NRP-1 results in abnormalities in the embryonic cardiovascular system, which includes excess capillaries and blood vessels, dilated blood vessels and malformations of the heart and also leads to deregulated angiogenesis and increased tumour growth (Kitsukawa et al., 1995; Miao et al., 2000). As may be expected, deletion of NRP-1 causes negative defects to the cardiovascular system such as impaired development of the aorta and other large blood vessels and aberrant yolk sac vascularisation (Suri et al., 1996).

1.13.2 *Heparan sulphate*

Heparan sulphate proteoglycans are found in the extracellular matrix and serve as an additional binding site for the heparin-binding isoforms of VEGF. Heparin and heparan sulphate are polysaccharides that can bind to some VEGFs and the extracellular domains of VEGF receptors 1 and 2. In addition, soluble VEGF receptor-1 shows affinity for heparin (Dougher et al., 1997) suggesting that this may be a method by which it can be retained by the

extracellular matrix. The binding of VEGF to VEGF receptor-1 is inhibited by heparin, however its kinase activity is increased by heparin treatment. In contrast the binding of VEGF to VEGF receptor-2 is stimulated by heparin indicating that heparin has different effects on different receptors (Terman et al., 1994). The heparin and heparan sulphate chains on the ECM sequester the larger VEGF isoforms soon after they are secreted from cells, hence VEGF does not circulate well in the blood stream. VEGF is therefore available only at sites close to its production. The sequestration of VEGF may provide a natural reserve of VEGF that can be released from the ECM by proteolytic digestion or a matrix that participates in the migration of endothelial cells at sites of angiogenesis.

Endothelial cells can adhere to and spread on immobilised VEGF₁₆₅ and VEGF₁₈₉ but not VEGF₁₂₁ (Hutchings et al., 2003). Adhesion to VEGF₁₈₉ was preferential and similar to that detected using fibronectin, one of the best adhesion substrates for endothelial cells. Fibronectin is usually found in the extracellular matrix and is a substrate for integrins; hence integrins mediate the interaction between endothelial cells and the extracellular matrix. In this report integrins are implicated in the interactions between extracellular matrix-bound VEGF and endothelial cells as combinations of RGD peptides and blocking antibodies to integrins inhibited all adhesion to immobilised VEGF (Hutchings et al., 2003). This integrin-mediated adhesion to VEGF promotes VEGF receptor-independent migration and survival of these cells. VEGF₁₆₅ and VEGF₁₈₉ were shown to directly associate with $\alpha\beta3$ integrin in an interaction that was as strong as that seen for vitronectin. VEGF₁₂₁ did not bind to $\alpha\beta3$ integrin indicating that the interaction occurs through the basic domain of VEGF encoded by exons 6 and 7. VEGF₁₈₉ supported endothelial cell migration to an extent comparable to fibronectin however migration on VEGF₁₆₅ was lower. The ligation of $\alpha\beta3$ to VEGF also generates a survival signal, as the pro-apoptotic action of tumstatin, an inhibitor of $\alpha\beta3$, was overridden by VEGF₁₆₅ and VEGF₁₈₉.

1.13.3 *Integrins*

Endothelial cell survival is maintained via two mechanisms, the first is the stimulation of cells by growth factors, for example VEGF, basic fibroblast growth factor and angiopoietin as discussed above, and the second is via contacts to the extracellular matrix. Integrins bind to components of the extracellular matrix and cluster with adapter molecules to form focal adhesions. The binding of integrins to the ECM activates intracellular kinases, by outside-in

signalling, including focal adhesion kinase (p^{125} FAK) (Masson-Gadais et al., 2003), Src (Courter et al., 2005; Jin et al., 2003; Wang et al., 2002), MAP kinase (Eliceiri et al., 1998), Jun kinase (Chen et al., 1999) and PKC (Friedlander et al., 1995; Ishida et al., 1997) thereby cooperating with growth factor signalling to control cell survival and proliferation.

Modifications to the cell cytoskeleton mediate changes in cell morphology and allow cells to migrate. The activation of integrins and the Rho GTPases, Cdc42 and Rac1, results in cell spreading and the formation of lamellopodia and filopodia by the reorganisation of actin (Hall, 1998; Price et al., 1998). In addition the activation of Rho, in a signalling pathway that does not involve Rac, results in the formation of actin stress fibres (Clark et al., 1998b). Endothelial cell migration requires the turnover of focal adhesions, which involves the phosphorylation of p^{125} FAK via VEGF receptor-2, HSP90 (heat shock protein 90) and RhoA-ROCK (Le Boeuf et al., 2004). The interaction between VEGF receptor-2 and $\alpha v\beta 3$ is required for the full activation of FAK. Inhibition of HSP90 by geldanamycin however impairs FAK phosphorylation. HSP90 is required as a chaperone protein to bind to VEGF receptor-2 to facilitate the phosphorylation of FAK on tyrosine 407. This then promotes the recruitment of paxillin and vinculin to build up new focal adhesions. In addition Src activities are required to phosphorylate tyrosine 861 of FAK, which is required to recruit vinculin, but not paxillin, to FAK.

The adherence of endothelial cells to vitronectin, a ligand for $\alpha v\beta 3$ integrin, increases VEGF-induced tyrosine phosphorylation of VEGF receptor-2 and augments the mitogenicity of VEGF as shown by an increased rate of proliferation of stimulated endothelial cells in the BrdU assay (Soldi et al., 1999). The development of a $\beta 3$ antibody, which does not interfere with endothelial cell adhesion to vitronectin or VEGF binding to the surface of endothelial cells, enabled Soldi et al to study the role of this integrin subunit. The addition of this antibody resulted in reduced tyrosine phosphorylation of VEGF receptor-2, and of the p85 subunit of PI3K, inhibition of the kinase activity of PI3K, a decrease in the number of migrating and polarised endothelial cells in response to VEGF, and a decrease in the rate of VEGF-induced proliferation of endothelial cells. Adhesion to the extracellular matrix induces the PI3K-dependent activation of Akt and the over-expression of either a constitutively active PI3K or a mutant of Akt inhibits detachment-induced apoptosis of epithelial cells.

The $\alpha v\beta 3$ integrin in particular is required for the survival and migration of endothelial cells. The growth factor- and integrin-mediated signalling pathways in endothelial cells appear to overlap and are probably integrated to ensure efficient signalling in response to growth factors and to changes in the adhesion status of cells.

The $\alpha v\beta 3$ integrin has been shown to cooperate with VEGF receptor-2 and positively influence VEGF receptor-2 activity. The $\beta 3$ integrin subunit has been shown to co-immunoprecipitate with VEGF receptor-2 in VEGF-stimulated endothelial cells and in resting and stimulated CHO cells transfected with αv , $\beta 3$ and VEGF receptor-2 (Soldi et al., 1999). In cells over-expressing αv and $\beta 3$ subunits this association is independent of activation of VEGF receptor-2 however in HUVE cells the VEGF-induced clustering of integrins at focal adhesions is required to increase their local concentration before association with VEGF receptor-2 is detected. By using either a mutant of $\beta 3$, lacking the cytoplasmic domain, or chimeric integrins, containing the extracellular domain of $\beta 1$ and the transmembrane and cytoplasmic domains of $\beta 3$, the association of $\beta 3$ with VEGF receptor-2 was shown to be dependent on its extracellular domain.

In contrast to the results discussed above, Reynolds et al have generated $\beta 3$ deficient mice that show elevated VEGF receptor-2 signalling and enhanced tumour and VEGF-specific angiogenesis (Reynolds et al., 2002). They present results showing that $\beta 3$ deficient cells have increased levels of VEGF receptor-2, increased phosphorylation of the receptor and of ERK and the ability to migrate and proliferate to a greater extent when compared to wild type cells. From this they propose that $\beta 3$ integrins control the amplitude of VEGF responses by controlling VEGF receptor-2 levels or activity. In two papers this group have shown that pathological angiogenesis and tumour growth is enhanced in $\beta 3$ integrin deficient mice or $\beta 3/\beta 5$ integrin doubly deficient mice and the later of the two papers attributes this to an increase in VEGF receptor-2 signalling (Reynolds et al., 2004). In consideration of the fact that the binding of $\beta 3$ to vitronectin has been shown to enhance VEGF receptor-2 signalling in response to VEGF, Reynolds et al suggest some situations in which $\beta 3$ may negatively regulate angiogenesis. It is possible that the binding of $\beta 3$ integrin to a ligand, other than vitronectin, reduces signalling through VEGF receptor-2, perhaps by enhancing the association of the receptor with phosphatases. In addition $\beta 3$ deficient mice do not respond to the endogenous angiogenesis inhibitor tumstatin and will not be affected by the initiation of

integrin-mediated death by ligand-free $\beta 3$ integrin. The absence of these mechanisms in $\beta 3$ null mice may therefore result in an increase in VEGF receptor signalling that supersedes the decrease in VEGF receptor activity due to $\beta 3$ binding to vitronectin. Further investigation is required to determine how $\beta 3$ integrin transduces signals that depress the expression of VEGF receptor-2.

1.13.4 *Cadherins*

The vascular endothelial- (VE-) cadherin protein, an adhesive protein, is found in adherens junctions between endothelial cells. Contact-inhibited cells have a reduced response to growth factors when they reach confluency due to the establishment of intercellular contacts and the initiation of negative intracellular signals. Cadherins, and complexes containing cadherin and catenin proteins, cluster at intercellular junctions and interact with growth factor receptors and their effectors (Hoschuetzky et al., 1994). Vascular-Endothelial Cadherin (VE-Cadherin) associates with VEGF receptor-2 in VEGF-stimulated cells as shown by co-immunoprecipitation experiments (Grazia Lampugnani et al., 2003). Co-immunoprecipitation does not occur however in β -catenin null cells. Stimulation of these cells results in an increase in VEGF receptor-2 phosphorylation, and endothelial cell proliferation, compared to wild-type cells.

The treatment of densely seeded cells with a neutralising VE-cadherin antibody results in increased VEGFR-2 tyrosine phosphorylation suggesting that VE-cadherin is inhibiting cell growth in a cell-contact dependent manner (Grazia Lampugnani et al., 2003). The inactivation of VE-cadherin in mice results in the collapse of vascular development, and the truncation of the protein in cells results in the abolition of both PI3K/Akt activation and up-regulation of anti-apoptotic proteins (Carmeliet et al., 1999). Proteins involved in endothelial cell-cell adhesions therefore prevent the overgrowth of cells and may also contribute to cell survival.

1.14 - Temporary inactivation of receptor tyrosine kinases by protein tyrosine phosphatases

Protein Tyrosine Phosphatases (PTPs) act on receptor tyrosine kinases to dephosphorylate tyrosine residues thereby attenuating receptor signalling. This action can result in either the complete abrogation of all signals or the modulation of selective signalling pathways. A broad reduction in signalling can be achieved by targeting all tyrosine residues. This is easily

achieved if the regulatory tyrosine residue in the activation loop of the receptor is dephosphorylated as this renders the kinase inactive and prevents autophosphorylation. Through this transient mechanism receptor tyrosine kinases regulate the strength and duration of growth factor signals. Alternatively, the signalling capacity of the receptors can be modulated by the dephosphorylation of key tyrosine residues. By selectively targeting residues that are used as docking sites for downstream signalling molecules specific pathways can be inactivated. This method is used to finely tune signal transduction.

1.15 - Regulation of VEGF receptor signalling by phosphatases

Many protein tyrosine phosphatases have been identified in endothelial cells and some of these are involved in VEGF receptor signalling.

- Receptor protein tyrosine phosphatases (rPTPs) such as rPTP μ , rPTP β , vascular-endothelial PTP (HPTP β) and DEP-1 (rPTP η /CD148) are membrane-associated phosphatases and are expressed in endothelial cells (Fachinger et al., 1999; Gaits et al., 1995; Grazia Lampugnani et al., 2003; Sui et al., 2005).
- A non-receptor, protein tyrosine phosphatase, PTP ϵ , is also highly, constitutively and preferentially expressed in endothelial cells and negatively regulates endothelial cell proliferation (Thompson et al., 2001).
- The Src homology phosphatases, SHP-1 and SHP-2, are non-receptor PTPs, also known as PTP1c and PTP1d respectively, and they associate with VEGF receptors. SHP-1 is predominantly expressed in haemopoietic cells and SHP-2 is ubiquitously expressed (Feng and Pawson, 1994; Kroll and Waltenberger, 1997).
- HCPTP-A (Human cytoplasmic protein tyrosine phosphatase)/Red cell acid phosphatase-1/LMW-PTP (Low Molecular Weight Protein Tyrosine Phosphatase) is constitutively expressed in endothelial cells and associates with VEGF receptor-2 (Huang et al., 1999).

1.15.1 *DEP-1 is up-regulated in response to increased endothelial cell density*

VE-cadherin expression and clustering at intercellular junctions of confluent cells is accompanied by a strong reduction of VEGF receptor-2 phosphorylation and blocks the proliferative response of endothelial cells to growth factors (Grazia Lampugnani et al., 2003). VE-cadherin acts by forming complexes with VEGF receptor-2 to concentrate the receptors within close vicinity to junctional phosphatases. The high cell density-enhanced phosphatase

DEP-1/CD148 is a general receptor kinase phosphatase that can act on VEGF receptor-2. The induction of contact inhibited cell growth by VE-cadherin has added a degree of complexity to the role that this protein plays in VEGF signalling. Previously the association of VE-cadherin with VEGF receptor-2 was attributed to an increase in signalling through PI 3-kinase and improved cell survival (Carmeliet et al., 1999). The dephosphorylation of VEGF receptor-2 by DEP-1 may therefore appear to contradict this role. It is possible however that the phosphatase activity of DEP-1 is specific to selective tyrosine residues within VEGF receptor-2. A possible explanation for both endothelial cell survival and growth inhibition is that some signalling pathways may be inhibited by DEP-1 (e.g. the PLC γ 1 and MAP kinase pathways) whereas others may remain unaffected (e.g. the PI 3-kinase pathway).

1.15.2 Vascular endothelial PTP interacts with the Angiopoietin receptor Tie-2 but not VEGF receptor-2

Receptor type PTPs from subclass III, to which both VE-PTP and DEP-1 belong, contain fibronectin type III repeats. These protein domains can interact with the extracellular matrix (ECM), promote cell adhesion and transduce signals generated by cell-cell contacts. VE-PTP co-immunoprecipitates with and dephosphorylates the angiopoietin receptor Tie-2 but not VEGF receptor-2 (Fachinger et al., 1999). The Angiopoietin/Tie-2 system is important for sprouting angiogenesis and vascular re-modelling. Fachinger et al therefore conclude that VE-PTP is a Tie-2-specific phosphatase expressed in endothelial cells to specifically modulate Angiopoietin/Tie-2 function.

1.15.3 Inhibition of VEGF receptor-2-mediated cell proliferation through activation of SHP-1 by tumour necrosis factor- α

Kroll and Waltenberger identified SHP-1 and SHP-2 as phosphatases that were expressed in PAE cells and co-immunoprecipitated with phosphorylated VEGF receptor-2 in VEGF-stimulated cells (Kroll and Waltenberger, 1997). From this they speculated that these phosphatases participate in the modulation of VEGF-induced signals and were possibly involved in the negative regulation of receptor signalling.

SHP-1 and SHP-2 are cytosolic non-receptor-like phosphatases containing 2 SH2 domains and a catalytic domain. SHP-1 can be a negative regulator of signalling through interactions with the erythropoietin receptor, T-cell antigen receptor and c-Kit (Schmidt et al., 1998; Sharlow et al., 1997; Yi and Ihle, 1993) and also a positive regulator of signalling acting

through src kinase (Roskoski, 2005). SHP-1 interacts with receptors via its SH2 domains and both domains are involved in the interaction of SHP-1 with the EGF receptor (Keilhack et al., 1998). SHP-1 is primarily expressed in haemopoietic tissue but is also expressed in endothelial cells as these cells have a common lineage.

Further to the studies by Kroll and Waltenberger, TNF (Tumour Necrosis Factor) was found to have a negative effect on VEGF receptor-2 phosphorylation in HUVE cells (Guo et al., 2000). TNF acutely inhibits the ability of VEGF to activate VEGF receptor-2 and MAP kinase and inhibits DNA synthesis in endothelial cells. These effects of TNF can be reversed by sodium orthovanadate, a tyrosine phosphatase inhibitor, leading the investigators to the hypothesis that a TNF activated protein tyrosine phosphatase impairs the ability of VEGF to activate VEGF receptor-2. The immunoprecipitation of VEGF receptor-2 from HUVE cells showed that a brief stimulation of cells with either VEGF or TNF could induce the association of SHP-1 with VEGF receptor-2. In addition the pre-treatment of cells with TNF for 3 hours followed by a 5 minute-stimulation with VEGF resulted in an increased association of SHP-1 with VEGF receptor-2. Nakagami et al also obtained many of the same results and also confirmed the ability of TNF α to act through SHP-1 by examining the effects of expressing a dominant negative SHP-1 protein in endothelial cells (Nakagami et al., 2002). The reduction in phosphatase activity of the dominant negative SHP-1 protein was shown using a PTP assay in which ^{32}P orthophosphate release from radiolabelled myelin basic protein is measured. This illustrated a reduction in SHP-1 activity in unstimulated cells, and only a slight increase in activity when cells were stimulated with TNF α . The over-expression of dominant negative SHP-1 reversed the inhibition of VEGF and EGF-induced ERK phosphorylation, as seen in cells pre-treated with TNF α and attenuated the effects of TNF α on thymidine incorporation and c-fos promoter activity. These observations provide one example of the activation of one receptor system communicating with, and impairing the activity of another, and identify SHP-1 as a mediator for the negative role of TNF in VEGF signal transduction.

1.15.4 *SHP-2 associates with phosphotyrosine residue 1213 in activated VEGF receptor-1*

VEGF stimulation of VEGF receptor-1 results in a small increase in its kinase activity and weak phosphorylation. The weak tyrosine phosphorylation of VEGF receptor-1 may be due to either the phosphorylation of few tyrosine residues with low stoichiometry or the tight

regulation of VEGF receptor-1 by phosphatases. By over-expressing VEGF receptor-1 six phosphorylation sites have been mapped to the intracellular domain of the receptor, four of which interact with downstream signalling molecules.

SHP-2 is a ubiquitously expressed protein tyrosine phosphatase and Kroll and Waltenberger showed its expression in endothelial cells by western blotting in PAE cells (Kroll and Waltenberger, 1997). Ito et al expressed a constitutively active VEGF receptor-1 protein using a baculovirus system and identified two major tyrosine phosphorylation sites, Tyr-1213 and Tyr-1242, two minor sites at Tyr-1327 and 1333 and showed the binding of SH2 domain-containing proteins to these residues (Ito et al., 1998). SHP-2 is able to co-immunoprecipitate with wild-type VEGF receptor-1, but not a receptor mutated at tyrosine 1213, and a yeast two-hybrid study showed that tyrosine 1213 of VEGF receptor-1 is a binding site for the N-terminal SH2 domain of SHP-2. In PAE cells over-expressing wild-type VEGF receptor-1 SHP-2 is phosphorylated in response to VEGF and also in response to PlGF, but to a lesser extent. The consequences of SHP-2 binding to VEGF receptor-1 is not known however we can speculate that as a phosphatase it may dephosphorylate VEGF receptor-1. In a recent study, comparing signal transduction via wild type and mutant VEGF receptor-1 proteins, Ito et al noted that VEGF receptor-1 containing an Y1213F mutation was kinase inactive. If the role of SHP-2 is to bind to and dephosphorylates tyrosine 1213 this could provide a mechanism to abrogate all VEGF receptor-1-mediated signals. Alternatively SHP-2 could mediate the indirect binding of Grb2 to VEGF receptor-1 in agreement with previous studies showing indirect binding of Grb2 to the PDGF receptor.

1.15.5 HCPTP-A/LMW-PTP regulates VEGF receptor-mediated cell migration and proliferation

LMW-PTP dephosphorylates and inactivates receptor tyrosine kinases, including the PDGF, insulin and ephrin receptors and docking proteins such as β -catenin (Chiarugi et al., 2004; Kikawa et al., 2002; Shimizu et al., 2001; Taddei et al., 2002; Taddei et al., 2000). In a yeast two-hybrid screen HCPTP-A (also known as LMW-PTP) was found to bind specifically to the autophosphorylated intracellular domain of VEGF receptor-2 but not to a mutated, kinase inactive domain (Huang et al., 1999). The details of the association are not clear as LMW-PTP lacks any identifiable phosphotyrosine binding domains. It can however associate with phosphotyrosine residue 857 in the activation loop of the PDGF receptor via its catalytic domain (Chiarugi et al., 2002). It is possible therefore that LMW-PTP binds to the activation

loop tyrosine in the kinase domain of VEGF receptor-2. At present, the evidence supporting this is that a recombinant GST fusion of the VEGF receptor-2 kinase domain, when incubated with recombinant LMW-PTP, is dephosphorylated in a manner that is proportional to the concentration of the phosphatase. In addition, VEGF receptor-2 phosphorylated *in vivo* in an endothelial cell line was also dephosphorylated *in vitro* after incubation with recombinant LMW-PTP or adenoviral LMW-PTP. Also in endothelial cells adenovirus-mediated over-expression of LMW-PTP impaired VEGF-mediated VEGF receptor-2 autophosphorylation, MAP kinase activation and endothelial cell migration and survival/proliferation. This data indicates that LMW-PTP is a negative regulator of VEGF receptor-2 phosphorylation and signal transduction. In the rat aortic ring assay inhibition of angiogenesis was evident from a reduction in the number and length of vascular sprouts from segments of rat aorta infected with LMW-PTP compared to those infected with mock virus. This result may well be a result of inhibited VEGF receptor signalling however we know from other studies that the PDGF receptor is a substrate for LMW-PTP and these receptors are also expressed in endothelial cells. In addition LMW-PTP does not interact with VEGF receptor-1, but interacts with Tie-2 in a yeast two-hybrid system, suggesting some specificity in the action of LMW-PTP on endothelial signalling pathways. Hence the inhibition of angiogenesis in the aortic ring assay may be due to the negative regulation of multiple, but specific, pathways.

1.16 - Sequestration of receptors from the plasma membrane negatively regulates signalling

Many areas of the plasma membrane in mammalian cells form flask-shaped pits that are typically 55-65nm in diameter. These structures were first visualised in the 1950s (Yamada, 1955) and further studies have revealed that they are composed of cholesterol and sphingolipids (Stan, 2002) and are characterised by a major constituent, caveolin-1 (Rothberg et al., 1992). Desensitisation by sequestration in caveolar vesicles has been reported for the EGF receptor, protein kinase C α , the norepinephrine transporter and the β -1 adrenergic receptor (Jayanthi et al., 2004; Matveev and Smart, 2002; Prevostel et al., 2000; Steinberg, 2004). Caveolin-1, the defining protein of caveolae, has been labelled as a general kinase inhibitor because its association with kinases such as c-Src/Fyn, EGFR, Neu, PKA and PKC holds them in an inhibited state in quiescent cells (Smart et al., 1999). This mechanism of suppressing activation differs from the action of phosphatases; the receptors are not modified

by caveolin-1, but are removed from their preferred signalling location and their activity is suppressed.

Matveev et al show that in cells stimulated with EGF for a short length of time (10 minutes) the EGF receptor is phosphorylated and localised in lipid rafts (Matveev and Smart, 2002). If stimulation is sustained for a longer time (60 minutes) the receptor is dephosphorylated and localised to caveolae. The sequestration of the receptor into a compartment that is inaccessible to external reagents prevents ligand-receptor interactions and therefore desensitises the cell. In these studies the PDGF receptor behaved in a similar manner and the desensitisation of one receptor type, the EGF receptor, caused the desensitisation of another, the PDGF receptor, a mechanism that they termed transmodulation. A resensitisation assay showed that this is a reversible process as receptors re-exposed to the extracellular environment were capable of binding ligands. The localisation of the receptor therefore depends on the length of time that the ligand is bound.

Caveolae are a ubiquitous feature of endothelial cells as they make up 95% of the cell surface and approximately 15% of endothelial cell volume (Predescu and Palade, 1993). VEGF receptor-2 is localised in endothelial caveolae and association with caveolin-1 negatively regulates its activation in unstimulated cells (Labrecque et al., 2003). This association has been shown by co-immunoprecipitation studies and is likely to be mediated by the scaffolding domain of caveolin-1. Additionally Ikeda et al show that the VEGF receptor-2-caveolin-1 complex also includes the small GTPases ARF6 and Rac1 (Ikeda et al., 2005). The inhibitory action of caveolin-1 is circumvented by its phosphorylation on tyrosine 14 in response to VEGF, which is mediated by Src and/or ARF6. The phosphorylation of VEGF receptor-2, and of a minor fraction of caveolin-1, results in the movement of the phospho-receptor and of phospho-caveolin-1 out of caveolae/lipid rafts and into vesicles localised to the edge of lamellopodia. These vesicles co-localise with vinculin indicating that they are focal complexes/adhesions. ARF6 has been shown to be important in the proper localisation of activated VEGF receptor-2 to these focal complexes and in consequential cell migration and proliferation. Phospho-caveolin-1 can interact with Grb7, LMW-PTP and Csk so may participate as a scaffolding protein in VEGF-mediated signalling.

As in other cell type's caveolin-1 is required to regulate the cholesterol content of endothelial plasma membranes. The absence of caveolae is noted in transformed cells and the disruption

of the caveolin-1 gene results in uncontrolled endothelial cell proliferation and suppression of capillary-like tube formation. Cholesterol depletion of endothelial cells removes caveolin-1 and VEGF receptor-2 from caveolae and results in increased basal and VEGF-induced phosphorylation of VEGF receptor-2 (Labrecque et al., 2003). In addition VEGF receptor-2 activation is prolonged and phosphorylation of PLC γ 1 is enhanced. In contrast however, VEGF-induced ERK activation is inhibited, as is endothelial cell migration, suggesting that the localisation of VEGF receptor-2 to caveolae is crucial for VEGF-mediated signalling. In the inverse situation, the over-expression of caveolin-1 blocked VEGF-dependent activation of Elk promoter activity. The endothelial nitric oxide synthase (eNOS) protein is enriched in caveolae and associates with the scaffolding domain of caveolin-1 (Li et al., 1996). This interaction holds eNOS in an inactive state therefore over-expression of caveolin-1 or its scaffolding domains results in a decrease in eNOS-dependent nitric oxide release (Bucci et al., 2000). The nitric oxide generated by eNOS is important for maintenance of systemic blood pressure, vascular remodelling, angiogenesis and wound healing.

1.17 - Down-regulation of receptor tyrosine kinases by an irreversible mechanism

A definitive mechanism used to attenuate receptor tyrosine kinase signalling is activation-dependent receptor degradation. Removal of the protein not only terminates the signalling in the short term it also generates a refractory time period during which the cell is unable to transmit another signal. This mechanism is initiated by the ubiquitination of activated receptors by ubiquitin ligase enzymes at the plasma membrane, which triggers their internalisation. This is then followed by the sorting of receptors into early endosomes, late endosomes and multivesicular bodies and concludes with the degradation of receptors in lysosomes (Dikic, 2003; Waterman and Yarden, 2001).

Internalisation, by clathrin coated pit- or caveolae-mediated endocytosis, is a mechanism used to remove receptors from the cell surface and is prerequisite for receptor degradation either in the lysosome or the proteasome. The internalisation of a receptor however is not sufficient to result in receptor inactivation. As discussed above inactivation may be transient, through either receptor dephosphorylation or recycling, or permanent by means of receptor degradation. It is possible therefore that signals from an endocytosed ligand-receptor complex are sustained, or new signals generated, before the receptor is inactivated. The epidermal

growth factor (EGF) receptor remains active for a considerable length of time after endocytosis and is hyperphosphorylated when compared to surface EGF receptors (Burke et al., 2001; McPherson et al., 2001; Wiley and Burke, 2001). In addition the association of proteins with EGF receptor is determined by the location of the receptor within the cell (Baass et al., 1995; Burke et al., 2001; Oksvold et al., 2000; Oksvold et al., 2001). If internal complexes can initiate signal transduction then intracellular trafficking may not be just a means of receptor regulation but may also control signal specificity.

Several reports suggest that MAP kinase activation is sustained from internalised receptor complexes (Oksvold et al., 2001; Pol et al., 1998; Qiu et al., 2004; Rizzo et al., 2001) and an alternative route is applied for the down-regulation of this pathway. The inhibition of insulin receptor internalisation, for example, results in a partial inhibition of Shc phosphorylation and MAP kinase down-regulation (Ceresa et al., 1998). The internalisation of insulin receptors may therefore have a positive affect on MAP kinase signalling. The hyper-phosphorylation of internalised receptors has been reported for EGF and PDGF receptors (Kapeller et al., 1993; Lai et al., 1989; Wada et al., 1992). This may be an indication that the phosphatases that act on cell surface receptors do not dephosphorylate those that have been internalised. The inactivation of MAP kinase signalling is achieved by the direct dephosphorylation of p42 and p44 MAP kinases by specific phosphatases (Camps et al., 2000).

1.17.1 The ubiquitination of receptors targets them for degradation

Several receptor tyrosine kinases, once activated, are covalently modified by the addition of ubiquitin molecules (Haglund et al., 2003). This is the initiating event in an irreversible down-regulatory mechanism as ubiquitin molecules label proteins for internalisation, followed by, either lysosomal or proteasomal degradation (Huibregtse, 2005; Mosesson et al., 2003). Phosphotyrosine residues found in activated receptors can be docking sites for ubiquitin ligase enzymes, which catalyses the conjugation of ubiquitin molecules to the targeted protein. Ubiquitination occurs through the action of E1, E2 and E3 classes of ubiquitin ligase proteins. E1 is the ubiquitin-activating enzyme that activates ubiquitin in an ATP-dependent manner. The activated ubiquitin is then transferred to E2, the ubiquitin-conjugating enzyme. E3 is the enzyme that denotes the specificity of the protein that is to be ubiquitinated as it transfers the ubiquitin molecule from the E2 enzyme to the substrate. The addition of multiple individual ubiquitin molecules (multi-monoubiquitination) initiates membrane protein internalisation and targets them for endosomal sorting, resulting in either receptor recycling or lysosomal

degradation. The alternative to monoubiquitination is the addition of chains of ubiquitin molecules (polyubiquitination) and this targets proteins to the proteasome resulting in protein unfolding and degradation. The sustained attachment of ubiquitin to receptors is essential for degradation, as it targets receptors in multivesicular bodies to the lysosome. If ubiquitin has been removed from the receptor during the trafficking of early endosomes to multivesicular bodies then the receptor follows the default pathway, and is recycled back to the plasma membrane (Katzmann et al., 2002).

The E3 family of ubiquitin ligases can be divided into 2 classes based on structural differences. The Cbl (Casitas B lineage lymphoma) family of ubiquitin ligases (c-Cbl, Cbl-b and Cbl-c/Cbl-3) contain RING (really interesting novel gene) finger domains that catalyse the direct transfer of ubiquitin from the E2 enzyme to the substrate. Cbl ligases contain a phosphotyrosine-binding domain for the direct binding to receptors but they can also bind indirectly through an association with Grb2.

Ubiquitination of VEGF receptor-2 by Cbl has been implicated in the down-regulation of the receptor in BAE cells (Duval et al., 2003). Cbl associates with VEGF receptor-2 and is tyrosine phosphorylated in response to VEGF stimulation of BAE cells. The production of NO is inhibited by the down-regulation of VEGF receptor-2 following sustained VEGF stimulation. The pre-treatment of BAE cells with VEGF desensitises cells, prevents further activation of VEGF receptor-2, Akt and endothelial nitric oxide synthase (eNOS) and hence inhibits NO production. The co-expression of VEGF receptor-2, Cbl and eNOS resulted in an inhibition of NO release from VEGF stimulated cells when compared to the NO release from cells expressing only the receptor and eNOS.

The role of Cbl has also been studied following integrin and VEGF receptor activation by shear stress (Wang et al., 2004; Wang et al., 2002). VEGF receptor-2 and Cbl association is increased by shear stress and this can occur independently of VEGF ligand binding. Cbl association is also induced in BAE cells plated on integrin ligands, vitronectin or fibronectin. The association of Cbl with VEGF receptor-2 could occur via two interactions, either Grb2 acts as a scaffold protein to couple the two proteins, or the PTB domain of Cbl interacts directly with phospho-tyrosine residues in VEGF receptor-2. The association of Cbl with VEGF receptor-2 is important for signalling to Akt and IKK (Inhibitor of Kappa-B Kinase). SU-1498, a VEGF receptor inhibitor, and a negative mutant of Cbl inhibit IKK activation,

which regulates NF kappa-B translocation to the nucleus. Cbl can bind to other adapter proteins e.g. p85, Grb2, Shc, Crk and Nck, and it is proposed that in this case Cbl recruits PI 3-kinase via p85 to activate Akt, which then activates IKK. VEGF receptor-2 can therefore form complexes upon shearing, or VEGF applications, and the phosphorylation of Cbl can initiate receptor degradation and signalling to IKK.

The second class of ligase contains a C-terminal HECT (homologous to E6-AP COOH terminus) catalytic domain. This domain includes a conserved cysteine residue to which the ubiquitin is transferred from E2 to create a high-energy intermediate. The activated ubiquitin moiety is then transferred to a lysine side chain ϵ -amino group in the ligase bound substrate. The Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) ubiquitin ligase E3 is a member of this family. The Nedd4 family of ligases contain several, usually three or four WW domains, which are protein-protein interaction modules containing two conserved tryptophan residues (Ingham et al., 2004). These domains are responsible for substrate recognition and bind to proline rich regions, including specific PPXY and PPLP motifs, and phosphoserine and phosphothreonine residues that precede a proline residue (Lu et al., 1999). In addition Nedd4 ubiquitin ligases contain a C2 domain, a calcium dependent phospholipid binding-domain (Plant et al., 1997). C2 domains function as membrane recruitment domains and have been shown to interact with a variety of phospholipids and proteins. This domain is therefore important for protein localisation.

It is possible that Nedd4 binds directly to receptor tyrosine kinases and transfers ubiquitin to these substrates (Rotin et al., 2000). Nedd4 has been shown to directly interact with a PPSY motif in the notch receptor and PY motifs in voltage gated sodium channels (Farr et al., 2000) however it may also bind indirectly to receptors via Grb10. Nedd4 has been implicated in the ligand-induced ubiquitination of the insulin-like growth factor-I (IGF-I) receptor (Vecchione et al., 2003). Grb10 interacts with Nedd4 in a phosphotyrosine and calcium independent manner and is not ubiquitinated (Morrione et al., 1999). This provided the first indications that Grb10 might act as an adapter protein to localise Nedd4 to its target proteins to promote their ubiquitination. Nedd4 and Grb10 have also been implicated in the degradation of VEGF receptor-2 however in this case, although Nedd4 is involved in VEGF receptor-2 degradation, it is not involved in its ubiquitination (Murdaca et al., 2004). This group also propose that Cbl ubiquitinates VEGF receptor-2. Grb10 binds constitutively to Nedd4 thereby inhibiting

Nedd4-mediated VEGF receptor degradation. Nedd4 may act to promote VEGF receptor-2 degradation by the ubiquitination of Eps15, a protein involved in the endocytic pathway.

The E3 ubiquitin ligases are active throughout the whole endocytosis process and Cbl remains associated with activated receptor tyrosine kinases throughout the endosomal compartments. The over-expression of Cbl results in an increase in EGF receptor degradation but not its rate of internalisation (Duan et al., 2003). This suggests that the ubiquitination of receptors is required primarily for receptor sorting after their internalisation from the plasma membrane. Multiple cycles of Cbl-mediated mono-ubiquitin transfer and the oligomerisation of CIN85-Cbl complexes results in the clustering of mono-ubiquitinated receptors and associated proteins (Haglund et al., 2002). This targets the receptor, as well as all of the proteins involved, for lysosomal degradation. Eps15, epsin and Hrs for example are ubiquitinated by Nedd4, and Endophilin is ubiquitinated by Itch (Angers et al., 2004; Katz et al., 2002; Polo et al., 2002). Cbl protein levels are also partially regulated by ubiquitination, which results in either the targeted degradation of Cbl or the co-ordinate degradation of Cbl with targeted proteins (Courbard et al., 2002; Magnifico et al., 2003). Nedd4 and HECT family ligases are probably activated in response to receptor tyrosine kinase-mediated signals in order to create these large multiply mono-ubiquitinated cargo complexes. The stoichiometry of multiple mono-ubiquitin molecules may correlate with the dynamics of receptor endocytosis, i.e. more mono-ubiquitin molecules may signal for a faster rate of degradation.

Down-regulation of VEGF receptor-2 is dependent on its C-terminal tail. Meyer et al used chimeric receptors; containing the transmembrane and cytoplasmic domains of VEGF receptor-1 or VEGF receptor-2 fused to the extracellular domains of CSF-1R/c-fms, to show that ligand dependent autophosphorylation and degradation of the VEGF receptor-2 chimera is impaired when tyrosines 1212 and 1175 are removed by the partial deletion of its C-terminal tail (Meyer et al., 2004b; Singh et al., 2005).

By comparison the VEGF receptor-1 chimera shows very poor ligand-stimulated phosphorylation, no significant receptor degradation and down-regulation and the deletion of the C-terminal tail does not improve this (Meyer et al., 2004a). Maximal activity of VEGF receptor-2 is required for its down-regulation (Dougher and Terman, 1999) and since VEGF receptor-1 demonstrates poor kinase activity it is possible that it does not undergo ligand-dependent down-regulation. The replacement of the C-terminal tail of VEGF receptor-1 with

that of VEGF receptor-2 results in ligand-dependent autophosphorylation and increased down-regulation similar to that seen for the VEGF receptor-2 chimera (Meyer et al., 2004a). This region of VEGF receptor-1 therefore restrains the ligand-dependent kinase activation and down-regulation of the receptor and may not contain the motif that directs VEGF receptor-2 to degradation. If VEGF receptor-1 has a role in sequestering VEGF, to act as a negative regulator of angiogenesis, then it is conceivable that it undergoes ligand-dependent recycling rather than down-regulation and degradation.

Kobayashi et al and Carter et al have showed down-regulation of phosphorylated VEGF receptor-1. The former group propose that Cbl is involved in this degradation and have shown recruitment of a Cbl complex to tyrosine 1333 of VEGF receptor-1 (Kobayashi et al., 2004). They also show that VEGF stimulation enhances this association, phosphorylates Cbl, ubiquitinates the receptor and results in receptor internalisation. The identification of a WW domain binding-site in VEGF receptor-1 however suggests that Nedd4 could bind directly to this receptor and may mediate its ubiquitination and degradation. Furthermore as associations via WW domains are independent of phosphotyrosine residues this mechanism would not require the kinase activity of the receptor.

1.17.2 A phosphoserine residue could target VEGF receptor-2 for degradation, an alternative to ubiquitination.

Singh et al propose that the down-regulation of VEGF receptor-2 is mediated by a distinct mechanism that requires tyrosine kinase activity of the receptor and involves non-classical protein kinase C (PKC) (Singh et al., 2005). In this model ubiquitin is not the indicator for internalisation and degradation, instead a phosphoserine residue is used. As seen previously down-regulation of VEGF receptor-2 is dependent on maximal receptor activation and turnover of a kinase dead chimeric receptor was inefficient, compared to that of the wild type chimera, in response to ligand stimulation. In this case the turnover of the mutant receptor was similar to that of the wild-type receptor in the absence of ligand. In their model Singh et al propose that selective activation of VEGF receptor-2 accelerates the rate of degradation because it activates isozymes of PKC. The specific PKC proteins involved however are not those that are dependent on PLC γ 1 activity as receptors that are unable to recruit and activate PLC γ 1 undergo normal down-regulation. 12-O-tetradecanoyl phorbol-13-acetate (TPA) is a phorbol ester that mimics the action of diacylglycerol and activates PKC. Treatment of PAE cells expressing VEGF receptor-2 with TPA induced receptor down-regulation with kinetics

similar those obtained from stimulation with VEGF indicating a role for PKC in this process. The use of TPA to directly activate PKC bypasses the requirement for VEGF receptor activation and as kinase dead chimeric receptors undergo accelerated down-regulation in response to short term TPA treatment this suggests that PKCs promote the turnover of receptors but does not increase receptor kinase activity. By generating receptors that lacked regions of the C-terminal tail and deletion mutants and point mutations within the receptor a region of 39 amino acids immediately C-terminal to the kinase domain and two serine residues within this region were identified as necessary for efficient receptor down-regulation. Serine 1188 is likely to play a major role, and Serine 1191 a minor role, in regulating ligand-dependent down-regulation of VEGF receptor-2. These observations suggest that a positive feedback mechanism could exist in that activation of non-classical PKC isozymes directs phosphorylation of serine residues in the C-terminal domain of VEGF receptor-2 thereby marking the receptor for internalisation and proteasomal degradation.

1.18 - Summary and Objectives

VEGF- A_{165} -induced signalling involves a multitude of receptors, adapter proteins, signalling enzymes and structural proteins for the integration of numerous signalling pathways and cell processes. In this study we investigate the involvement of PI3K in VEGF-initiated cell signalling. We aim to elucidate the cellular localisation of the p85 adapter protein subunit, and that of the lipid product of the PI3K catalysed reaction, in VEGF stimulated endothelial cells. We also aim to confirm that ShcB/Sck, a recently identified component of VEGF receptor-2 signalling complexes, is an adapter protein used for VEGF receptor signalling. For these purposes we aim to validate, primary endothelial cells and endothelial cell lines for their capacity to mediate VEGF receptor-2 signalling and show that VEGF signals can be transduced via recognized pathways in these cells. The endothelial cells chosen will therefore provide us with a representative model in which fundamental VEGF signalling mechanisms are conserved. In these cells we hope to confidently identify a role for PI3K and Sck in VEGF signalling. In addition, with regards to Sck, we aim to attribute function(s) to the SH2 and PTB domains and also to the conserved tyrosine phosphorylation residues, located within Grb2 binding sites.

The second aim is to identify the route of VEGF receptor-2 trafficking during VEGF- A_{165} -induced degradation. This has been studied extensively for other receptor tyrosine kinases so

we hope to determine whether VEGF receptor-2 follows the conventional endosomal route. In addition the ubiquitin ligases, Cbl and Nedd4, mediate the degradation of activated receptors and proteins involved in the endocytic process. Nedd4 has been identified as a potential mediator of VEGF receptor-1 degradation and by using a chimeric receptor system we aim to confirm the interactions between Nedd4 and VEGF receptor-1 and identify any consequences of this.

Chapter 2

Materials and Methods

Chapter 2 - Materials and Methods

2.1 - Suppliers

AbCam, Cambridge, UK

Amersham, Little Chalfont, Buckinghamshire, UK

Astrazeneca, Alderley Park, Cheshire, UK

BD Biosciences, Cowley, Oxfordshire, UK

Biogenesis, Poole, Dorset, UK

Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK

Cell Signalling Technologies, Beverly, MA, USA

Chemicon International, Chandlers Ford, Hampshire, UK

Clontech, Palo Alto, CA, USA

First Link, Brierley Hill, Birmingham, UK

Flowgen, Ashby de la Zouch, Leicestershire, UK

Gibco BRL Life Technologies, Paisley, UK

Invitrogen, Paisley, UK

Jackson Immunoresearch Laboratories, West Grove, PA, USA

Life Technologies, Paisley, UK

New England Biolabs, Hitchin, Hertfordshire, UK

Oxoid, Basingstoke, Hampshire, UK

Pierce, Cramlington, Northumberland, UK

Promega, Southampton, UK

Qiagen, Crawley, West Sussex, UK

R&D Systems, Abingdon, Oxon, UK

Roche Diagnostics, Lewes, East Sussex, UK

Santa Cruz Biotechnology, Santa Cruz, CA, USA

Schleicher & Schuell, Dassel, Germany

Sigma GenoSys, Pampisford, Cambridge, UK

Southern Biotechnology, Birmingham, AL, USA

Stratagene, West Cedar Creek, TX, USA

Tebu-bio, Peterborough, Cambridgeshire, UK

UK HGMP Resource Centre, Cambridge, UK

Upstate Biotechnology, Milton Keynes, UK

2.2 - Materials

2.2.1 *General chemicals and reagents*

All chemicals were of analytical grade and were obtained from Sigma or Fisher Scientific unless otherwise stated.

2.2.2 *DNA plasmids and bacterial cultures*

Plasmids were obtained from the following individuals or companies: pMT2-PLC γ 1 (Dr Matilda Katan, Institute of Cancer Research, London, UK), Shc, expression vector not known (Dr. Edward Skolnik, Skirball Institute, New York, USA), pcDNA3-GFP-p85 (Prof. William Gullick, University of Kent, Canterbury, Kent, UK) pcDNA3-HA-Cbl and pc-DNA3-HA-Cbl70Z (Prof. Yosef Yarden, Weizmann Institute of Science, Rehovot, Israel), pcDNA-HA-Nedd4 and pc-HA-Nedd4C744S (Dr. Allan Weissman, National Cancer Institute, Frederick, MD, USA), pEGFP-C1, pEGFP-N1 and pDsRed2-C1 (Clontech), pcDNA-DEST53 Gateway™ Vector (Invitrogen), pFLAG-CMV™-2 (Sigma), Bacterial image clone 1970831 (4841-k24) expressing mouse Grp-1 (UK HGMP Resource centre, Hinxton, Cambridge, UK), Bacterial R34739 (R23N6) LLNL Human Chromosome clone (MRC UK HGMP Resource centre, Babraham, Cambridge, UK).

2.2.3 *Molecular Biology materials*

Reagents used in experiments involving Molecular biology techniques included: Bacterial cell culture reagents (Oxide), synthetic oligonucleotides (Invitrogen and Sigma Genosys), QIAquick gel extraction and PCR purification kits (Qiagen), Restriction enzymes (NEB or Roche), T4 polynucleotide kinase (NEB), Taq (Promega), Expand high fidelity PCR system (Roche), QuikChange® site directed mutagenesis kit (Stratagene), dNTPs (Invitrogen).

2.2.4 *Tissue culture materials*

PAE/VEGF receptor-2 cells were obtained from Dr. Lena Claesson-Welsh (University of Uppsala, Uppsala, Sweden), NIH3T3/HER4 cells were obtained from Prof. William Gullick (University of Kent, Canterbury, Kent, UK), HEK293 cells were a gift from Dr. Terry Herbert (University of Leicester, Leicester, UK), HUVE cells were isolated from umbilical cords obtained from Leicester Royal Infirmary (Leicester, UK), For the maintenance of mammalian cell cultures the following reagents were used: Cell culture media, foetal calf serum, penicillin and streptomycin, OptiMEM® (GIBCO BRL life technologies), endothelial cell growth

supplement – ECGS (Tebu Bio), FuGENE™6 transfection reagent (Roche Diagnostics), VEGF (Tebu Bio) Heregulin (R & D systems), G418 sulphate (GIBCO BRL life technologies), gelatin, collagenase and heparin (Sigma).

2.2.5 *Protein biochemistry materials*

Reagents used in experiments involving protein manipulations included: Protease inhibitor cocktail (Sigma), Protein G Sepharose beads (Sigma), ProtoFlowGel (30% w/v acrylamide, 0.8% w/v bisacrylamide solution, Flowgen) protein molecular weight markers (Amersham and Bio-rad), nitrocellulose transfer membrane (Schleicher and Schuell) Bovine Serum Albumin (BSA, First Link). The suppliers of antibodies for western blotting and immunofluorescence are detailed in Tables 2.1, 2.2 and 2.3.

2.2.6 *Imaging materials*

Hoechst 33342 (Sigma), DABCO (1,4-Diazabicyclo(2,2,2)octane, sigma)

2.3 - Solutions

2.3.1 *Common buffers*

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.4

Tris buffered saline (TBS): 50 mM Tris, 15 mM NaCl; pH 7.4

2.3.2 *Bacterial cell culture and DNA manipulations*

Luria-Bertani medium (LB): Bacto-tryptone 10g/L, Bacto-yeast extract 5g/L, NaCl 10g/L; pH 7.0

LB Agar: Bacto-trytone 10g/L, Bacto-yeast extract 5g/L, NaCl 10g/L, Bacto-Agar 15g/L; pH 7.0

SOC medium: Bacto-trytone 20g/L, Bacto-yeast extract 5g/L, 10 mM NaCl, 25 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose; pH 7.0

SOB medium: Bacto-trytone 20g/L, Bacto-yeast extract 5g/L, 8.6 mM NaCl, 25 mM KCl; pH 7.0

TNE: 10 mM Tris, 100 mM NaCl, 1 mM EDTA; pH 8.0

Alkaline lysis buffer I: 50 mM Glucose, 10 mM EDTA, 25 mM Tris, 100 µg/ml RNase; pH 7.5

Alkaline lysis buffer II: 0.2 M NaOH, 1 % w/v SDS

Alkaline lysis buffer III: 3 M potassium acetate, 5M glacial acetic acid; pH5.5

Tris EDTA (TE): 10 mM Tris, 1 mM EDTA; pH 7.4

50X TAE: 2 M Tris, 1 M sodium acetate, 0.05 M EDTA; pH8.2

3x Agarose gel loading buffer: 5 mM EDTA, 30 % v/v glycerol, 0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol FF

2.3.3 *Western blotting*

Cell lysis buffer: 50 mM Tris, 1 % v/v Triton X-100, 5 mM EGTA, 150 mM NaCl, 25 mM benzamidine; pH 7.4

3x laemmli sample buffer: 12 % w/v SDS, 36 % v/v glycerol, 150 mM Tris, 0.01 % w/v bromophenol blue; pH 6.8

Gel buffer: 3 M Tris, 0.3 % w/v SDS; pH 8.45

Resolving gel: 6-10 % w/v acrylamide, 0.15-0.26 % w/v bisacrylamide, 33.3 % v/v gel buffer, 11 % v/v glycerol, 0.05 % w/v ammonium persulphate (APS), 0.05 % v/v tetramethylenediamine (TEMED)

Stacking gel: 4 % w/v acrylamide, 0.1 % w/v bis-acrylamide, 25 % v/v gel buffer, 0.08 % w/v APS, 0.08 % v/v TEMED

Cathode buffer: 0.1 M Tris, 0.1 M tricine, 0.1 % w/v SDS; pH 8.25

Anode buffer: 0.2 M Tris; pH 8.9

Transfer buffer: 48 mM Tris, 39 mM glycine, 20 % v/v methanol, 0.0375 % w/v SDS

Blot strip buffer: 62.5 mM Tris, 2 % w/v SDS, 0.8 % v/v β -mercaptoethanol; pH 6.7

TBST: 1x TBS, 0.1 % v/v Tween 20

PBST: 1x PBS, 0.05 % v/v Tween 20

2.4 - Methods

2.4.1 Molecular Biology

2.4.1(i) *Amplification of DNA by Polymerase chain reaction*

To amplify sequences of DNA a double stranded DNA template was denatured at 94-95°C. An initial period of 5 minutes at this temperature ensures that all template DNA is denatured. The temperature was then lowered to 55-60°C to allow primers, containing sequences that flank the DNA sequence of interest, to anneal to the denatured template. The temperature was increased again to 72°C, which facilitates polymerase activity. Polymerases, such as Taq,

synthesise sequences that initiate from the oligonucleotide primers and are complementary to the template DNA. The use of two primers ensures that both the sense and anti-sense strands are duplicated. The newly synthesised strands are then used as templates in the subsequent cycles of the reaction. An additional elongation period of 10 minutes was added at the end of the reaction to ensure that DNA synthesis was completed for all strands.

PCR reactions take place in thin walled PCR tubes. A total reaction volume of 50 μ l typically contained 5-50 ng template DNA, 1 μ M primers, 200 μ M of each dNTP, 1.5 mM MgCl₂, 10x reaction buffer and 2.5 units of Taq DNA polymerase. For the high fidelity PCR system several amounts of template DNA were used. A total reaction volume of 50 μ l typically contained 5-100 ng template DNA, 1 μ M primers, 200 μ M of each dNTP, 1.5 mM MgCl₂, 10x reaction buffer and 2.6 units of Expand High Fidelity enzyme mix. For PCR reactions to amplify GC rich sequences of DNA, in Sck for example, the PCR reaction mix was supplemented with 5 % v/v DMSO and 1 M Betaine (N,N,N-trimethylglycine). In addition the annealing temperature in the PCR reaction conditions was reduced slightly.

2.4.1(ii) *Generation of N-terminally-tagged DNA constructs*

DsredII-Grp-1

Full-length mouse Grp-1 DNA was isolated from bacterial image clone 1970831 (4841-k24, UK HGMP) and amplified by PCR using primers to incorporate an EcoRI restriction site at the 5' end of Grp1 and a BamHI restriction site at the 3' end of Grp-1.

PCR Primers for full length Grp-1

Forward- 5' GCT AGA ATT CAA TGG ACG AAG GCG GTG GCG G 3'

Reverse- 5' GCT AGG ATC CTA TTT CTT ATT GGC AAT CCT CC 3'

<u>Programme</u>	Denaturation	94°C; 1min	
	Annealing	55°C; 1min	
	Elongation	72°C; 2min	30 cycles

The 1200 base pair PCR fragment and the DsredII-C1 vector (Clontech) were cut with EcoRI and BamHI restriction enzymes. Grp-1 was ligated into DsredII-C1.

GFP-Shc and Flag-Shc

Full length, wild type, mouse Shc DNA was amplified by PCR from a construct containing Shc (vector not known) provided by E. Skolnik. Primers were used to incorporate an EcoRI restriction site at the 5' end of Shc and a KpnI restriction site at the 3' end of Shc.

PCR Primers for Shc

Forward- 5' GAG CGA ATT CTA ACA AGC TGA GTG GAG GCG GC 3'
 Reverse- 5' GCT TGG TAC CTC ACA CTT TCC GAT CCA CGG GTT G 3'

Denaturation 94°C; 1min
 Annealing 55°C; 1min
 Elongation 72°C; 2min 30 cycles

The 1400 base pair PCR product, pEGFP-C1 (Clontech) FLAG-CMV™-2 (Sigma) was cut with EcoRI and KpnI. Shc was ligated into pEGFP-C1 and pFLAG-CMV™-2 vectors.

GFP-Sck and Flag-Sck

Cosmid DNA was isolated from R34739 (R23N6) LLNL Human Chromosome clone (UK HGMP). This provided a template for the 5' fragment of Sck. A partial fragment of Sck had previously been cloned into the Dsred-N1 vector by Dr Sally Prigent, which provided the template for the 3' fragment of Sck. The two fragments of Sck were amplified by PCR from these templates, to give the 5' fragment (bases 1-397) and the 3' fragment (bases 351-1749), using primers that incorporate AttB sites at the ends of the full-length Sck cDNA sequence.

PCR Primers for 5' fragment of Sck

Forward- 5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GAC
 GCA GGG TCC GGG CGG GCG CGC 3'

Reverse- 5' GGA TGA AGC TGC CCT TCC GGA TCC 3'

Denaturation 94°C; 1.5min
 Annealing 58°C; 1.5min
 Elongation 72°C; 2min 33 cycles

PCR Primers for 3' fragment of Sck

Forward- 5' GGA CGC CGC CGC CGC CGC CGA GTG GAT CC 3'
Reverse- 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA
GGG CTC CCG TGA GAC CAC GCC 3'
Denaturation 94°C; 1.5min
Annealing 60°C; 2min
Elongation 72°C; 2min 30 cycles

PCR fragments of 400 base pairs and 1400 base pairs were cut with the restriction enzyme BspMII and ligated together. The high fidelity PCR system was used to amplify full length Sck using the forward primer for the 5' fragment of Sck, the reverse primer for the 3' fragment of Sck and the PCR conditions for the 5' fragment of Sck. Full length Sck flanked by AttB sites was cloned into the gateway vector pDONR™201 (invitrogen) by a recombination reaction. A second recombination reaction inserted full length Sck into the pDEST53 (invitrogen) and FLAG-CMV™-2 vectors. Dr. Eilis Byrne inserted an AttB cassette into the FLAG-CMV™-2 Vector (Sigma) at the NotI restriction site in the multiple cloning region to generate the latter vector.

GFP-SckΔPTB and FLAG-SckΔPTB

SckΔPTB DNA was amplified by PCR from GFP-Sck (pDEST53-Sck) using primers designed to incorporate AttB sites at the 5' and 3' ends.

PCR Primers for SckΔPTB

Forward- 5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CCT
GCA CAG CCC GCC CAA GGT 3'
Reverse- 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA
GGG CTC CCG TGA GAC CAC GCC 3'
Denaturation 94°C; 1.5min
Annealing 58°C; 1.5min
Elongation 72°C; 2min 33 cycles

The 1300 base pair PCR fragment was cloned into the gateway vector pDONRTM201 (Invitrogen) by a recombination reaction. A second recombination reaction inserted Sck Δ PTB into the pDEST53 (Invitrogen) and FLAG-CMVTM-2 (Sigma) + AttB cassette (Invitrogen) vectors.

GFP-PLC γ 1

Full length wild type bovine PLC γ 1 was amplified using the high fidelity PCR system from pMT2-PLC γ 1 (provided by M Katan) and primers designed to incorporate AttB1 sites at the 5' and 3' ends of the PCR product.

PCR Primers for PLC γ 1

Forward- 5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GGC
GGG CGC CGC GTC CCC TTG C 3'

Reverse- 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CTA
GAG GCG GTT GTC TCC ATT 3'

Denaturation 94°C; 0.5min

Annealing 58°C; 1min

Elongation 72°C; 2.75min 30 cycles

The 3900 base pair PCR fragment was cloned into the gateway vector pDONRTM201 (Invitrogen) by a recombination reaction. A second recombination reaction inserted PLC γ 1 into the pDEST53 (Invitrogen) and FLAG-CMVTM-2 (Sigma) + AttB cassette (Invitrogen) vectors.

2.4.1(iii) Site directed mutagenesis to generate GFP- and Flag-tagged Sck Δ SH2, SckY391F and SckY315/316F

Mutations to the DNA sequence that codes for Sck were made in the partial Sck construct cloned into DsredN-1 using the QuikChange[®] site-directed mutagenesis kit. Specific mutagenesis of double-stranded DNA requires a set of two complementary primers encoding the desired mutation. The primers used for the three different mutations are given below. The wild type DNA sequence was used as a DNA template in the PCR based method. The template DNA was denatured and the oligonucleotide primers anneal to the site of mutagenesis. This initiates the polymerase chain reaction, which was catalysed by the Taq

polymerase, Pfu Turbo. The resultant DNA contains the desired mutation and nicks that are staggered along the circular DNA. The restriction enzyme DpnI digests the methylated, non-mutated template DNA, leaving the mutated DNA intact. The mutated DNA was transformed into XL1-Blue super competent cells, which repair the nicks in the mutated plasmid.

PCR Primers for SckY315/316F

Sense strand - 5' GGA CTC TTT GGA GCA CAA TTT CTT CAA CAG CAT
CCC GGG G 3'

Anti-sense strand- 5' CCT GAG AAA CCT CGT GTT AAA GAA GTT GTC
GTA GGG CCC C 3'

Denaturation	95°C; 0.5min	
Annealing	55°C; 0.5min	
Elongation	68°C; 12min	18 cycles

PCR Primers for SckY391F

Sense strand - 5' CGA GGA GCA CCT GTT TGT CAA CAC CCA GGG 3'

Anti-sense strand- 5' GCT CCT CGT GGA CAA ACA GTT GTG GGT CCC 3'

Denaturation	95°C; 0.5min	
Annealing	55°C; 0.5min	
Elongation	68°C; 12min	18 cycles

PCR Primers for Sck Δ SH2

Sense strand - 5' GAA CAG CTG CGT CAG TAG CCC TGG TAC CACG 3'

Anti-sense strand- 5' CTT GTC GAC GCA GTC ATC GGG ACC ATG GTG C 3'

Denaturation	95°C; 0.5min	
Annealing	55°C; 0.5min	
Elongation	68°C; 12min	16 cycles

DNA fragments containing the mutated sites to code for Y315/316F and Y391F of Sck were cut out of Dsred-Sck using BstEII and KpnI restriction enzymes. Full length Sck in pDEST53-Sck was also cut with these enzymes and the wild type fragment was replaced with the mutated fragment. Likewise, the same mutated fragments were cut out of Dsred-Sck using BstEII and Tth111I and replaced the wild type Sck fragment in FLAG-CMVTM-2-Sck.

The DNA fragment containing the mutated site that codes for a premature stop codon to produce Sck Δ SH2 was cut out of Dsred-Sck using BstEII and BbsI. Full length Sck in pDEST53-Sck was also cut with these enzymes and the wild type fragment was replaced with the mutated fragment. Likewise, the same mutated fragment was cut out of Dsred-Sck using BstEII and Tth111I and replaced the wild type Sck fragment in FLAG-CMVTM-2-Sck.

2.4.1(iv) *Generation of chemically competent bacteria*

DH5 α cells (200 μ l or one colony) were cultured in 200 ml of LB at 37°C overnight, with agitation at 200 rpm. Four 200 ml cultures of LB were inoculated with 4 ml of the overnight culture and incubated at 37°C, with agitation at 200 rpm, until the OD₆₀₀ was between 0.3 and 0.5 (approximately 2 hours). Cells were kept on ice in-between the subsequent stages. The cultures were transferred aseptically into 250 ml autoclaved centrifuge pots and the bacteria were pelleted at 4000 rpm for 10 minutes at 4°C. Each pellet was resuspended in 20 ml of sterile transforming buffer 1 (30 mM Potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂ and 15 % w/v glycerol) and transferred to a chilled 50 ml falcon tube. Another 20 ml of sterile transforming buffer 1 was added and the suspensions were kept on ice for 30 minutes. The cells were pelleted by centrifugation as before and then each pellet was resuspended in 4.5mls sterile transforming buffer 2 (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl and 15 % w/v glycerol). The cells were incubated on ice for 15 minutes, aliquoted and snap frozen in liquid nitrogen. Aliquots were stored at -80°C.

2.4.1(v) *Transformation of chemically competent bacteria*

1 ng of plasmid DNA, or 1 μ l of a ligation reaction, was mixed with 100 μ l of competent DH5 α bacteria and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 45 seconds and then returned to ice for a further 2 minutes. Cells were recovered in 500 μ l LB, warmed to 42°, and incubated at 37°C, with agitation at 225 rpm, for 20 minutes. 200 μ l of the culture was plated onto LB-Amp (100 μ g/ml) or LB-Kan (25 μ g/ml) and grown at 37°C overnight.

2.4.1(vi) *Generation of electro-competent bacteria*

One colony of DH5 α cells was used to inoculate 50 ml of SOB medium and the culture was grown up overnight at 37°C with agitation at 225 rpm. A 500 ml culture of SOB was inoculated with 5 ml of the overnight culture and incubated at 37°C, with agitation at 200

rpm, until the OD₆₀₀ was between 0.5 and 0.7 (approximately 4 hours). Cells were kept on ice in-between the subsequent stages. The cultures were transferred aseptically into 400 ml autoclaved centrifuge pots and the bacteria were pelleted at 4000 rpm for 15 minutes at 4°C. Each pellet was resuspended in 400 ml of ice-cold wash buffer (sterile 10 % v/v glycerol in PBS). The cells were pelleted by centrifugation and washed as before. Finally cells were pelleted and resuspended in 2mls wash buffer, aliquoted and snap frozen in liquid nitrogen. Aliquots were stored at -80°C.

2.4.1(vii) *Transformation of electro-competent bacteria*

DH5 α cells were gently thawed on ice, and then added to 1-2 μ l of ligation mix in a chilled eppendorf tube. The DNA and bacteria were mixed well and incubated on ice for 30-60 seconds. The DNA-bacteria mix was transferred to a cold electroporator cuvette and the mix was subjected to one pulse (~5 msec) at 1.8 kV. The cuvette was removed from the apparatus immediately and 1 ml of SOC medium, at 42°C, was added to the cells. The cell suspension was incubated at 37°C for 1 hour with agitation at 225 rpm. 250 μ l of the culture was plated onto LB-Amp (100 μ g/ml) or LB-Kan (25 μ g/ml) and grown at 37°C overnight.

2.4.1(viii) *Small-scale plasmid preparation*

3 ml of LB-Amp (100 μ g/ml) or LB-Kan (25 μ g/ml) was inoculated with one colony of DH5 α and the culture was grown up at 37°C, with agitation at 225 rpm, overnight. 1.5 ml of the overnight culture was centrifuged at 12,000 x g for 1 minute at room temperature. The cells were resuspended in 100 μ l of TNE. 150 μ l of mixed phenol/chloroform/isoamyl alcohol (25:24:1 v/v, Invitrogen) was added to the solution and mixed by inversion several times. The solution was centrifuged at 14,000 x g for 2 minutes. 100 μ l of the upper, aqueous phase was transferred into a fresh tube and the DNA was precipitated from this solution using 10 μ l sodium acetate (3 M) and 250 μ l absolute ethanol. The solution was incubated on ice for 5 minutes to facilitate DNA precipitation. The DNA was pelleted by centrifugation at 14,000 x g for 15 minutes at 4°C. The DNA pellet was washed with 0.5 ml of 70 % ethanol, the DNA was pelleted again, the supernatant was removed and the pellet was left open to the air to dry. The pellet was resuspended in 15 μ l TE containing 20 ng/ μ l RNase. This method was only used for screening, as it does not yield high quality DNA.

2.4.1(ix) *Large-scale plasmid preparation*

5 ml of an overnight DH5 α culture was added to 500 ml of LB-Amp (100 μ g/ml) or LB-Kan (25 μ g/ml) and the bacteria were grown up at 37°C, with agitation at 225 rpm, overnight. The 500 ml culture was centrifuged in two sterile 250 ml centrifuge pots at 6000 x g for 5 minutes at 4°C. The bacterial pellets were resuspended in a total of 10 ml of alkaline lysis buffer I. 20 ml of freshly prepared alkaline buffer II was added and the solutions were mixed gently by swirling. 15 ml of alkaline lysis buffer III was added and the solutions were mixed by inversion. The precipitated proteins were pelleted at 8000 x g for 30 minutes at 4°C. The supernatant was removed and filtered through Whatman paper if necessary. 27 ml of room temperature isopropanol was added to the supernatant, the solutions were mixed by swirling and left at room temperature for 2 minutes before centrifuging at 6000 x g for 30 minutes at 4°C. The supernatant was removed, the pellet was dried for 5 minutes and the resuspended in 5.5 ml of TE. This solution was transferred to a 15 ml falcon tube and 275 μ l of 10 mg/ml ethidium bromide and 6 g of CsCl₂ were added. The CsCl₂ was dissolved and the solution was centrifuged at 4000 rpm for 5 minutes. The clear solution was loaded into two Beckman Quickseal ultra-centrifuge tubes and the tubes were balanced and heat-sealed. The solutions were centrifuged at 80,000 rpm overnight at 20°C. The plasmid DNA was identified as the lower of the two ethidium bromide-labelled bands of DNA. The top of the ultra-centrifuge tube was pierced to create a vent and the DNA band was removed using a needle and syringe. The ethidium bromide was extracted using water saturated isobutanol. To the remaining solution an equal volume of water was added and 6 volumes of absolute ethanol. This solution was centrifuged at 11,000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet was washed with 70 % ethanol. The DNA was resuspended in 500 μ l TE.

2.1.4(x) *Purification of small amounts of DNA*

Small amounts (up to 10 μ g) of double stranded DNA (100-10,000 base pairs) were routinely purified using QIAquick gel purification and PCR purification kits. The spin columns in these kits contain silica-gel membranes that bind DNA fragments larger than 100 base pairs but not short fragments such as oligonucleotide primers in PCR reactions. In addition DNA can be purified from solutions containing buffers, agarose gels and enzymes.

2.4.1(xi) *Restriction enzyme digests*

Up to 5 µg of DNA were digested in a 20-30 µl reaction-volume, containing 10 units of restriction enzyme with the corresponding buffer. For the digestion of multiple sites by different restriction enzymes a single reaction was used only when the buffers for each enzyme were compatible. In this case 10 units of each restriction enzyme were used. In circumstances where the buffers were incompatible two sequential reactions were set up. The DNA was purified from the first reaction using QIAquick columns.

2.4.1(xii) *DNA ligations*

Ligation reactions often contained approximately the same molar amounts of insert DNA and plasmid vector. In some cases, increasing amounts of insert DNA were added to the same amount of plasmid vector for multiple attempts at ligation. In a 15 µl ligation reaction, 1.5 units of T4 DNA ligase with the appropriate buffer, supplemented with 5 mM ATP, were added. The ligations were performed at 16°C overnight.

2.4.1(xiii) *Separation of DNA by agarose gel electrophoresis*

DNA samples were separated by electrophoresis in 0.6-1.5 % agarose gels in TAE containing 0.6 µg/ml ethidium bromide. 3x agarose gel loading buffer was added to the samples before they were loaded into the gel. Electrophoresis was performed at 90 V in TAE buffer. Low-intensity UV radiation facilitated the visualisation of DNA bands and these were cut out of the gel using a clean scalpel blade. DNA was purified from the gel fragments using the QIAquick gel extraction kit.

2.4.1(xiv) *Sequencing*

Sequencing of PCR products or vectors was carried out by Lark Technologies Inc, Takeley, Essex, UK.

2.4.2 - Mammalian Cell Culture

2.4.2(i) *Isolation and maintenance of HUVE cells*

HUVE cells were isolated from human umbilical cords by a previously described method (Marin et al., 2001). In brief, PBS supplemented with 100 U/ml penicillin and 0.1mg/ml streptomycin was flushed through the vein of the cord to remove blood and cell debris. One end of the cord was then clamped and the vein was filled with 0.5mg/ml collagenase in HEPES buffered (pH 7.4) DMEM medium. The collagenase-filled cord was incubated at

37°C for 15 minutes and then agitated to promote cell detachment. The collagenase solution was drained and Medium 199 with glutamax-1 supplemented with 20 % foetal calf serum (FCS) was added to stop the collagenase activity. The isolated cells were then centrifuged at 500 x g for 5 minutes and resuspended in Medium 199 with glutamax-1 supplemented with 20 % FCS, 5 U/ml heparin and 0.5 mg/ml ECGS. HUVE cells were initially cultured in a gelatin-coated (1 % w/v gelatin) T25 flask. The following day medium, containing non-adherent blood cells, was removed and HUVE cells were washed with PBS before fresh medium was added. Upon reaching confluence HUVE cells were passaged and expanded into T75 flasks. Subsequent passages expand the cell population further however cells were not used beyond passage seven.

2.4.2(ii) *Maintenance of cell lines*

BAE and HEK-293 cells were cultured in DMEM with glutamax 1 supplemented with 10 % foetal calf serum (FCS), 100 IU/ml penicillin and 100 IU/ml streptomycin. PAE cells were cultured in Ham's nutrient mix F12 with L-glutamine supplemented with 10 % FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin. NIH3T3/HER4 cells were cultured in DMEM with glutamax 1 supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 0.4 mg/ml G418 sulphate.

All cells were passaged upon approaching confluence. BAE, PAE, HUVE and NIH3T3/HER4 cells were washed with PBS and incubated with Trypsin-EDTA for 2 minutes at 37°C. HEK 293 cells were agitated with medium to lift them from the tissue culture plastic. Cells were pelleted by centrifugation at 500 x g for 5 minutes and resuspended in medium. For the maintenance of cells for future experiments BAE, PAE and NIH3T3/HER4 cells were split at a ratio of 1:10 and HUVE cells at a ratio of 1:4. Cell required for imminent experiments were counted and seeded at the appropriate density.

2.4.2(iii) *Storage of cells in liquid nitrogen*

Cells were frozen in liquid nitrogen for long-time storage. Cells cultured in T75 tissue culture flasks were grown until they approached confluence. The cells were trypsinised and pelleted at 500 x g for 5 minutes. The medium was poured off of the cells and the cells were gently resuspended in 1 ml of sterile FCS. An equal volume of medium containing 20 % DMSO was

added to the cell suspension. Cells were aliquoted and placed in an insulated box at -80°C before being transferred to liquid nitrogen.

Cells were thawed quickly by incubating the vials in a 37°C water bath. The cell suspension was added to 12 ml of complete medium. The following day, the medium was replaced to remove traces of DMSO.

2.4.2(iv) *Transfection of cell lines*

PAE/VEGF receptor-2 and NIH3T3/HER4 cell lines were transfected using FuGENE™6. Cells were seeded at a density of 1×10^5 cells per well of a 6 well plate 18-24 hours prior to transfection. 100 μl (minus the volume of FuGENE™6 to be used) of serum free medium (OptiMEM®) was added to an eppendorf. FuGENE™6 reagent (typically 4 μl) was added directly to the serum free medium. The solutions were mixed and incubated at room temperature for 5 minutes. The DNA (typically 2 μg) was then added directly to the FuGENE™6 solution. The solutions were mixed and incubated at room temperature for 20 minutes. During this incubation, the cells were washed with PBS and fresh medium was added. The FuGENE™6-DNA solution was then added dropwise to the cell medium and the 6 well plates was swirled to mix. The cells were transfected for 24 hours. The ratios of DNA:FuGENE™6 are given below and the volumes of OptiMEM® and FuGENE™6 were adjusted depending on the size of the cell culture.

Ratios of DNA (μg) : FuGENE™6 (μl)

pEGFP-N1	1 : 1		
GFP-p85	1 : 2	DsredII-Grp-1	1 : 3
GFP-Shc	1 : 2	Flag-Shc	1 : 3
GFP-Sck (+ mutants)	1 : 3	Flag-Sck (+ mutants)	1 : 3
GFP-PLC γ 1	1 : 2	PEGFP-C1	1 : 2
HA-Cbl	1 : 2	HA-Cbl70Z	1 : 2
HA-Nedd4	1 : 3	HA-Nedd4C744S	1 : 6

2.4.2(v) *Transduction of cell lines*

BAE cells were seeded at a density of 1×10^5 cells per well of a 6 well plate 6 hours prior to transduction. 28 μl of AdKDR (Wild type VEGF receptor-2 virus, made by Dr. J Carter) in 2

ml of complete medium was added to the cells. Cells were transduced overnight, then washed and incubated in complete medium for 7.5 hours. PAE cells were seeded at a density of 5×10^5 cells/9cm-diameter plate 6 hours prior to transfection and then transfected as required overnight. Transfected cells were transduced with 30 μ l AdCMV (Mock virus, made by Dr. J Carter) or 20 μ l Admyc.GyrFlt-1 (Myc-tagged GyraseB-Flt-1 virus, made by Dr. J Carter) in complete medium for 6 hours and then incubated in fresh complete medium overnight.

2.4.2(vi) *Serum starvation of cells and stimulation with VEGF*

Cells were serum-starved (or incubated in 2 % serum for HUVE cells) overnight prior to stimulation. Cells were stimulated with VEGF in serum-free medium at the concentrations stated and for varying lengths of time as detailed for each experiment.

2.4.2(vii) *Treatment of cells with coumermycin*

Infected cells were washed with PBS and serum-starved for 3 hours with 5 ml of serum free medium prior to treatment with coumermycin. 5 ml of 10 μ M or 2 μ M coumermycin in serum free medium was typically added to the 5 ml of serum free medium already added to the cells. This results in a final concentration of 5 μ M and 1 μ M of coumermycin (this was adjusted for different doses) and eliminates the need to replace the medium, which can activate certain signalling pathways E.g. ERK via a fluid-shear stress mechanism. Cells were typically treated with coumermycin for 15 minutes (or times as stated for each experiment).

2.4.2(viii) *Treatment of cells with inhibitors*

Cells were treated with inhibitors 1 hour prior to stimulation with VEGF. The inhibitors were present throughout the time-course of stimulation. The VEGF receptor-2 inhibitor (Astrazeneca) was used at 100 nM and the PP1 Src inhibitor (gift from Dr JM Willets, University of Leicester, UK) was used at 10 μ M.

2.4.3 - Protein Biochemistry

2.4.3(i) *Immunoprecipitation*

Cells were lysed in ice-cold 1 % Triton X-100 cell lysis buffer with NaF (100 mM) and Na_3VO_4 (1 mM) phosphatase inhibitors and 20 μ l/ml of a mammalian protease inhibitor cocktail (containing- 104mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 80 μ M Aprotinin, 2.1mM Leupeptin, 3.6mM Bestatin, 1.5mM Pepstatin A and 1.4mM 1-

transepoxy succinyl-L-leucylamido (4-guanidino) butane (E-64), Sigma) for 10 minutes at 4°C. Cell debris was removed by centrifugation at 25,000 x g at 4°C for 5 minutes. The cell lysates were added to ~2.5 mg protein-G Sepharose beads pre-coated with 2 µg anti-VEGF receptor-2 (Flk-1(A3), Santa Cruz), 5 µg anti-FLAG (M2, Sigma) or 0.4 µg anti-HA (3F10, Roche) and rotated at 4°C for at least 3 hours. For immunoprecipitation using the antibody against HER4 (gift from Prof. W. Gullick (Srinivasan et al., 1998)) ~2.5mg protein-A Sepharose beads were pre-coated with 2µg HER4 antibody. The beads were then pelleted and the supernatant was removed. The beads were washed four times with 1 ml of 0.1 % TritonX-100 in PBS and then 30 µl of 1.5x sample buffer, freshly supplemented with 100 mM dithiothreitol (DTT), was added to elute the immunoprecipitated proteins from the beads. Samples were either loaded onto an SDS-PAGE gel immediately or stored at -20°C. To re-run immunoprecipitation samples an additional 20 µl of 1.5x sample buffer, freshly supplemented with 100 mM DTT, was added to the beads.

2.4.3(ii) *Whole cell lysate preparation*

Cells were lysed in boiling 2x sample buffer with NaF (100 mM) and Na₃VO₄ (1 mM) phosphatase inhibitors and 100 mM DTT. For the detection of phosphorylated proteins samples were loaded onto an SDS-PAGE gel immediately. Other samples were either run straight away or stored at -20°C.

2.4.3(iii) *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

Whole cell lysate and immunoprecipitated proteins were separated on 6-10 % acrylamide mini-gels (Bio-rad), depending on the size of the proteins of interest, using the method described by Schagger and von Jagow. Electrophoresis was performed at 50 V, until the samples had entered the stacking gel, and then 120 V for 1.5-2 hours as necessary.

2.4.3(iv) *Electrophoretic transfer of proteins*

The gels were soaked in transfer buffer for 20 minutes prior to the transfer of proteins onto nitrocellulose membranes. This was achieved by placing 6 sheets of 3MM paper, soaked in transfer buffer, onto the transfer apparatus (Bio-rad). A single sheet of nitrocellulose membrane, soaked in transfer buffer, was placed on top of the paper. The gel was then placed on top of the membrane and 6 more sheets of 3MM paper, also soaked in transfer buffer, were

placed on to top of the gel. The lids of the transfer apparatus were put on and the apparatus was set to transfer at 22 V for 80 minutes.

2.4.3(v) *Western blotting*

Membranes were blocked for at least 1 hour in 5 % BSA or powered milk (Marvel) in TBST as indicated in Table 2.1. The membranes were incubated in primary antibody also detailed in Table 2.1. The membranes were washed four times in TBST for at least 10 minutes per wash. The membranes were then incubated in a secondary antibody conjugated to horseradish peroxidase, details of which are detailed in Table 2.2. This was followed by at least four more washes in TBST each for at least 10 minutes.

2.4.3(vi) *Enhanced Chemi-Luminescence (ECL) development of blots*

The ECL solution used to detect the immunolabelled proteins contains 0.2 mM p-coumaric acid, 1.25 mM luminol and 0.009 % hydrogen peroxide in 0.1 M Tris buffer at pH8.5. This solution was added to the membrane for 1 minute. The membrane was dried and exposed to x-ray film in the dark. If required scanned blots were quantified using Image Quant software.

2.4.3(vii) *Re-probing of blots*

After exposure to one set of primary and secondary antibodies, if the blot needed to be re-probed the antibodies were removed by incubation of the blot in blot strip buffer supplemented with 0.8 % β -mercaptoethanol. The blot was incubated at 60°C for 1 hour. The blot was then washed several times repeatedly in de-ionised water and four times with TBST for about 5 minutes per wash. The blots were then incubated in blocking buffer as required for the next primary antibody.

Primary Antibody	Company	Cat No.	Species	Blocking conditions	Dilution	Incubation conditions
Phospho-Tyr (PY99)	Santa Cruz Biotechnology	Sc-7020	Mouse	5% BSA, O/N	1 in 500	1 hr, RT
Flk-1 (A-3)	Santa Cruz Biotechnology	Sc-6251	Mouse	5% milk, 1 hr	1 in 1000	O/N, 4°C
Phospho-MAPK E10	New England Biolabs	9106	Mouse	5% milk, 1 hr	1 in 2000	O/N, 4°C
ERK 1 (K-23)	Santa Cruz Biotechnology	Sc-94	Rabbit	5% BSA, O/N	1 in 5000	1 hr, RT
Vinculin	Gift from Norman et al.	-		5% milk, 1 hr	1 in 1000	O/N, 4°C
Phospho-Y317 Shc	Upstate Biotechnology	07-206	Rabbit	5% milk, 1 hr	1 in 250	O/N, 4°C
Phospho-Y239 Shc	Upstate Biotechnology	07-209	Rabbit	5% milk, 1 hr	1 in 2000	O/N, 4°C
Shc (PG-797)	Santa Cruz Biotechnology	Sc-967	Mouse	5% BSA, O/N	1 in 1000	1 hr, RT
Shc (C-20)	Santa Cruz Biotechnology	Sc-288	Rabbit	5% BSA, O/N	1 in 1000	1 hr, RT
Sck	Leicester University	-	Rabbit	5% milk, 1 hr	1 in 250	O/N, 4°C
HA (Y-11)	Santa Cruz Biotechnology	Sc-805	Rabbit	5% BSA, O/N	1 in 1000	1 hr, RT
Flag (M2)	Sigma	F3165	Mouse	5% BSA, O/N	1 in 1000	1 hour, RT
GFP	Abcam	Ab 6556	Rabbit	5% BSA, O/N	1 in 5000	1 hr, RT
Myc (9B11)	Cell signalling technology	2276	Mouse	5% milk, 1 hr	1 in 1000	O/N, 4°C
Nedd4 (WW2)	Upstate Biotechnology	07-049	Rabbit	5% milk, 1 hr	1 in 20,000	3 hrs, RT
Cbl (C-15)	Santa Cruz Biotechnology	Sc-170	Rabbit	5% milk, 1 hr	1 in 200	O/N, 4°C
PLC γ 1 (1249)	Santa Cruz Biotechnology	Sc-81	Rabbit	5% milk, 1 hr	1 in 500	1 hr, RT
Phospho-PLC γ 1	Cell signalling technology	2821	Rabbit	5% milk, 1 hour	1 in 1000	O/N, 4°C in BSA
Phospho-Src Y416	Cell signalling technology	2101	Rabbit	5% milk, 1 hour	1 in 1000	O/N, 4°C in BSA
pp60 c-Src	Upstate Biotechnology	06-143	Rabbit	5% milk, 1 hr	1 in 250	O/N, 4°C
PI3K p85	Upstate Biotechnology	06-195	Rabbit	5% milk, 1 hr	1 in 2000	O/N, 4°C

Table 2.1 - Summary of primary antibodies used for western blotting

Secondary Antibody	Company	Catalogue number	Dilution	Incubation conditions
HRP- α mouse	Jackson Immunoresearch Laboratories	115-035-146	1 in 2000	1 hr, RT
HRP- α rabbit	Jackson Immunoresearch Laboratories	111-035-144	1 in 2000	1 hr, RT

Table 2.2 - Summary of secondary antibodies used for western blotting

2.4.4 - Enzyme Linked Immunosorbant Assay (ELISA)

2.4.4(i) *Preparation of ELISA plate*

The day before the experiment an ELISA plate (Maxisorb 96 well plate, Life Technologies) was initially prepared by the addition of 50 μ l of 5 μ g/ml anti-mouse IgG (BD Biosciences) in Na₂CO₃ buffer (0.05 M, pH 9.6) to each well that was to be used. This was incubated at 4°C, with slight rocking, overnight. On the day of the experiment the ELISA plate was washed four times with PBST and 50 μ l of 5 μ g/ml anti-VEGF receptor-2 (Flk-1(A3), Sc-6251) was added to all wells, except those that were used as a negative control for non-specific binding to IgG, for these wells 50 μ l of buffer alone was added. This was incubated at 4°C for at least 1 hour. The ELISA plate was then washed as before and blocked with 5 % BSA in PBST. The plate was incubated for at least 1 hour at room temperature. When the cell lysates were prepared the ELISA plate was washed again and 50 μ l of cell lysate was added to each well (in duplicate for each plate of cells). The plate was incubated at 4°C with slight rocking overnight.

2.4.4(ii) *Preparation of Cells*

On the day before the experiment HUVE or PAE/VEGF receptor-2 cells were seeded in 9cm plates at a density of 5×10^5 cells per plate. On the day of the experiment, the cells were washed twice with 2 x 10ml of ice-cold PBS on ice. 10 ml of serum free medium at 12°C was added and the cells were incubated at 37°C for 30 minutes. The cells were then washed again as before and 3ml of NHS-Biotin (0.2 mg/ml in PBS, Pierce) was added to each plate. The cells were incubated at 4°C with slight rocking for 30 minutes. At this stage several plates were designated as controls. At least two plates were used as an indication of the total level of receptors at the cell surface and at least another two plates were used as the control for non-specific binding of proteins to IgG. These control plates were left on ice. 5 ml of serum free medium at 12°C was added to all other plates and these were incubated at 37°C for 20

minutes to internalise biotin-labelled cell surface receptors. At this point VEGF was added to half of the plates, to a final concentration of 50 ng/ml, and all plates were incubated at 37°C for a further 20 minutes. The cells were then lysed in 100 µl of 1.5 x lysis buffer (200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na₃VO₄, 7.5 mM EDTA, 7.5 mM EGTA, 1.5 % v/v TritonX-100, 0.75 % Igepal CA-630), supplemented with 50 µg/ml aprotinin, 50 µg/ml leupeptin and 1mM AEBSF, scrapped off of the plate and passed through a 27G needle. Cell debris was removed by centrifugation at 13,000 x g at 4°C for 10 minutes. Each cell lysate was added in duplicate to coated wells of the prepared ELISA plate.

The above protocol describes the procedure for the VEGF receptor-2 degradation assay. A modified protocol was used for the VEGF receptor-2 internalisation assay and is described below.

On the day before the experiment HUVE or PAE/VEGF receptor-2 cells were seeded in 9cm plates at a density of 5×10^5 cells per plate. On the day of the experiment, the cells were washed twice with 2 x 10 ml of ice-cold PBS on ice. 10 ml of serum free medium at 12°C was added and the cells were incubated at 37°C for 30 minutes. The cells were then washed again as before and 3 ml of NHS-Biotin (0.2mg/ml in PBS, Pierce) was added to each plate. The cells were incubated at 4°C with slight rocking for 30 minutes. At this stage several plates were designated as controls as described previously. These control plates were left on ice. 5 ml of serum free medium at 12°C or serum free medium containing VEGF, to a final concentration of 50ng/ml, was added to the remaining plates as required. The cells were incubated at 37°C for 1-40 minutes. After the designated incubation times the cells were washed in ice cold PBS and incubated at 4°C on ice. When all the cells were treated they were washed with buffer (50 mM Tris, 100 mM NaCl; pH8.6). 3 ml of this buffer and 1 ml of MesNa solution (15 mg/ml MesNa in pH8.6 buffer supplemented with 15 mM NaOH) was added to each plate of cells. The cells were incubated at 4°C for 20 minutes. At this stage 1 ml of IAA solution (17 mg/ml IAA-iodoacetamide in PBS) was added to each plate and the cells were incubated at 4°C for 10 minutes. The cells were then lysed in 100 µl of 1.5 x lysis buffer (200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na₃VO₄, 7.5 mM EDTA, 7.5 mM EGTA, 1.5 % v/v TritonX-100, 0.75 % Igepal CA-630), supplemented with 50 µg/ml aprotinin, 50 µg/ml leupeptin and 1 mM AEBSF, scrapped off of the plate and passed through a 27G

needle. Cell debris was removed by centrifugation at 13,000 x g at 4°C for 10 minutes. Each cell lysate was added in duplicate to coated wells of the prepared ELISA plate.

2.4.4(iii) *Developing ELISA plate*

The day after the experiment the unbound material was washed off of the ELISA plate with four washes of PBST. 50 µl of streptavidin-conjugated horseradish peroxidase (1 in 1000 dilution, Amersham) in PBST supplemented with 1 % BSA was added to each well and the plate was incubated for at least 1 hour at 4°C. The plate was then washed again and biotinylated proteins were detected using ortho-phenylenediamine (0.56 mg/ml in a buffer containing 25.4 mM Na₂HPO₄, 12.3 mM citric acid, pH 5.4) supplemented with 0.003% hydrogen peroxide. When a brown/yellow colour was visible the reaction was stopped using 8M H₂SO₄. The absorbance of the solution in each well was read at 490nm.

2.4.5 - Imaging

2.4.5(i) *Coverslip preparation*

Glass coverslips (22mm diameter, thickness No.1) were incubated in 1 M HCl for 30 minutes. The acid was then washed off using distilled water and absolute ethanol was added for a further 30 minutes. The coverslips were then dried and incubated in a 121°C oven overnight.

2.4.5(ii) *Imaging of cells expressing fluorescently-tagged proteins*

Cells were seeded onto glass, acid treated coverslips contained within a well of a 6 well plate, transfected as described above and serum starved. Cells were either left resting or stimulated with 50 ng/ml VEGF for various time points. The cells were then fixed using cold 4 % paraformaldehyde for 20 min at room temperature. The cell nuclei were stained with Hoechst (0.07 µg/ml in PBS) for 10 minutes and the coverslips were mounted onto slides using 2.5 % DABCO as an anti-bleaching agent in 90 % glycerol.

2.4.5(iii) *Immunofluorescence*

PAE/VEGF receptor-2 or HUVE cells were grown on acid-treated, glass coverslips contained within a well of a 6 well plate. PAE/VEGF receptor-2 cells were transfected as described above if required. Cells were serum starved overnight and were either left resting or stimulated with 50 ng/ml VEGF for various time points. The cells were fixed using cold 4 % paraformaldehyde and incubated at room temperature for 20 minutes. Cells were

permeabilised for 5 minutes with 0.5 % Triton X-100 in PBS and blocked with 10 % BSA in PBS for at least 10 minutes. The cells were exposed to primary antibody by placing the coverslips, cell-side down, onto a 40 µl droplet of antibody solution. The dilutions for the antibodies used are detailed in Table 2.3. The coverslips were exposed to primary antibody for 1 hour and then returned to the 6 well plate for washing. The cells were washed four times in TBS/0.1 % TritonX-100 for 5 minutes per wash. The coverslips were exposed to secondary antibodies in the same way as they were for the primary antibodies. Details of dilutions are given in Table 2.3 and cells were exposed to the secondary antibodies for no longer than 1 hour. The cells were washed as before and were finally stained with Hoechst (0.07 µg/ml) for 10 minutes and mounted onto slides, using 2.5 % DABCO as an anti-bleaching agent in 90 % glycerol.

Primary Antibody	Company	Catalogue number	Dilution	Secondary Antibody	Dilution
Flk-1 C1158	Santa Cruz Biotechnology	Sc-504	1 in 50	Rhodamine Red- α rabbit or FITC- α rabbit	1 in 200 1 in 200
Paxillin	BD Biosciences	P13520	1 in 500	Texas Red- α mouse	1 in 200
HA (High affinity)	Roche	1-867-431	1 in 500	AMCA- α rat	1 in 50
Myc 9B11	Cell signalling technology	2821	1 in 2000	Texas Red- α mouse	1 in 200
EEA-1	BD Biosciences	610456	1 in 200	FITC- α mouse	1 in 200
CD63 (RFAC4)	Chemicon International	CBL553	1 in 200	FITC- α mouse	1 in 200
CD61	BD Biosciences	555752	1 in 100	Texas Red- α mouse	1 in 200
Caveolin N20	Santa Cruz Biotechnology	Sc-894	1 in 200	FITC- α rabbit	1 in 200
Phospho- MAPK	New England Biolabs	9106	1 in 400	Texas Red- α mouse	1 in 200

Table 2.3 - Summary of antibodies used for immunofluorescence

2.4.5(iv) *Real-time imaging*

PAE/VEGF receptor-2 cells were seeded onto coverslips, transfected as described above and serum starved overnight. The coverslips were carefully sandwiched between two lubricated metal rings that fit into a heated stage. 0.5ml of medium was added to the cells and oil was also added dropwise on top of the medium to prevent evaporation. The cells were incubated at 37°C in the heated stage under the microscope. A cell expressing the fluorescently tagged

protein of interest was chosen and the automated programme in Openlab was started to record the cell. The automation was designed so that we capture 5 focal planes around the original plane of focus (5 μm) to detect slight vertical movements of the proteins. The growth factor stimulus was slowly injected into the cell medium using a Gilson pipette. The Openlab programme was used to capture images at the desired time points and to compile a movie of the cell.

2.4.5(v) *BrdU assay*

PAE/VEGF receptor-2 cells were seeded onto coverslips and transfected as described above. The cells were serum starved for 16 hours and then stimulated with 50ng/ml VEGF for 24 hours. 7 hours after stimulation with VEGF, BrdU was added to a final concentration of 10 μM in 50 ng/ml VEGF/serum free medium. The cells were then washed in three times in PBS and fixed in ice-cold ethanol containing 15 mM glycine. Cells were incubated in fixative overnight at -20°C. The cells were washed four times in PBS for 5 minutes per wash. Immunofluorescence staining of the cells was performed as above using the primary antibody for BrdU from the BrdU labelling kit supplied by Roche. The anti-BrdU antibody was diluted 1 in 100 in the incubation buffer supplied and the cells were exposed to this for 1 hour. The cells were washed four times in PBS for 5 minutes per wash and then incubated in anti-mouse texas red secondary antibody (details in Table 2.3). Finally the cells were washed again as before, stained with Hoechst (0.07 $\mu\text{g}/\text{ml}$) for 10 minutes and mounted onto slides, using 2.5 % DABCO as an anti-bleaching agent in 90 % glycerol.

2.4.5(vi) *Microscopy*

The majority of images were taken using an inverted Nikon eclipse TE300 microscope, a 60x objective lens (1.4 aperture) DAPI, TRITCI and FITCI filter blocks and a Hamamatsu ORCA-ER digital camera. These images were processed using Openlab computer package. For visualisation of proteins immunostained with the AMCA- (blue dye) conjugated secondary antibody an Axioskop2 Zeiss microscope was used with a quantix photometrics camera and a Zeiss, plan neofluar, 40x objective (0.75 aperture). Images taken with this microscope were processed using Smartcapture 2 software.

Chapter 3

Analysis of VEGF receptor signalling in different cell types

Chapter 3 – Analysis of VEGF receptor signalling in different cell types

3.1 - Introduction

There are a number of cell types that are available to study signalling pathways downstream of the VEGF receptors. Primary cells are the favoured choice, as they will reflect endothelial cell biology more closely than immortalised cell lines or non-endothelial cells. Primary cells are particularly valuable for the study of endogenous proteins and their interactions with each other. It is now possible to over-express proteins in these cells using recombinant DNA; however the efficiency of the technique used is often poor. As there are limitations in the experiments that can be achieved using primary cells we sought to investigate other cell systems that can be used as an alternative. In addition to analysing VEGF signalling in alternative cell types we also considered the best cells to use for microscopy-based and biochemical-based experiments. The predominant techniques used in this study are immunoprecipitation, western blotting and immunofluorescence imaging therefore the properties of an alternative cell line must include the ability to over-express proteins of interest and good cell morphology.

From the cells that we have available human umbilical-vein endothelial (HUVE) cells are used as an example of primary endothelial cells. In this study the characteristics of these cells are compared to those of several endothelial cell lines and non-endothelial cells. The following cell lines were investigated - bovine aortic endothelial (BAE) cells, BAE cells transduced with adenoviral AdKDR resulting in the over-expression of VEGF receptor-2, porcine aortic endothelial (PAE) cells stably expressing VEGF receptor-2 and human embryonic kidney-293 (HEK-293) cells transiently transfected with recombinant DNA so that they express VEGF receptor-2.

3.2 - Results

In order to study the signalling pathways downstream of VEGF receptor-2 we first investigated the activation of VEGF receptor-2 in response to VEGF. Before cells were stimulated with VEGF they were first serum starved. This eliminates any growth factors from the complete serum-containing cell media and induces cells into a quiescent state. Throughout this study serum starved cells are referred to as either resting or unstimulated. The tyrosine phosphorylation levels of VEGF receptor-2, in response to stimulation with VEGF-containing

media, was analysed in all cell types by immunoprecipitating either endogenous VEGF receptor-2 from HUVE cells, or over-expressed VEGF receptor-2 in the other cell lines. Proteins in the immunoprecipitates were run on SDS-PAGE gels and probed with phosphotyrosine- and receptor-specific antibodies. The receptor-specific antibody used for immunoprecipitation and western blotting (Flk A3, sc-6251) binds to the C-terminus, in the intracellular domain, of VEGF receptor-2 (amino acids 1158-1345 of murine VEGF receptor-2).

As phosphorylation of VEGF receptor-2 by VEGF results in the activation of a mitogen activated protein (MAP) kinase pathway we also chose to study the extracellular regulated kinases (ERKs). The ERKs consist of a family of proteins however we have studied only two members, ERK1 and ERK2. ERK1, a 44 kDa protein and ERK2, a 42 kDa protein are both expressed endogenously in all cell types and can be readily detected in whole cell lysates (WCL). Human ERK-1 is phosphorylated at key tyrosine (Y204) and threonine (T202) residues in response to extra-cellular mitogens and growth factors.

3.2.1 *Human Umbilical-Vein Endothelial (HUVE) cells*

HUVE cells are primary cells that express VEGF receptor-2 at high levels, a small amount of VEGF receptor-1 and all of the components necessary for VEGF signal transduction. This makes these cells ideal for studying VEGF signalling. Both receptors bind VEGFA₁₆₅ and we assume therefore that downstream signalling events are regulated by cross talk between the two receptors. The presence of VEGF receptor-1 is an important feature as it can regulate the amount of VEGF that can binds to VEGF receptor-2 and can signal through the transactivation of VEGF receptor-2.

In serum starved HUVE cells tyrosine phosphorylation of VEGF receptor-2 was not detected (Figure 3.1). This indicates that the basal level of phospho-VEGF receptor-2 is very low. Upon stimulation with VEGF however key tyrosine residues are phosphorylated resulting in the activation of the receptor. Figure 3.1A shows the phosphorylation levels of VEGF receptor-2 in response to 50 ng/ml VEGF after 1, 2, 5, 10 and 20 minutes of stimulation. The peak of tyrosine phosphorylation as shown here occurs after 1-2 minutes. We show that the reduction in receptor protein level is due to down-regulation and this will be discussed in more detail in Chapter 6.

Phosphorylation of ERK 1 and ERK 2 is detected in HUVE cells after 2 minutes of VEGF stimulation (Figure 3.1B). ERK activation, for both kinases, peaks at 5 minutes and then falls close to basal levels during 20 minutes of stimulation. The maximum level of ERK phosphorylation in response to VEGF is comparable to that resulting from stimulation with phorbol 12-myristate 13-acetate (PMA), a compound that mimics diacylglycerol and therefore activates protein kinase C isoforms and consequently ERK 1/2.

In addition to studying the levels of tyrosine phosphorylation of VEGF receptor-2 in response to a time course of VEGF stimulation we also analysed the response of HUVE cells to different doses of VEGF. VEGF receptor-2 is phosphorylated in response to VEGF at concentrations from 10-100 ng/ml and the levels of both phospho-VEGF receptor-2 and of the receptor are constant over this range (Figure 3.1C). The levels of phospho-ERK however do change depending on the dose of VEGF (Figure 3.1D). Both ERK 1 and 2 are maximally phosphorylated at 50 ng/ml although ERK 2 is activated at a similar level over the 25-100 ng/ml range.

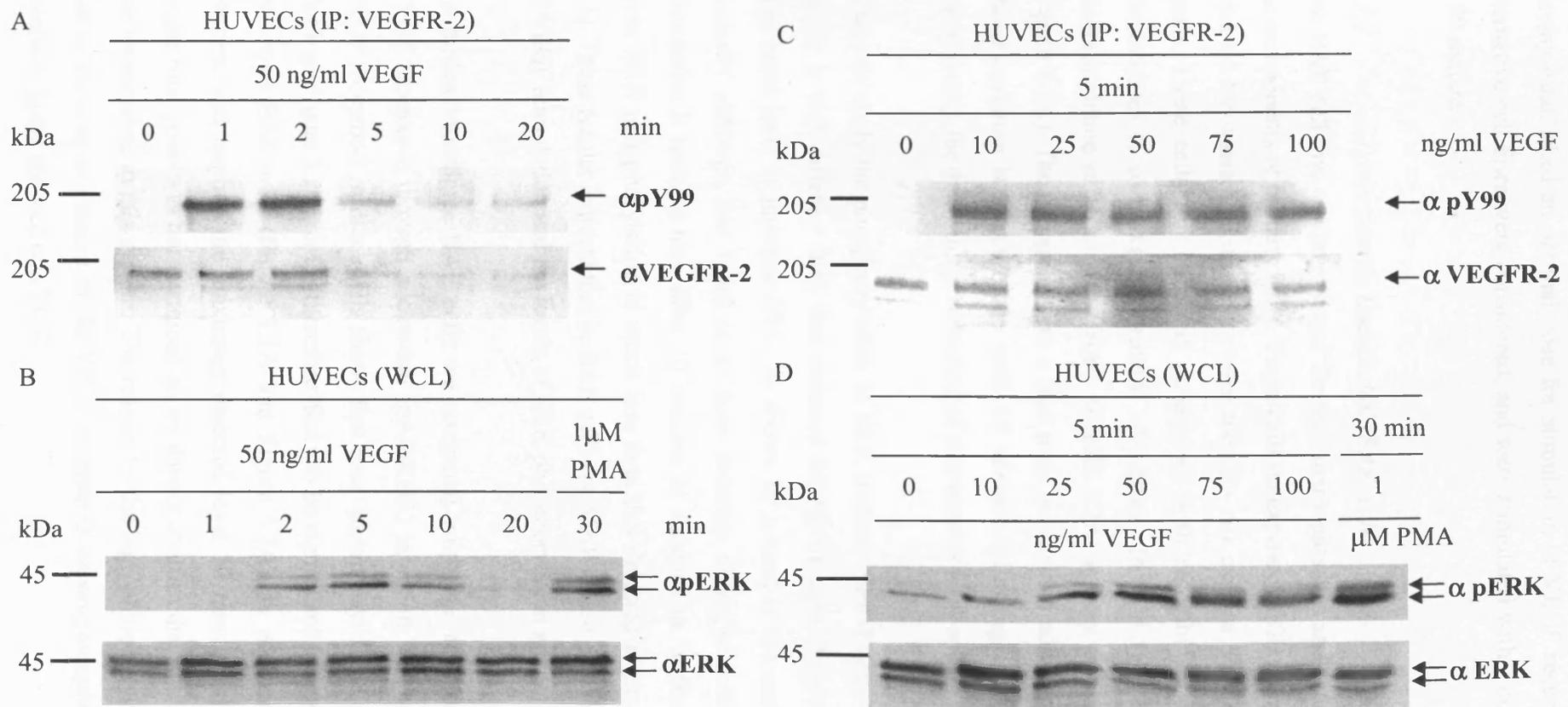


Figure 3.1 - Detection of VEGFR-2 and ERK phosphorylation levels in HUVECs by western blotting.

HUVECs were left unstimulated or stimulated with either 50ng/ml VEGF or 1 μ M PMA for the times indicated (A and B). Cells were also left unstimulated or stimulated for 5 minutes with varying doses of VEGF or with PMA (C and D). VEGFR-2 was immunoprecipitated from resting and stimulated HUVECs and the level of receptor phosphorylation was determined by western blotting using an anti-phosphotyrosine antibody (pY99). The level of receptor in the immunoprecipitates was analysed by blotting for VEGFR-2 (A and C). The phosphorylation of ERK, in response to VEGF stimulation, was determined from HUVEC whole cell lysates (B and D). Arrows indicate positions of VEGFR-2 (A and C) or ERK1/2 (B and D). These blots are representative of at least three independent experiments.

Having established an optimal dose for stimulation of VEGF receptor-2 in HUVE cells, the alternative cell lines were considered, and were stimulated with a dose of 50 ng/ml VEGF for 0-20 minutes.

3.2.2 *Bovine Aortic Endothelial (BAE) cells*

The BAE cell line can be cultured through many passages and therefore may not contain all the components of primary cells. These cells do express VEGF receptor-2 at levels that can be detected by western blotting however they do not express VEGF receptor-1 at detectable limits. These cells can be easily transfected with recombinant DNA or transduced with adenoviruses to over-express proteins of interest. In wild type BAE cells the profile for phosphorylation of VEGF receptor-2 (Figure 3.2A) is very similar to that for HUVE cells (Figure 3.1A). The exceptions are a later peak in maximal activation of VEGF receptor-2, as phosphorylation is increased up until 10 minutes following stimulation in these cells, and consequently the delay in the reduction of the receptor level occurs after this point.

When we study the phosphorylation of ERK in these cells (Figure 3.2B) we observe that the profile is very different from that obtained for HUVE cells (Figure 3.1B). Firstly, there is a high basal level of phospho-ERK, as shown by a band in the unstimulated cell lysate, and secondly although this basal level does increase throughout the time course of VEGF stimulation it remains high after 20 minutes of stimulation. In the HUVE cells at this time point ERK phosphorylation is much less than that detected at maximum activation (Figure 3.1). These results indicate that in BAE cells the MAP kinase pathway is active in the absence of VEGF stimulation and the levels of ERK phosphorylation are not efficiently regulated.

To further investigate BAE cells we compared wild type cells with cells over-expressing VEGF receptor-2 through adenoviral (pAd-KDR) infection. The phosphorylation profile of VEGF receptor-2 in these cells shows that basal phosphorylation can be detected by western blotting (Figure 3.2C) and is therefore likely to be significantly higher than that in HUVE and wild type BAE cells (Figure 3.1A and Figure 3.2A). In addition phosphorylation is less transient and reached the maximum detected level 10 minutes after VEGF stimulation. A longer time point was not examined so we cannot confirm that this is the maximum level that can be achieved in this system. The reason for the high phosphorylation levels is likely to be due to the over expression of the VEGF receptor-2 causing autophosphorylation of localised receptors in the absence of VEGF.

The profile for phospho-ERK (Figure 3.2D) is similar to that of wild type cells (Figure 3.2B). Treatment of these cells with PMA results in a level of phospho-ERK similar to that seen in serum starved cells (Figure 3.2D). This suggests that the MAP kinase pathway is activated in BAE cells expressing high levels of VEGF receptor-2 even in the absence of exogenous stimuli.

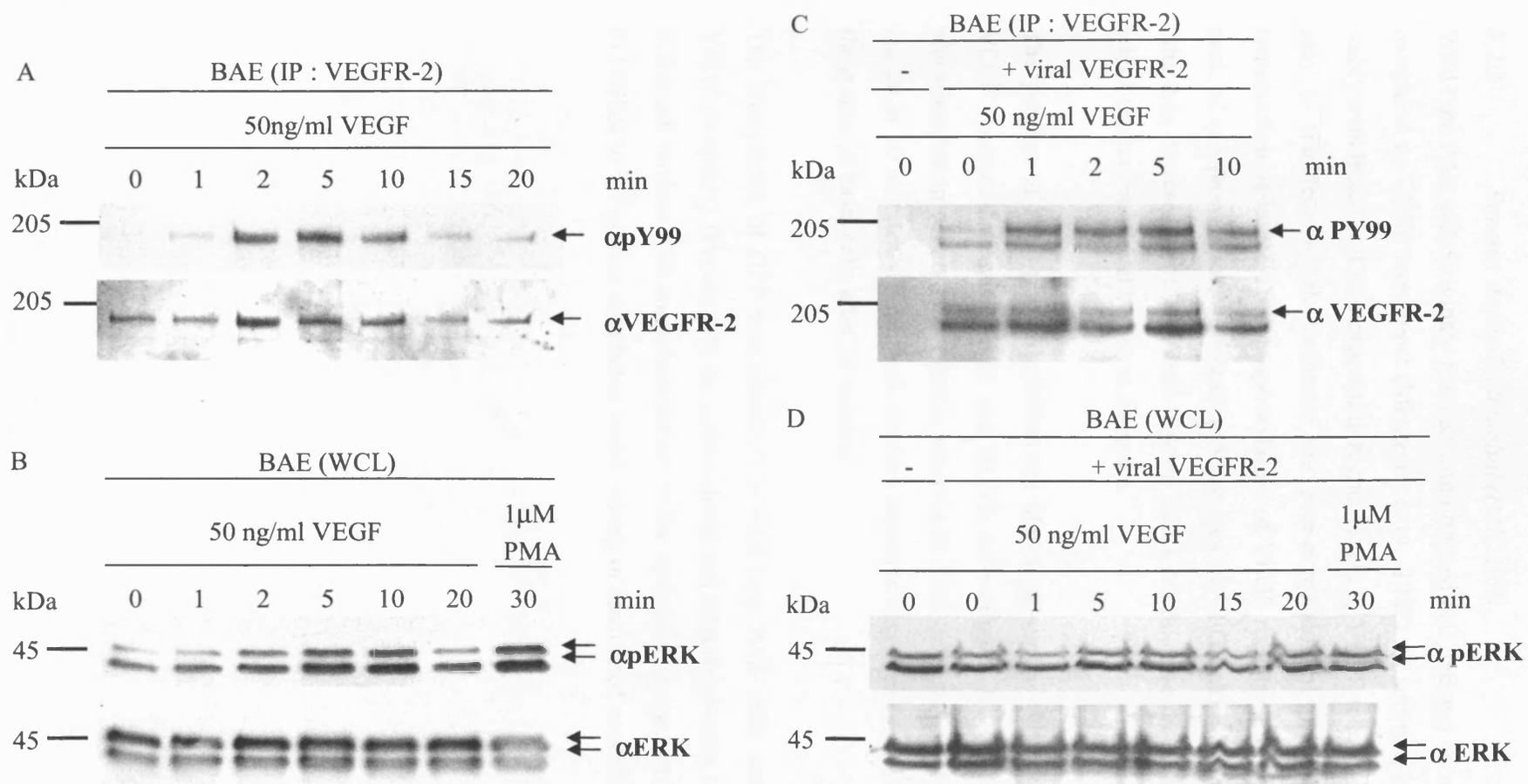


Figure 3.2 - Detection of VEGFR-2 and ERK phosphorylation levels in BAE cells and BAE cells transduced with viral VEGFR-2 by western blotting. BAE cells (A and B) and BAE cells transduced with viral VEGFR-2 (C and D) were left unstimulated or stimulated with either 50ng/ml VEGF or 1 μM PMA for the times indicated. VEGFR-2 was immunoprecipitated from resting and stimulated cells and the level of receptor phosphorylation was determined by western blotting using an anti-phosphotyrosine antibody (pY99). The level of receptor in the immunoprecipitates was analysed by blotting for VEGFR-2 (A and C). The phosphorylation of ERK, in response to VEGF stimulation, was determined from whole cell lysates of wild-type BAE cells or BAE cells expressing viral VEGFR-2 (B and D). Arrows indicate positions of VEGFR-2 (A and C) or ERK1/2 (B and D). Blots of wild type BAE cells are representative of two independent experiments. Blots of BAE cells transduced with viral VEGF receptor-2 are from a single experiment.

3.2.3 *Porcine Aortic Endothelial (PAE) cells*

Wild type PAE cells originate from an immortalised cell line and do not contain either VEGF receptor-1 or VEGF receptor-2 (Miyazono et al., 1987). In addition we also have available a stably transfected cell line expressing higher levels of VEGF receptor-2 only. These cells are easy to transfect, which facilitates the over-expression of proteins in the VEGF signal transduction pathway. The phosphorylation of VEGF receptor-2 in PAE cells shows that the peak of receptor activation occurs at 5 minutes of VEGF stimulation (Figure 3.3A), a little later than detected for HUVE cells (Figure 3.1A), but this also coincides with the time point at which the receptor level begins to decrease.

The profiles of ERK phosphorylation are also very similar between PAE cells expressing VEGF receptor-2 (Figure 3.3B) and HUVE cells (Figure 3.1B). In HUVE cells maximal phosphorylation occurs at 5 minutes whereas in PAE cells expressing VEGF receptor-2 this occurs at 10 minutes. These levels decline however very rapidly and return to levels close to those seen in basal cells after 20 minutes.

The localisation of GFP was observed in wild type PAE cells and PAE cells expressing VEGF receptor-2 (Figure 3.4). In unstimulated and stimulated cells GFP is mainly localised to the cell nucleus with some localisation in the cytoplasm. Importantly GFP does not appear to localise to the plasma membrane under resting or stimulated conditions.

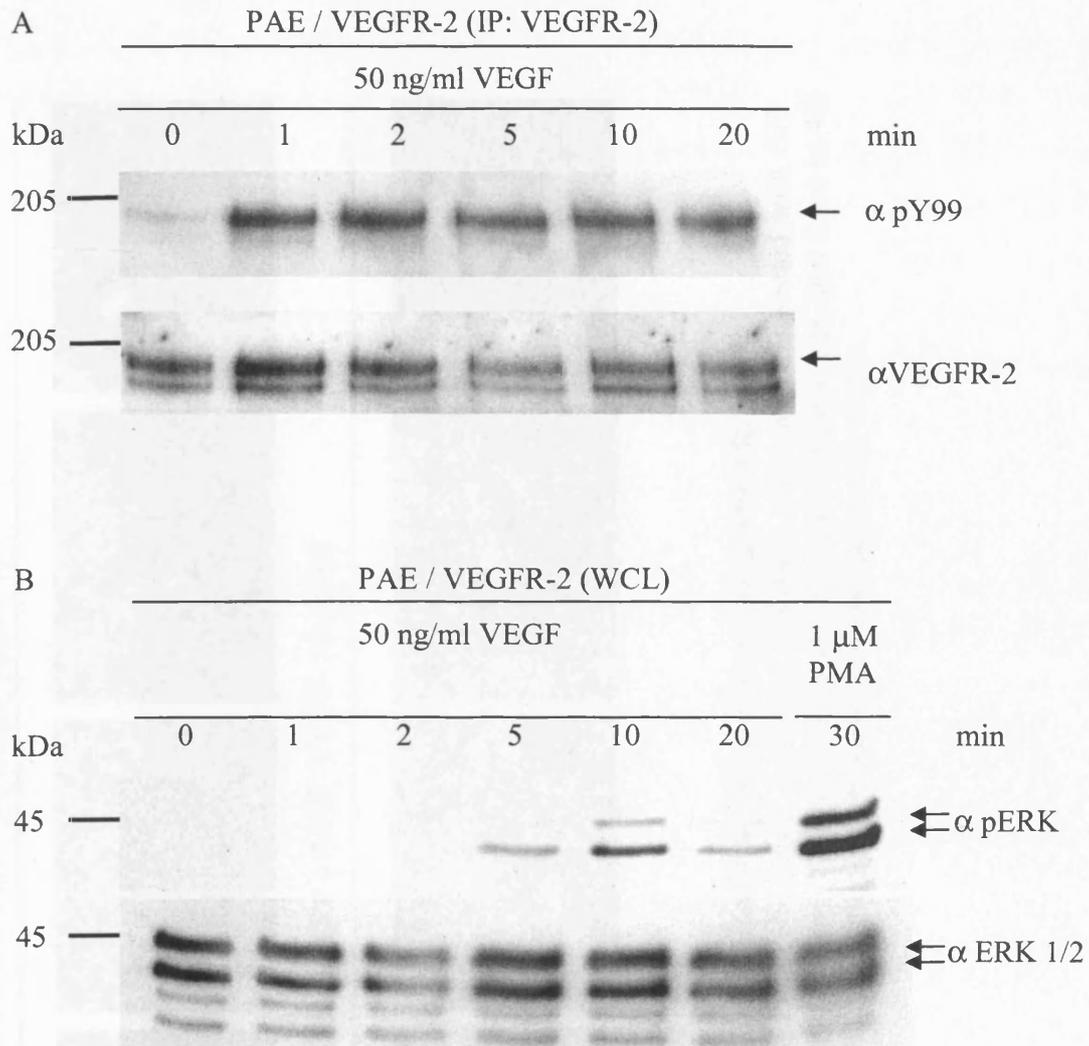


Figure 3.3 - Detection of VEGFR-2 and ERK phosphorylation levels in PAE cells expressing VEGFR-2 by western blotting.

PAE cells expressing VEGFR-2 were left unstimulated or stimulated with either 50ng/ml VEGF or 1 μM PMA for the times indicated. VEGFR-2 was immunoprecipitated from resting and stimulated cells and the level of receptor phosphorylation was determined by western blotting using an anti-phosphotyrosine antibody (pY99). The level of receptor in the immunoprecipitates was analysed by blotting for VEGFR-2 (A). The phosphorylation of ERK, in response to VEGF stimulation, was determined from whole cell lysates using an antibody detecting phosphorylated, active ERK(B). For comparison the blot was stripped and reprobed with an antibody recognising ERK independent of phosphorylation. Arrows indicate positions of VEGFR-2 (A) or ERK 1/2 (B). These blots are representative of at least three independent experiments.

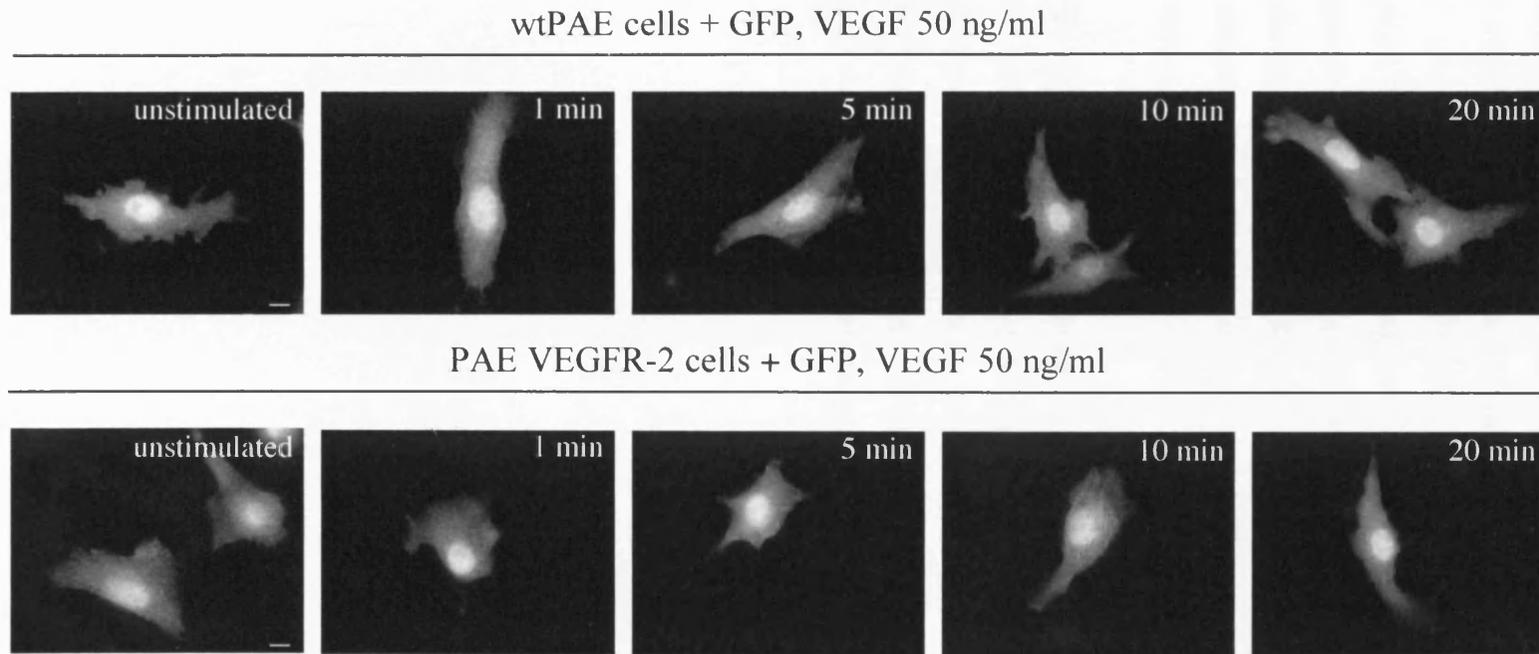


Figure 3.4. Localisation of GFP in wild type PAE cells and in PAE cells expressing VEGFR-2

Images shown are of wild type PAE cells and PAE cells stably expressing VEGFR-2 transiently transfected with GFP. Serum starved cells were unstimulated or stimulated with 50 ng/ml VEGF for the times indicated and then fixed in 4% paraformaldehyde. These are representative cells from a single experiment. Scale bar represents 10 μ m.

3.2.4 *Human Embryonic Kidney (HEK)-293 cells*

HEK-293 cells are not endothelial cells but can be transfected very easily at high efficiency to express multiple proteins of interest at high levels (Thomas and Smart, 2005). This is particularly useful for biochemical analysis such as co-immunoprecipitation. VEGF receptor-2, when expressed in HEK-293 cells, is phosphorylated by VEGF although there is also a slight basal level of phospho-VEGF receptor-2 in unstimulated cells (Figure 3.5A), which probably reflects spontaneous dimerisation due to very high receptor levels. The level of phospho-VEGF receptor-2 peaks at about 10 minutes and then decreases after 15 minutes of stimulation, which again is later than that seen in HUVE cells (Figure 3.1A), but follows the same pattern.

We were able to detect phospho-ERK in response to VEGF indicating that these cells contain the components necessary to signal through the MAP kinase pathway from the VEGF receptors. Figure 3.5B shows that phospho-ERK can be detected after 10 minutes of VEGF stimulation; this represents the peak of phosphorylation as after this phosphorylation levels decrease to basal levels, which is complete after 20 minutes of VEGF stimulation.

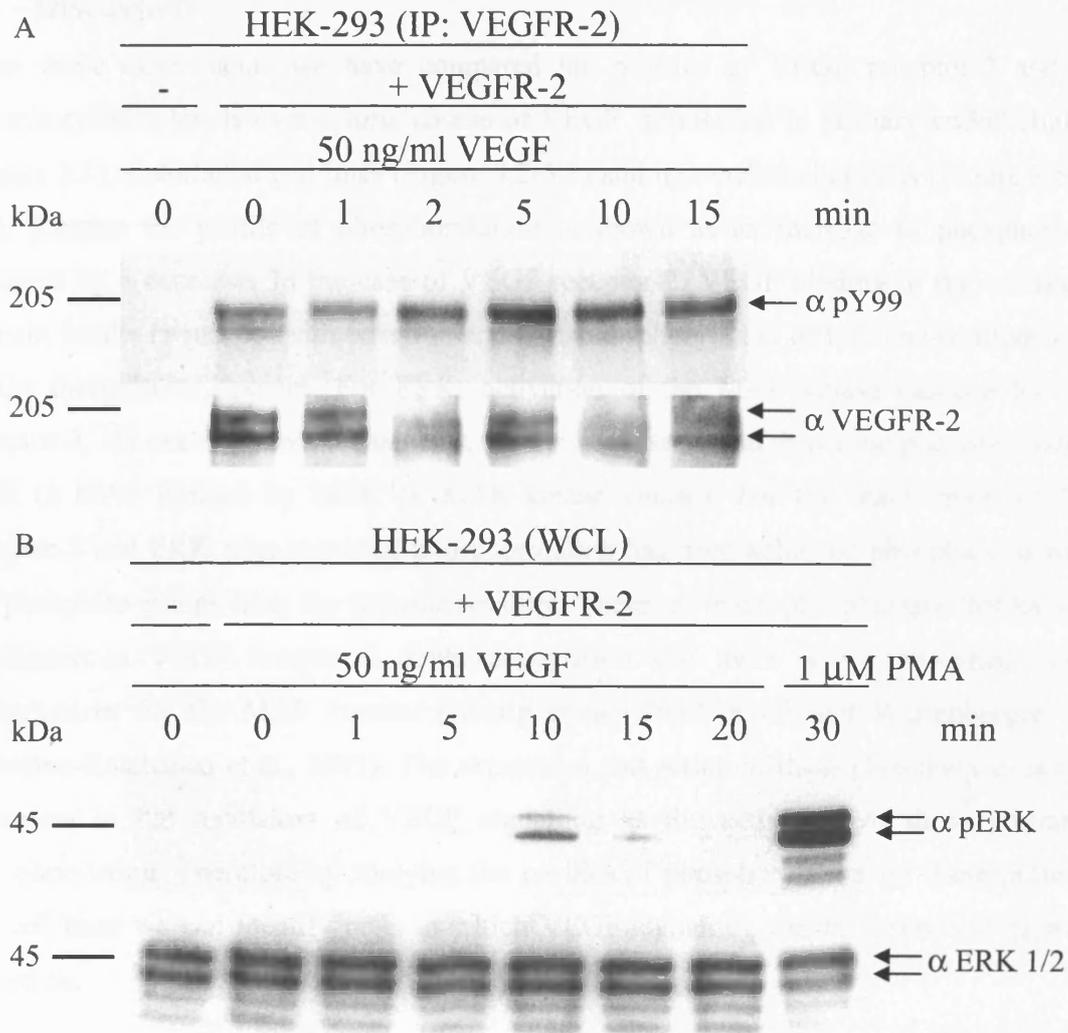


Figure 3.5 - Detection of VEGFR-2 and ERK phosphorylation levels in HEK-293 cells expressing VEGFR-2 by western blotting.

HEK-293 cells expressing VEGFR-2 were left unstimulated or stimulated with 50ng/ml VEGF or 1μM PMA for the times indicated. VEGFR-2 was immunoprecipitated from resting and stimulated cells and the level of receptor phosphorylation was determined by western blotting using an anti-phosphotyrosine antibody (pY99). The level of receptor in the immunoprecipitates was analysed by blotting for VEGFR-2 (A). The phosphorylation of ERK, in response to VEGF stimulation, was determined from whole cell lysates (B). Unstimulated, wild-type HEK-293 cells were used as a negative control. Arrows indicate positions of VEGFR-2 (A) or ERK 1/2 (B). These blots are representative of at least two independent experiments.

3.3 - Discussion

From these experiments we have compared the profiles of VEGF receptor-2 and ERK phosphorylation levels over a time course of VEGF stimulation in primary endothelial cells (Figure 3.1), endothelial cell lines (Figure 3.2-3.3) and non-endothelial cells (Figure 3.5). For both proteins the profile of phosphorylation is shown as an increase in phosphorylation followed by a decrease. In the case of VEGF receptor-2, VEGF binding to the extracellular domain results in receptor dimerisation and transphosphorylation of tyrosine residues located in the intracellular domain. For ERK, activation of the MAP kinase cascade by VEGF receptor-2, via one or more mechanisms, results in tyrosine and threonine phosphorylation of ERK (a MAP kinase) by MEK (a MAP kinase kinase). For the inactivation of VEGF receptor-2 and ERK after maximal phosphorylation has been achieved phosphatases remove the phosphate groups from the tyrosine residues. There are many phosphatases that have been implicated in VEGF receptor-2 dephosphorylation and there is an individual class of phosphatases for the MAP kinases (Huang et al., 1999; Kroll and Waltenberger, 1997; Toledano-Katchalski et al., 2003). The expression and action of these phosphatases is just as important to the regulation of VEGF signalling as the activation of these proteins by phosphorylation. Therefore by studying the profiles of phosphorylation for these proteins in the cell lines we can identify cells in which VEGF signalling can be turned off as well as turned on.

HUVE cells, as primary cells, are a convenient *in vitro* alternative to *in vivo* endothelial cells; however experiments are limited when using these cells. The over expression of exogenous proteins in HUVE cells using a nucleofection system for example is inefficient and inconsistent. Co-immunoprecipitation experiments require proteins to be expressed at high levels and the irregularity with regards to expressing tagged and/or mutated proteins in HUVE cells makes these experiments difficult. In the experiments described within this dissertation we have therefore used HUVE cells where possible, and for other experiments we have used a representative cell line that can be engineered to over-express components of the VEGF receptor-2 signalling pathway. PAE cells were chosen as representative cells. In particular, PAE cells are useful for microscopy work and signalling components tagged to GFP have been over-expressed in these cells, as they are relatively easy to transfect. In addition, for

proteins of particular interest we have expressed wild type and mutant proteins in these cells to determine the relative importance of certain domains or amino acids.

The BAE cell line showed a typical response to VEGF when analysing the profile of phospho-VEGF receptor-2 in comparison to that detected in HUVE cells. The levels of the receptor are also down regulated in these cells with prolonged VEGF stimulation. In BAE cells over-expressing VEGF receptor-2 through use of viral delivery we also observed a time-dependent increase and peak of phospho-VEGF receptor-2 levels. However this cell line, with and without the over-expression of VEGF receptor-2 by viral infection, displays a high level of basal MAP kinase activity, and the level of active ERK does not appear to be regulated very efficiently in a VEGF-dependent manner. The high basal activity of ERK in these cells may be due to over-expression of secreted growth factors resulting in autocrine and paracrine signalling, or expression of kinases with unusually high activity, or conversely expression of phosphatases with poorer activity. Irrespective of the mechanism, the high level of activity of signalling pathways in serum starved BAE cells may complicate the analysis of VEGF induced signalling.

The PAE cell line expressing VEGF receptor-2 responds to VEGF stimulation giving results that are very close to those obtained for HUVE cells, although responses are a little delayed. The maximum level of phospho-VEGF receptor-2 occurs at 1 and 5 minutes in HUVE and PAE cells respectively, the level of phospho-VEGF receptor-2 declines after these time points in both cell types, the level of the receptor decline throughout the time course indicating that the receptors are down regulated in response to VEGF and the profiles for ERK activation are also very similar. The availability of the wild type PAE cell line, in addition to PAE cells that express VEGF receptor-2, permits the comparison of two cell lines that differ only in the absence or presence of the VEGF receptor-2.

The generation of DNA constructs, to add a GFP tag to proteins of interest, has allowed the expression and visualisation of these tagged proteins in cells. Another advantage of the PAE cell lines is that they are easily transfected which allows us to study GFP-tagged proteins and to introduce mutant proteins in these cells. Control experiments have been carried out however to ensure that VEGF does not affect the localisation of GFP. The expression of GFP alone in wild type PAE cells and PAE cells expressing VEGF receptor-2 shows that this protein is mainly localised in the cell nuclei with a little localised to the cytoplasm (Figure

3.4). The localisation of GFP does not appear to change as cells are stimulated with VEGF, indicating that GFP does not associate with proteins that re-localise in response to stimulation. GFP does not appear to localise to the plasma membrane in these cells, under the conditions studied, which is a crucial when considering the conclusions that we would like to make from observations of protein re-localisation in response to VEGF. These observations suggest that this is a good cell line in which to study the re-localisation of GFP-tagged proteins in response to VEGF.

HEK-293 cells transiently transfected with VEGF receptor-2 showed peak phosphorylation of VEGF receptor-2 after 10 minutes of VEGF stimulation, which coincides exactly with maximal phosphorylation of ERK (Figure 3.5). Some reports suggest that VEGF is unable to signal to the MAP kinase pathway in non-endothelial cells such as fibroblasts (Takahashi and Shibuya, 1997). HEK cells are not endothelial cells and may lack some components specific to VEGF, or endothelial cell signalling so therefore they must be used with caution. The ability of VEGF receptor-2 to activate ERK in these cells however suggests that essential components are present. A major limitation with HEK 293 cells is that the morphology of these cells is not ideal for microscopy. GFP-tagged proteins can be expressed at high levels in HEK 293 cells however the determination of the cellular localisation of proteins is difficult because the nucleus of these cells occupies the majority of the cytoplasm. As many of our aims include the characterisation of protein localisation to the plasma membrane this cell line proved unsuitable for this study.

In conclusion HUVE cells are the preferred choice of endothelial cells and have therefore been used in this study whenever possible. These cells express both VEGF receptor-1 and VEGF receptor-2 and are the best cells available in which to study VEGF signalling. HUVE cells are difficult to work with however, as duplicate experiments often give variable results due to the different batches and passages of cells. In addition the expression of exogenous proteins using conventional transfection methods is inefficient in HUVE cells and the success of nucleofection is unpredictable. PAE cells expressing VEGF receptor-2 show a receptor phosphorylation profile that is similar to that of VEGF receptor-2 expressed in HUVE cells. In addition different batches and passages of cells show more consistent results and they are relatively easy to transfect. PAE cells expressing VEGF receptor-2, and engineered to express other proteins of interest, have therefore been used as an alternative to HUVE cells in studying signalling via VEGF receptor-2.

Chapter 4

Signalling via PLC γ 1 and PI3K

Chapter 4 – Signalling via PLC γ 1 and PI3K

4.1 – Introduction

As mentioned previously we sought to clarify the involvement of Phosphatidylinositol 3-kinase (PI3K) in VEGF-receptor-2-mediated signalling. In the previous chapter we identified PAE cells expressing VEGF receptor-2 as a representative cell line in which to study VEGF signalling, and here we show that in VEGF stimulated PAE/VEGF receptor-2 cells VEGF receptor-2 associates with the downstream signalling proteins, phospholipase C γ 1 (PLC γ 1) and the p85 subunit of PI3K, resulting in their activation. In this chapter we use the association of PLC γ 1 with VEGF receptor-2 as a positive control and as a guide to the best possible results that we can expect from our experimental systems. From these test experiments we have learned essential details about the PAE/VEGF receptor-2 cell line, such as, the number of cells required to provide enough protein for co-immunoprecipitation studies and the morphology of cells transfected with GFP-tagged proteins. The knowledge gained from these positive control experiments has enabled us to make judgements about the involvement of p85 and PI3K in VEGF receptor signalling. The involvement of PI3K in VEGF signalling is generally accepted however there are elements of the signalling mechanisms that remain controversial. Here we show that the p85 subunit of PI3K is found in a complex with VEGF receptor-2 and we show by the localisation of a reporter protein the activation of PI3K at the plasma membrane in VEGF stimulated cells. In addition we show the localisation of p85 at focal adhesions in resting and stimulated cells.

Signalling via PLC γ 1 to initiate DNA synthesis is well established and is currently the most characterised downstream signalling pathway of VEGF signalling (Sakurai et al., 2005; Takahashi et al., 2001). More recent data has emerged however showing that PLC γ 1 is also required for tubulogenesis and differentiation of endothelial cells (Meyer et al., 2003). Both lines of evidence show the recruitment of PLC γ 1 to VEGF receptor-2, although the tyrosine residues implicated in this recruitment were different, and both also show phosphorylation of PLC γ 1. We feel confident that this is a good signalling molecule to use as a positive control. We show similar profiles for association of PLC γ 1 with phospho-VEGF receptor-2 and phosphorylation of PLC γ 1 in response to VEGF in both HUVE cells and PAE cells expressing VEGF receptor-2. GFP-PLC γ 1 also shows some co-localisation with VEGF receptor-2 in stimulated PAE cells.

4.1.1 *Signalling via PLC γ 1*

Tyrosine 1175 is a major autophosphorylation site and is contained within a YIVL motif, a possible binding site for the SH2 domain of PLC γ 1. Tyrosine 801 and the three amino acids that follow, YLSI, also constitutes a potential binding site for the SH2 domain of PLC γ 1. Takahashi et al propose that a single autophosphorylation site, tyrosine 1175, is essential for VEGF dependent activation of PLC γ 1 (Takahashi et al., 2001). A mutant receptor in which tyrosine 1175 is mutated to phenylalanine shows an inability to phosphorylate PLC γ 1 and shows a significantly reduced level of MAP kinase phosphorylation and DNA synthesis. In support of this an antibody to phosphotyrosine 1175 and its SH2 domain consensus sequence, when injected into primary sinusoidal endothelial cells, reduces the ability of these cells to incorporate BrdU into newly synthesised DNA. This antibody is specific for the region in VEGF receptor-2, which is distinguishable from phosphorylation sites in other receptors that bind PLC γ 1. This paper demonstrates that phosphotyrosine 1175 is crucial for engaging VEGF-induced stimulation of DNA synthesis and shows that PLC γ 1 is activated through binding to this site. Although Takahashi et al show that tyrosine 1175 is important for signalling via the MAP kinase pathway (Takahashi et al., 2001) and ERK can be activated via PLC γ 1-mediated activation of PKC (Takahashi et al., 1999b), it is possible that the decrease in MAP kinase activation and DNA synthesis could be due to the inhibition of another pathway involving a protein, other than PLC γ 1, binding to this site. The adapter protein Sck for example has been shown to associate with tyrosine 1175 (Warner et al., 2000) and may provide an additional mechanism to couple VEGF receptor-2 to the MAP kinase pathway.

Meyer et al, using a VEGF receptor-2 chimeric system in which VEGF receptor-2 mutants are selectively activated by CSF-1, suggest that activation of PLC γ 1 promotes endothelial cell differentiation and is mediated by binding to tyrosine 1006 of VEGF receptor-2 (Meyer et al., 2003). This group show that mutation of tyrosines 779 and 1173 (the mouse equivalent to residues 801 and 1175) did not affect the ability of VEGF receptor-2 to activate PLC γ 1. Instead they propose that tyrosine 1006, located in the kinase domain of mouse VEGF receptor-2, recruits PLC γ 1 and promotes tubulogenesis and differentiation, thus preventing proliferation. A tyrosine to phenylalanine mutant of 1006 is still able to signal to MAP kinase therefore the kinase activity of the receptor is not impaired. In fact this mutant showed a greater amount of proliferation as shown by ^3H thymidine incorporation but severely

impaired the ability of this receptor to stimulate endothelial cell differentiation and tubulogenesis.

4.1.2 *Signalling via PI3K*

Dayanir et al report that in CSF-1 stimulated PAE cells the p85 subunit of PI3K is also recruited to the same chimeric VEGF receptor-2 as that used by Meyer et al (Dayanir et al., 2001; Meyer et al., 2003). In this case phosphotyrosine residues 801 and 1175 in VEGF receptor-2 have been shown to be important for either direct, or indirect, associations between the receptor and p85, and are also required for the production of phosphatidylinositol 3-phosphate by PI3K. Although the amino acids surrounding these residues do not conform to the classical p85 binding sites, GST-tagged N-terminal and C-terminal SH2 domains of p85 formed stable complexes with the phosphorylated chimeric receptor. These tyrosine residues may therefore be novel p85 docking sites. The recruitment of p85 to VEGF receptor-2 following stimulation, and hence receptor phosphorylation, is controversial. Another group shows, by co-immunoprecipitation, a constitutive association of p85 with exogenously expressed VEGF receptor-2 in HUVE cells (Thakker et al., 1999). An increase in tyrosine phosphorylation of p85 and increased PI3K activity were detected on VEGF stimulation by *in vitro* kinase assays suggesting that the p110 catalytic subunit is activated upon cell stimulation. Furthermore Qi et al report that there is no direct interaction between VEGF receptor-2 and PI3K (Qi et al., 1999). Immunoprecipitation using VEGF receptor-2, phosphotyrosine or p110 α antibodies and cell lysates from VEGF stimulated PAE cells expressing VEGF receptor-2 were subjected to an *in vitro* lipid kinase assay. Phosphatidylinositol was used as a substrate and PI3K activity was assessed by the production of phosphatidylinositol phosphate (PIP). VEGF stimulation led to a fourfold increase in PIP formation when using phosphotyrosine or p110 α antisera whereas no induction was seen in VEGF receptor-2 immunoprecipitates. Furthermore in co-immunoprecipitation experiments no complex formation was detected between VEGF receptor-2 and either p85 or p110 α . The specific sequence motif required for binding of the SH2 domain of p85, pTyr-X-X-Met, is absent in VEGF receptors-1 and -2. It is therefore conceivable that binding and/or activation are mediated by an intermediary protein and VE-cadherin, β -catenin, 125 PFAK and Src have been proposed as potential candidates.

4.1.3 *PH Domains*

Receptor-stimulated accumulation of PtdIns (3,4,5) P₃ by PI3K can very quickly lead to the recruitment of proteins containing a pleckstrin homology (PH) domain, a structural motif first defined in the cytoskeletal protein pleckstrin, which anchors it to the membrane. PH domains provide proteins with an affinity for membrane phospholipids and consequently they can be recruited to receptor-activated complexes at the plasma membrane. Many proteins containing PH domains are therefore targets of PtdIns (3,4,5) P₃ (Cullen and Chardin, 2000) and some examples of these targets include the small G proteins (Rho, Rac, Cdc-42 and Arf) and their guanine nucleotide exchange factors (Arno and Grp-1), which are activated by PtdIns (3,4,5) P₃ (Venkateswarlu et al., 1998a; Venkateswarlu et al., 1998b). PH domains have been identified in many proteins and this has revealed that different PH domains have distinct phospholipid-binding specificities thereby adding further to the complexity of cell signalling pathways.

Incubation of HUVE cells with VEGF leads to the activation of the anti-apoptotic kinase Akt. Akt is able to interact with PtdIns (3,4,5) P₃ via its PH domain (Franke et al., 1997) and PtdIns (3,4,5) P₃-activated protein kinase (PDK-1) (Alessi et al., 1997; Andjelkovic et al., 1997), another PH domain containing protein, phosphorylates, and hence activates, Akt. Activation of Akt occurs by phosphorylation of threonine 308 in its activation loop by PDK-1 (Alessi et al., 1997; Stephens et al., 1998; Stokoe et al., 1997) and of serine 473 in its C-terminus by PKD-2 (Alessi et al., 1997; Dong and Liu, 2005). Threonine 308 is basally phosphorylated in HUVE cells and VEGF stimulation does not increase this. Phosphorylation of serine 473 is detected in response to VEGF stimulation of HUVE cells however this is inhibited by PKC inhibitors, PKC down-regulation induced by PMA, wortmannin and LY294002, indicating that PKC-mediated phosphorylation of Akt is also PI3K dependent (Glikli et al., 2002). In addition VEGF-mediated phosphorylation of threonine 505 in the activation loop of PKC δ is inhibited by LY294002. Inhibition of PI3K, PKC α or PKC δ results in a strong reduction in branching tubulogenesis induced by VEGF.

PI3K activity is critically required for the regulation of endothelial cell survival, migration and mitogenesis, but not differentiation during angiogenesis in the chick embryo (Qi et al., 1999). In a representative primary cell culture model (bovine capillary endothelial cells) PI3K is activated and Akt is phosphorylated by VEGF and FGF-2. PI3K activity is required for the migration of, and synthesis of DNA in these cells, and although activity is not required for

tubular morphogenesis, survival of these structures is dependent on PI3K activity. PtdIns (3,4,5) P₃-mediated activation of Rac and subsequent actin reorganisation is likely to result in the motogenic effects of PI3K (Ridley et al., 1992), whereas the mitogenic effects are likely to require signalling pathways involving Ras (Joneson and Bar-Sagi, 1997) and many other signalling proteins (Varticovski et al., 1994). The Akt pathway can result in the phosphorylation of Caspase-9, BAD and FKHR1, which inhibit apoptosis and stimulate cell survival (Brunet et al., 1999; Cardone et al., 1998). Gerber et al show that VEGF signalling displays a high level of survival activity by inhibiting apoptosis of serum starved HUVE cells (Gerber et al., 1998). They propose that PI3K and Akt play a major part in this as the protective effects of VEGF are blocked by the PI3K inhibitors, wortmannin and LY294002, and by a dominant negative form of Akt. Furthermore this group show that a constitutively active form of Akt is sufficient for the survival of serum starved endothelial cells. Akt is also responsible for phosphorylating serine 1177 of eNOS resulting in its activation and increased NO production. Dimmeler et al show that inhibition of the PI3K/Akt pathway or mutation of serine 1177 prevents eNOS activation (Dimmeler et al., 2000). Also Fulton et al, by using adenovirus-mediated gene transfer, show that activated Akt increases basal NO release from endothelial cells and activation-deficient Akt attenuates NO production stimulated by VEGF (Fulton et al., 1999). NO produced by endothelial cells is responsible for maintaining systemic blood pressure, vascular remodelling and angiogenesis (Fulton et al., 1999; Morbidelli et al., 2003).

4.1.4 *Imaging signalling proteins*

In this study we have taken an imaging approach to visualise the cellular localisation of signalling proteins within resting and VEGF stimulated endothelial cells. In this chapter and the subsequent chapter we make use of DNA constructs that encode for DsRedII- and GFP-tagged proteins to observe changes in protein localisation between unstimulated and stimulated cells and in some cases the dynamics of proteins trafficking within live cells. DsRedII-tagged Grp-1 is used here as a reporter protein and facilitates the visualisation of PtdIns (3,4,5) P₃. We have therefore interpreted the localisation of this protein as the site of PI3K activity.

4.2 - Results

4.2.1 *GFP-PLC γ 1 co-immunoprecipitates, and shows some evidence of co-localisation, with VEGF receptor-2*

The probable association of PLC γ 1 with VEGF receptor-2 was investigated in HUVE cells, which express both proteins endogenously, and PAE cells expressing VEGF receptor-2, also transiently transfected with GFP-PLC γ 1. VEGF receptor-2 was immunoprecipitated from lysates of unstimulated and stimulated HUVE and PAE cells and proteins contained within the immunoprecipitates were resolved on an SDS-PAGE gel. Western blotting of whole cell lysates shows the expression of PLC γ 1, either endogenous or both endogenously and exogenously expressed proteins, in all cell samples (Figure 4.1). Immunoprecipitates show that PLC γ 1 and GFP-PLC γ 1 only associate with VEGF receptor-2 in stimulated cells. In HUVE cells phosphorylation of VEGF receptor-2 is evident after 0.5 minutes of VEGF stimulation and association of endogenous PLC γ 1 is evident after 1 minute of VEGF stimulation (Figure 4.1A). Similarly in PAE cells, the association of endogenous PLC γ 1 and GFP-tagged PLC γ 1 with VEGF receptor-2, and phosphorylation of the receptor occurs after 1 minute of VEGF stimulation (Figure 4.1C). In VEGF treated cells PLC γ 1 is also phosphorylated, as shown in whole cell lysate blots (Figure 4.1B and D). In HUVE cells endogenous PLC γ 1 is phosphorylated after 0.5 minutes of VEGF stimulation (Figure 4.1B) and in PAE cells expressing VEGF receptor-2 an increase in endogenous and GFP-tagged PLC γ 1 phosphorylation is evident after 1 minute of VEGF stimulation (Figure 4.1D). Figure 4.1 B and D also show an equal expression of PLC γ 1 proteins in all samples.

Figure 4.2 shows that in PAE cells expressing VEGF receptor-2, stimulation with VEGF for 1 minute results in the relocalisation of GFP-PLC γ 1 to areas of the cell periphery that resemble membrane ruffles. In addition PLC γ 1 co-localises with VEGF receptor-2 in these areas, which is supported by the co-immunoprecipitation data detailed above.

Figure 4.2 – A small proportion of GFP-PLC γ 1 co-localises with phospho-VEGF receptor-2 in areas of the cell periphery. PAE cells stably expressing VEGF receptor-2 were transiently transfected with GFP-PLC γ 1. Images shown are of cells that were serum starved (Unstimulated - A) and stimulated with 50 ng/ml VEGF for 1 minute (B). VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to rhodamine red. These are representative cells from a single experiment. The scale bar represents 10 μ m.

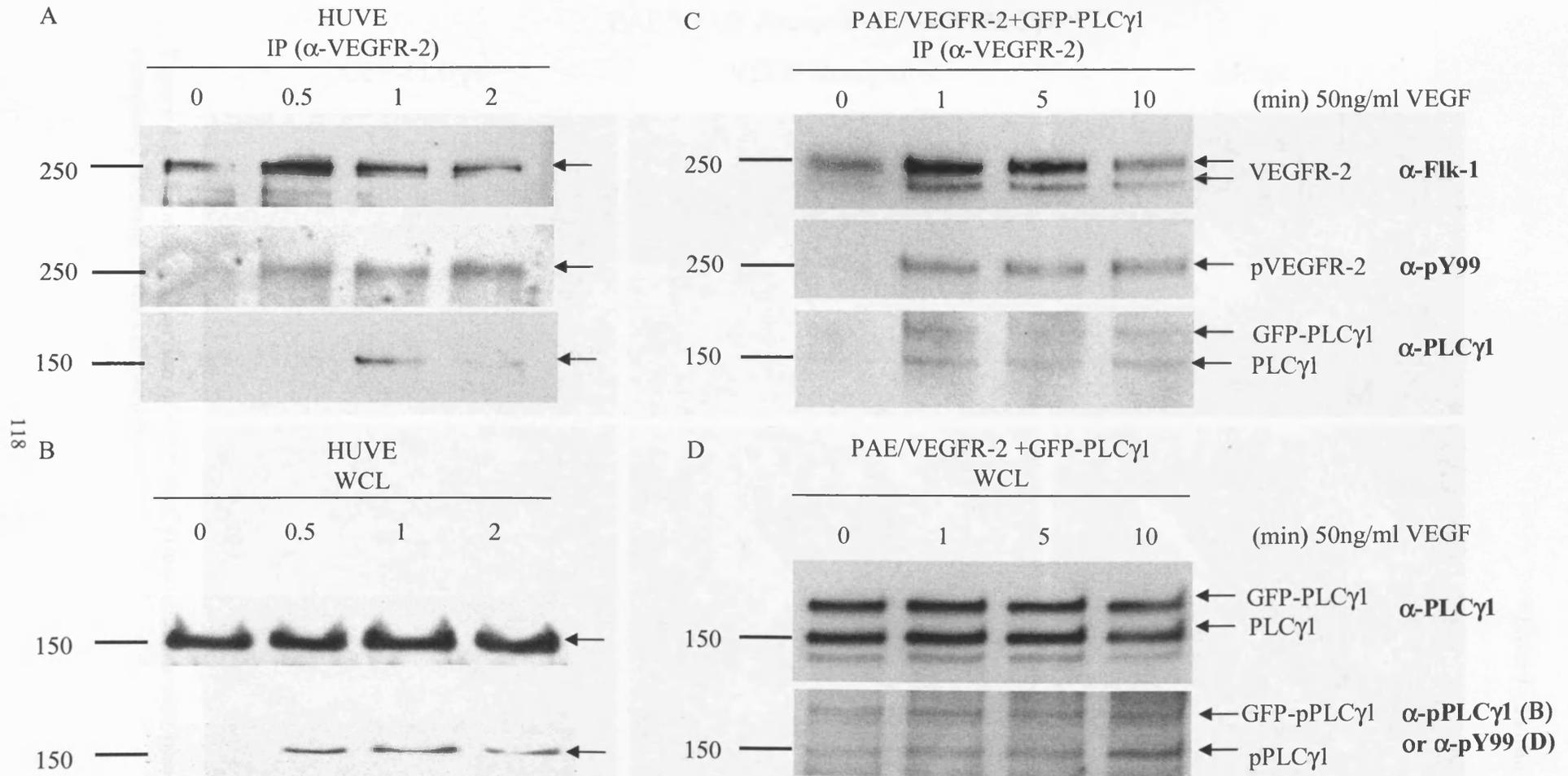


Figure 4.1 – GFP-PLC γ 1 and endogenous PLC γ 1 associate with phospho-VEGFR-2 Proteins in immunoprecipitates of VEGFR-2 from HUVE cells(A) or PAE/VEGFR-2 cells transfected with GFP-PLC γ 1(C) were separated using SDS-PAGE and were detected by western blotting. Panels A and C show that VEGFR-2 was precipitated, VEGFR-2 was phosphorylated in response to VEGF and PLC γ 1 (and GFP-PLC γ 1 in C) co-immunoprecipitated with phospho-VEGFR-2. Whole cell lysate blots (panels B and D) show expression of PLC γ 1 and GFP-PLC γ 1 and that PLC γ 1 (and GFP-PLC γ 1 in D) are phosphorylated in response to VEGF stimulation of HUVE (B) and PAE/VEGFR-2 (D) cells. These blots are from a single experiment.

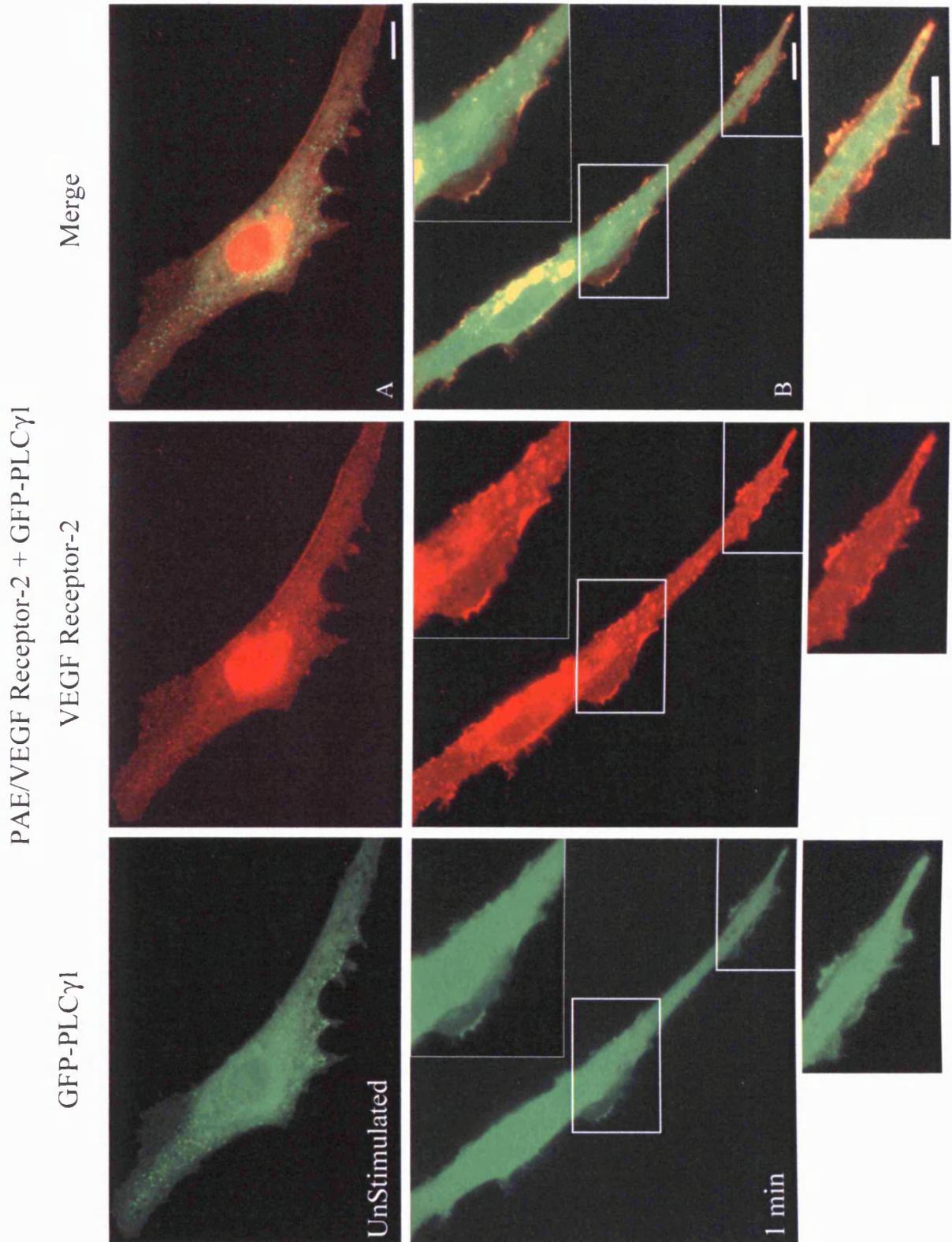


Figure 4.2 - A small proportion of GFP-PLC γ 1 co-localises with phospho-VEGF receptor-2 in areas of the cell periphery

4.2.2 *GFP-p85 associates with the EGF receptor-family member HER4 and localises to focal complexes in response to receptor activation*

PI3K signalling downstream of the EGF receptor family members has been well characterised (Grant et al., 2002; Hellyer et al., 2001) and here we show the association of p85 with activated ErbB4/HER4. Co-immunoprecipitation studies, in NIH3T3 cells expressing GFP-p85 and HER4, suggest an increase in the direct association between these proteins following HER4 activation by heregulin (Figure 4.3A). Phosphorylation of HER4 and an apparent decrease in HER4 protein levels in response to heregulin stimulation are also shown in whole cell lysate blots (Figure 4.3B).

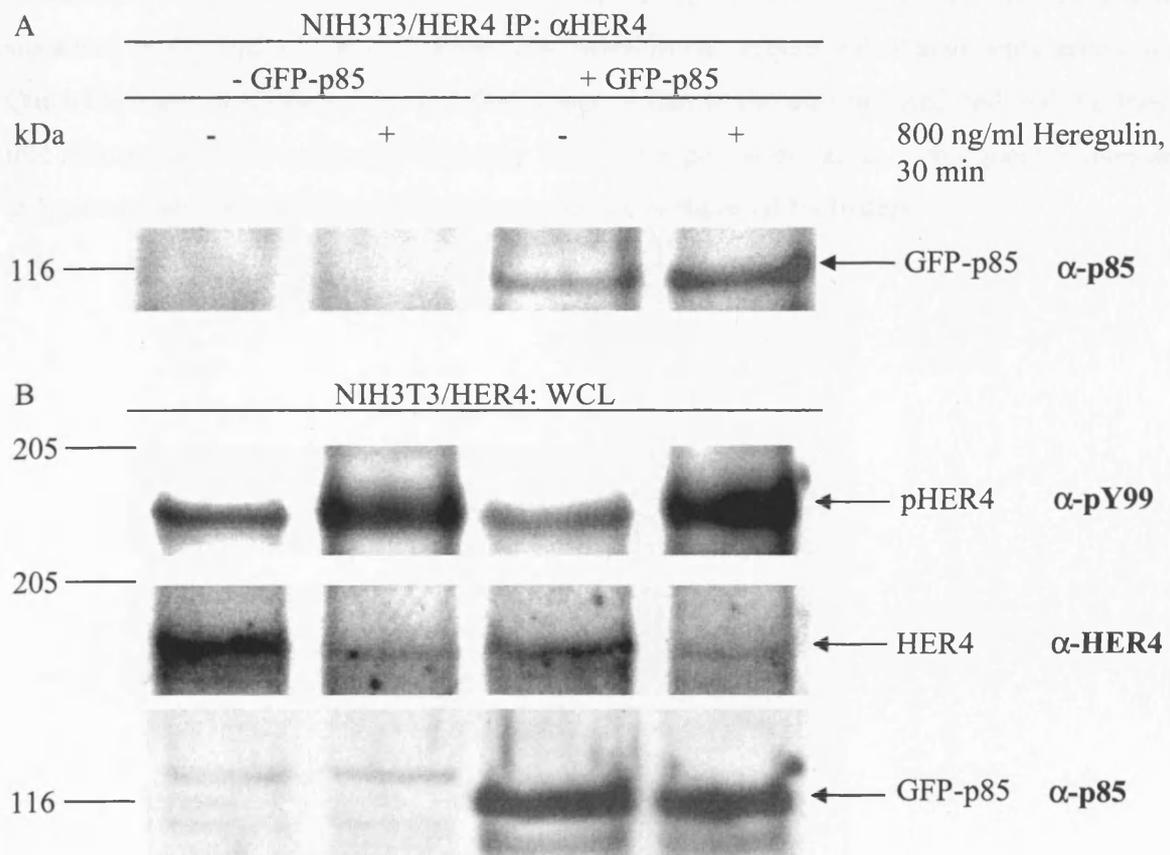
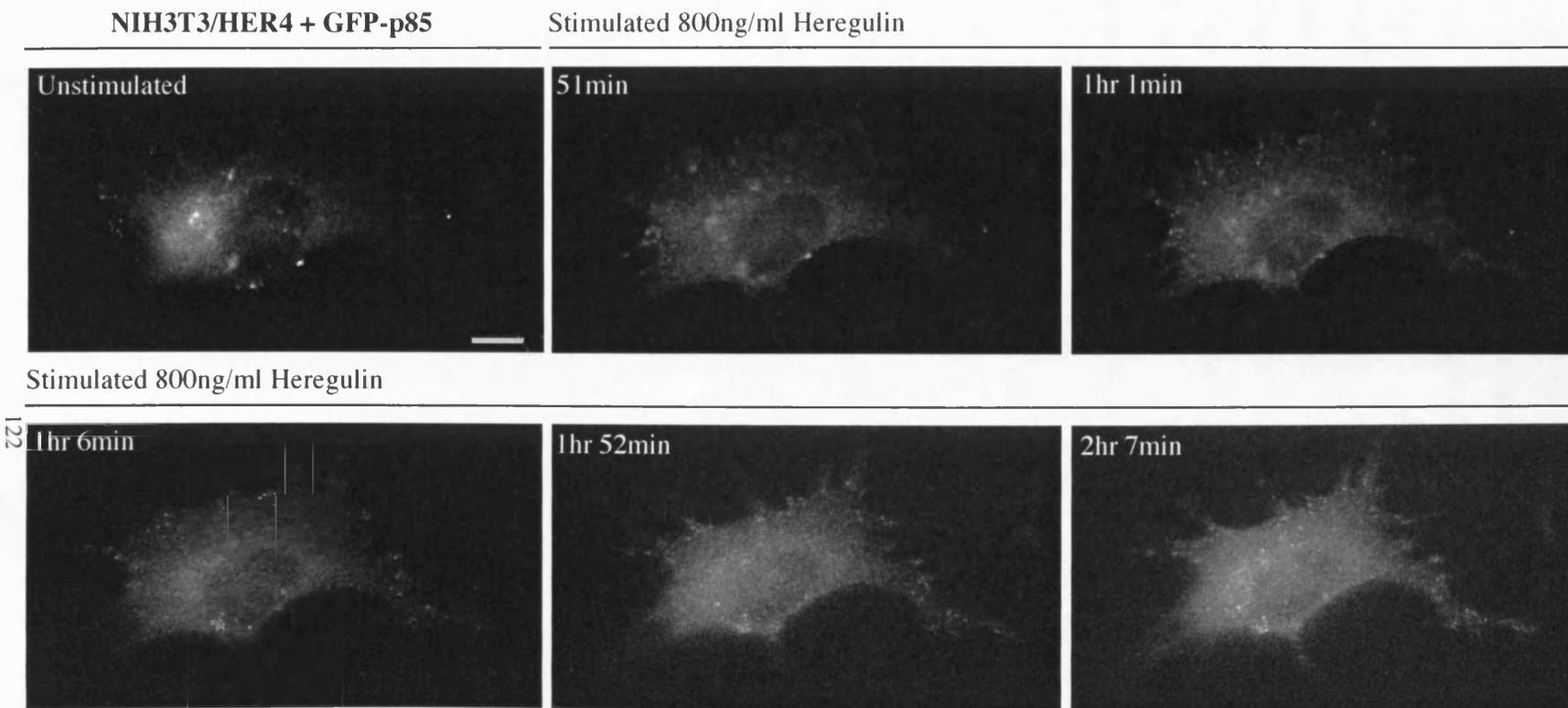


Figure 4.3 GFP-p85 associates with phospho-HER4. Proteins in immunoprecipitates of HER4 from NIH3T3/HER4 cells transfected with GFP-p85 (A) were separated using SDS-PAGE and were detected by western blotting. Panel A shows an increase in the level of GFP-p85 in immunoprecipitates from heregulin-stimulated cells compared with unstimulated cells. Whole cell lysate blots (panel B) shows phosphorylation of HER in response to heregulin stimulation and HER4 and GFP-85 protein levels. These blots are from a single experiment.

From personal communications with Prof. William Gullick, we were advised that stimulation of HER4 results in a prominent relocalisation of GFP-p85. We have therefore used this system as a positive control for protein relocalisation in response to activated growth factor receptors. In this study we have used a real time imaging technique to show the formation of GFP-p85 clusters in live NIH3T3 cells stably expressing HER4 and transiently transfected with GFP-p85. Figure 4.4 shows an unstimulated cell and images showing the re-localisation of p85 in the same cell during a 2 hour period of stimulation with 800ng/ml heregulin. The localisation of GFP-p85 remains relatively unchanged during the first 50 minutes post-stimulation and therefore images of the cell during this time period are not shown. The formation of GFP-p85 clusters was visualised during the next 15 minutes and these were sustained in the cell for up to 2 hours post-stimulation. Figure 4.4 is also represented as a QuickTime movie (Figure 4.5). The first image is that of the unstimulated cell and the frame that follows shows the cell approximately 50 minutes post stimulation. Subsequent images are at 5 minute intervals and show the dynamic nature of these GFP-clusters.



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Figure 4.4 - GFP-p85 localises to distinct clusters in heregulin stimulated NIH3T3 cells. NIH3T3 cells stably expressing HER4 were transiently transfected with GFP-p85. The images shown are of a serum starved cell (Unstimulated) and the same cell at various time-points post stimulation with 800 ng/ml heregulin. This is a representative cells from a total of five cells studied. The scale bar represents 10 μ m.

4.2.3 *A small amount of GFP-p85 can associate with phospho-VEGF receptor-2*

In experiments similar to those described above we show that a small amount of GFP-p85 associates with phospho-VEGF receptor-2 in PAE cells expressing VEGF receptor-2 (Figure 4.6). Immunoprecipitates from PAE cells expressing VEGF receptor-2 show that VEGF receptor-2 is phosphorylated after 1 minute of VEGF stimulation and that this coincides with co-immunoprecipitation of GFP-p85 (Figure 4.6A). Endogenous p85 and GFP-p85 is expressed equally in all samples in PAE/VEGF receptor-2 cells as shown by whole cell lysate blot (Figure 4.6B), however endogenous p85 was not detected in immunoprecipitates of VEGF receptor-2 in these cells. Additionally phosphorylation of GFP-p85, but not endogenous p85, was detected in stimulated cells (Figure 4.6B).

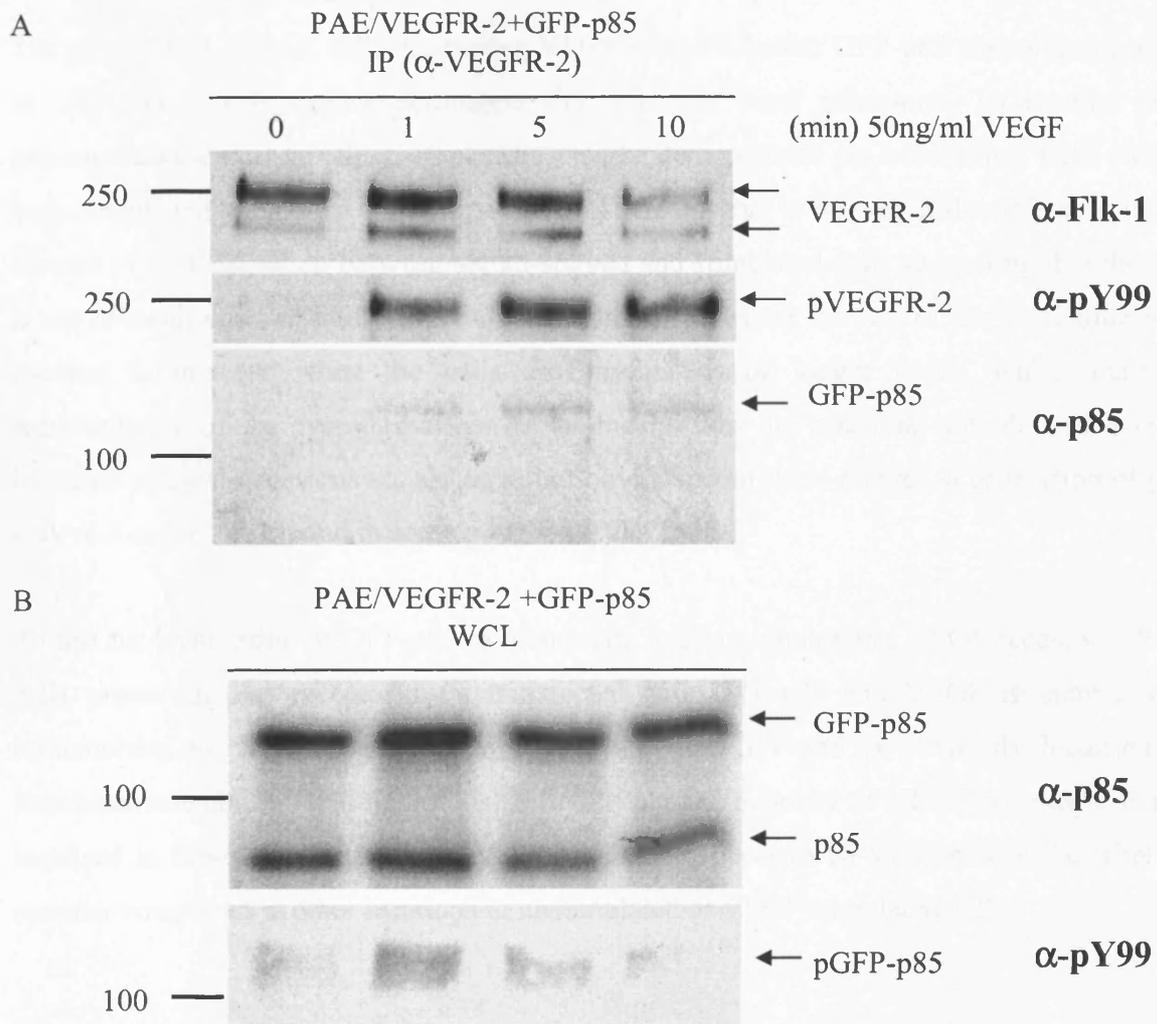


Figure 4.6 – GFP-p85 associates with phospho-VEGFR-2 Proteins in immunoprecipitates of VEGFR-2 from PAE/VEGFR-2 cells transfected with GFP-p85 (A) were separated using SDS-PAGE and were detected by western blotting. Panel A shows that VEGFR-2 was precipitated, VEGFR-2 was phosphorylated in response to VEGF and GFP-p85 co-immunoprecipitated with phospho-VEGFR-2. The whole cell lysate blots (B) show protein expression of endogenous p85 and GFP-p85 and that GFP-p85 is phosphorylated in response to VEGF in PAE/VEGFR-2 cells. These blots are from a single experiment.

4.2.4 *GFP-p85 co-localises with immunofluorescently labelled paxillin but not VEGF receptor-2*

The transfection of PAE cells expressing VEGF receptor-2 with GFP-p85 shows localisation of GFP-p85 to cell surface complexes that resemble focal adhesions. To confirm this, immunofluorescence labelling of paxillin clearly demonstrates co-localisation with p85 in both resting and stimulated cells (Figure 4.7). There appears to be very little difference in the amount of co-localisation between serum-starved and stimulated cells suggesting that the p85 is not re-localised in response to VEGF. Despite this however the size of the focal adhesions appears to increase when the cells are stimulated for longer times, which may be representative of an overall increase in co-localisation. In addition, not all GFP-p85 is localised to focal adhesions so, although not obvious from these images, a proportion of p85 may re-localise, or respond in some other way, to VEGF.

To test for localisation of GFP-p85 to membrane fractions containing VEGF receptor-2 PAE cells expressing this receptor were transfected with GFP-p85 and VEGF receptor-2 was immunolabelled. It is apparent from Figure 4.8 that GFP-p85 is obviously localised in structures resembling focal adhesions as before but the majority of VEGF receptor-2 is not localised to these structures, and GFP-p85 does not appear to co-localise with the labelled receptor complexes at other locations in unstimulated or VEGF stimulated cells.

PAE/VEGF Receptor-2 + GFP-p85

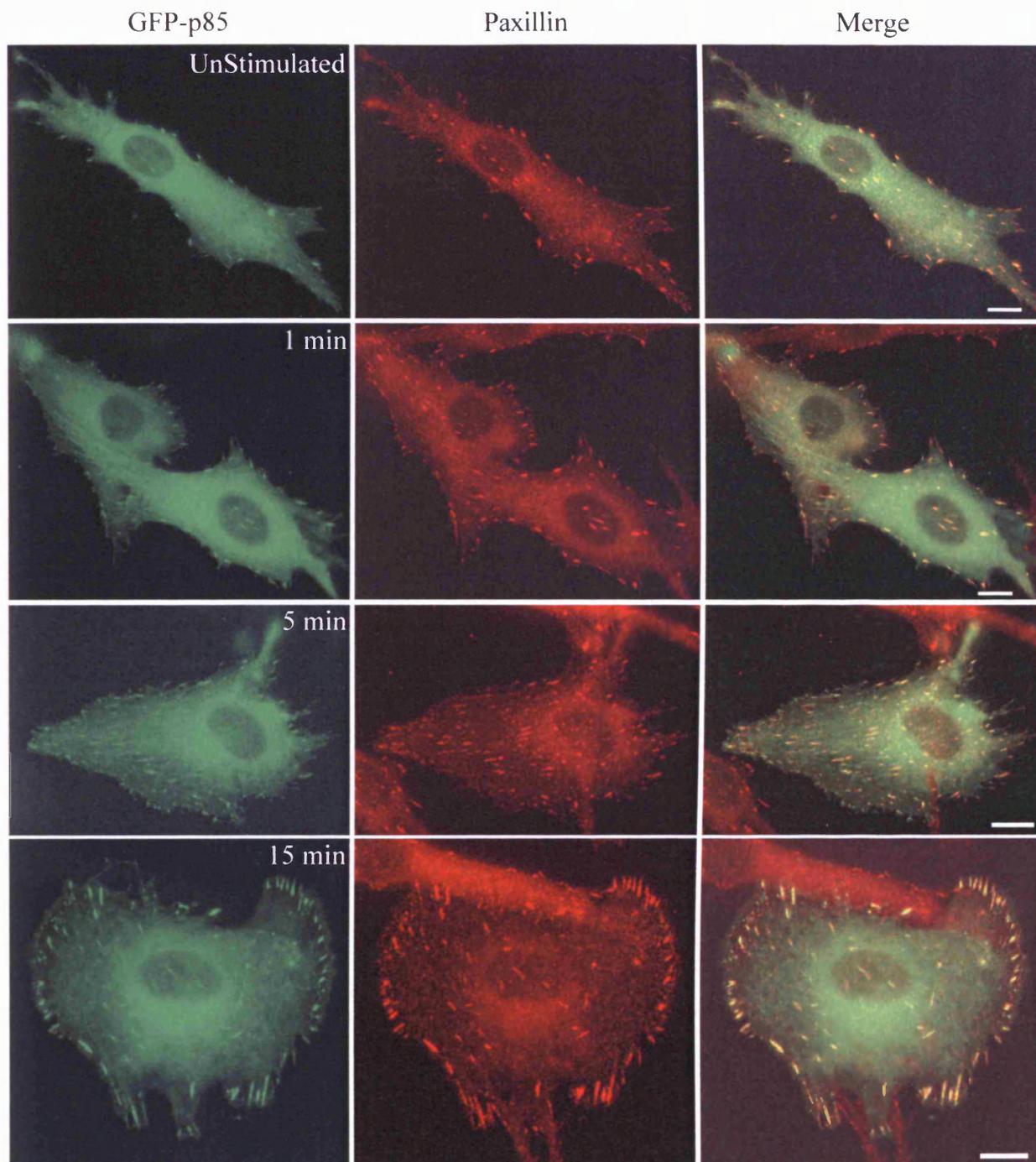


Figure 4.7 - GFP-p85 co-localises with immunofluorescently labelled paxillin in serum starved and VEGF stimulated PAE/VEGF receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were transiently transfected with GFP-p85. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for the times indicated. Paxillin is immunofluorescently labelled using an antibody against paxillin and a secondary antibody conjugated to Texas Red. These are representative cells from a single experiment. The scale bar represents 10 μ m.

PAE/VEGF Receptor-2 + GFP-p85

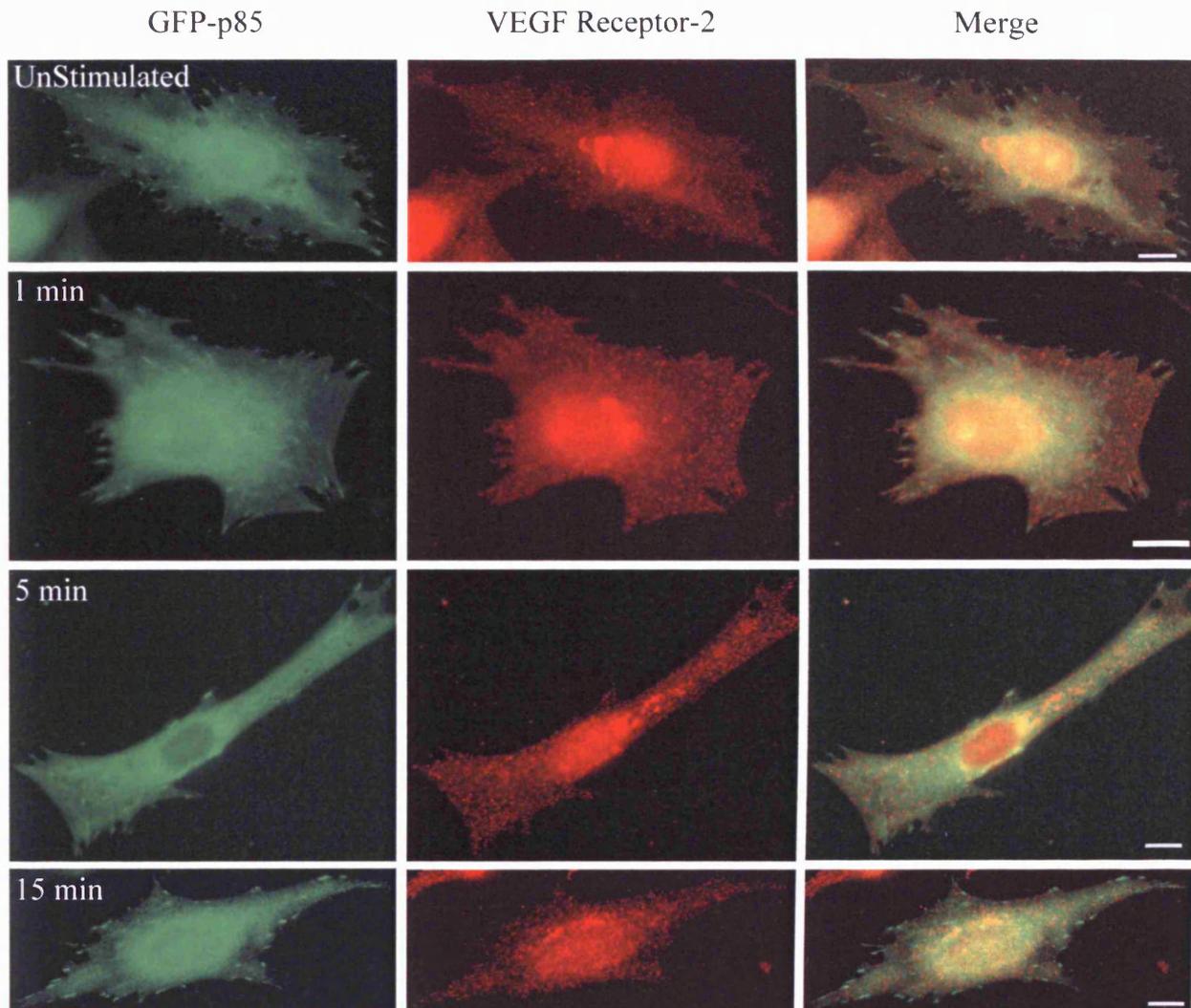


Figure 4.8 - GFP-p85 and VEGF receptor-2 do not co-localise in serum starved or VEGF stimulated PAE/VEGF receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were transiently transfected with GFP-p85. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for the times indicated. VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to Rhodamine Red. These are representative cells from a single experiment. The scale bar represents 10 μ m.

4.2.5 *The p85 subunit of PI3K co-localises with Grp-1, a reporter for the phospholipid product of PI3K activity*

DsredII-tagged Grp-1 has been used in this study as marker for PI3K activity. As Grp-1 detects the lipid product of PI3K (Klarlund et al., 1997; Klarlund et al., 1998; Klarlund et al., 2000), DsredII-Grp-1 and GFP-p85 were predicted to co-localise in VEGF stimulated PAE/VEGFR-2 cells. Figure 4.9 illustrates that both proteins are localised to the same areas of the cell membrane in stimulated cells (Figure 4.9B) however membrane localisation is not evident in unstimulated cells (Figure 4.9A).

4.2.6 *DsredIIGrp1 localises to the plasma membrane in VEGF stimulated PAE cells expressing VEGF receptor-2*

Grp-1 is a guanine nucleotide exchange factor for the ARF (ADP-ribosylation factors) family and has been implicated as a regulator of ARF-1 signalling (Klarlund et al., 1998). The Grp-1 protein contains a PH domain that binds, with high affinity, to the inositol head group of PtdIns (3,4,5) P₃. In addition it has been demonstrated that Grp-1 binds PtdIns (3,4,5) P₃ in preference to Phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5) P₂) and Phosphatidylinositol 3,4-bisphosphate (PtdIns (3,4) P₂) (Klarlund et al., 1998). Membrane localisation of Grp-1 has been demonstrated by the use of a GFP tag. The subcellular localisation of PtdIns (3,4,5) P₃ has been studied by the use of GFP-Grp-1 in PDGF or insulin stimulated 3T3-L1 adipocytes (Oatey et al., 1999) and nerve growth factor or epidermal growth factor stimulated PC12 cells (Venkateswarlu et al., 1998a). Here we used Grp-1 tagged to a red fluorescent protein, DsredII, and show that this protein is also localised to the membrane of stimulated endothelial cells.

The use of Grp-1 as a PtdIns (3,4,5) P₃ marker permits the indirect study of PI3K activity. This is especially useful because the current methods of PtdIns (3,4,5) P₃ detection only allow measurements of its total cellular synthesis and mass. To investigate the response of dsredIIGrp1 to VEGF, PAE cells expressing VEGF receptor-2 were transfected with dsredIIGrp1 recombinant DNA. Resting and stimulated cells were imaged to determine the localisation of the fluorescently tagged Grp1 protein. As figure 4.10A illustrates unstimulated cells show little membrane localisation of dsredIIGrp1. In stimulated cells dsredIIGrp1 is located to a greater extent at the membrane. The membrane localisation is more evident at the cell periphery however in some cells Grp-1 also appears to be localised to foci (Figure 4.10B).

PAE/VEGF Receptor-2 + DsRedII-Grp-1 + GFP-p85

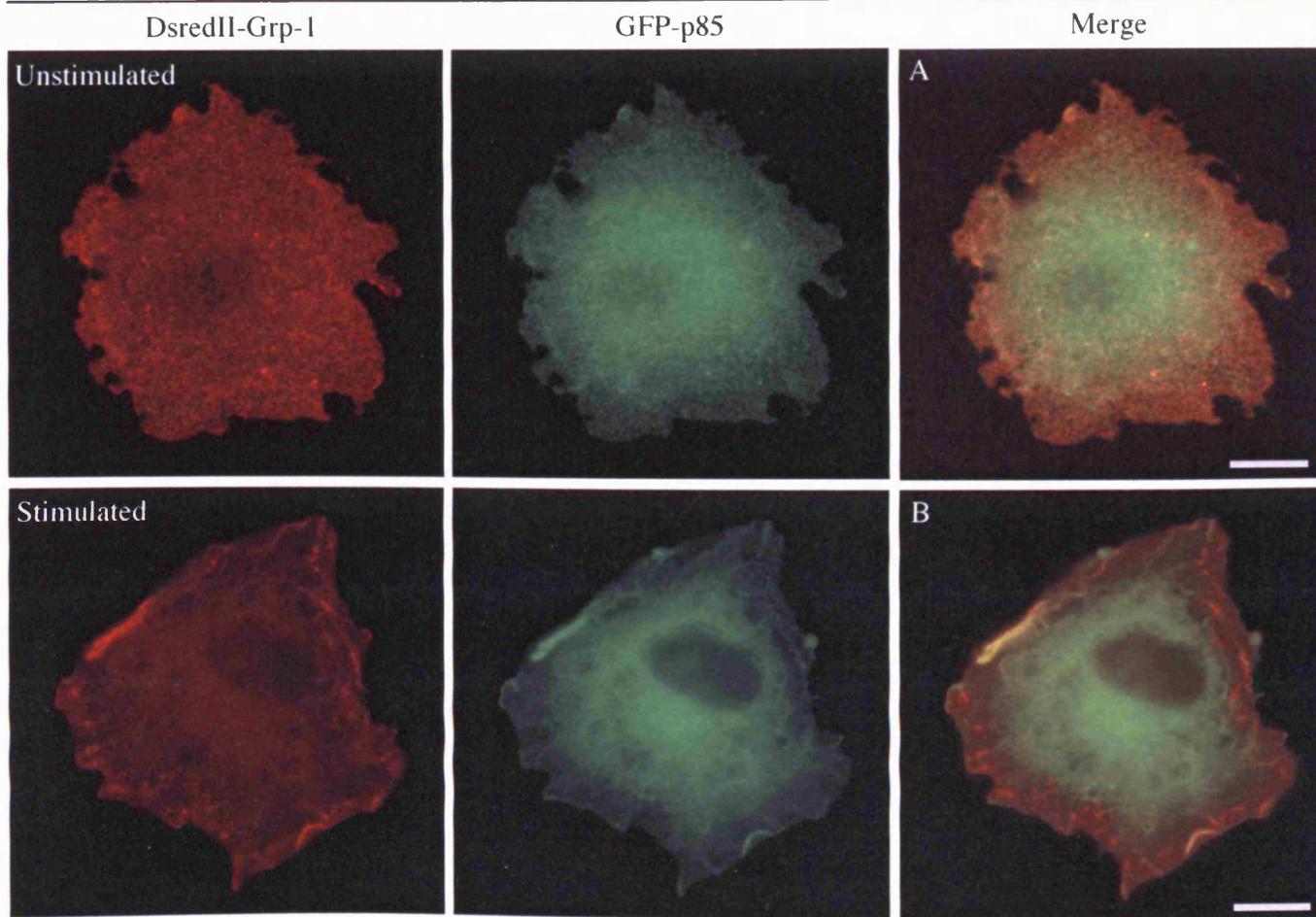


Figure 4.9 - DsRedII-Grp-1 co-localises with GFP-p85 at the plasma membrane of VEGF stimulated PAE/VEGF Receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were transiently transfected with DsRedII-Grp-1 and GFP-p85. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for 5 minutes. These are representative cells from a single experiment. Scale bar represents 10 μ m.

PAE/VEGF Receptor-2 + DsRedII-Grp-1

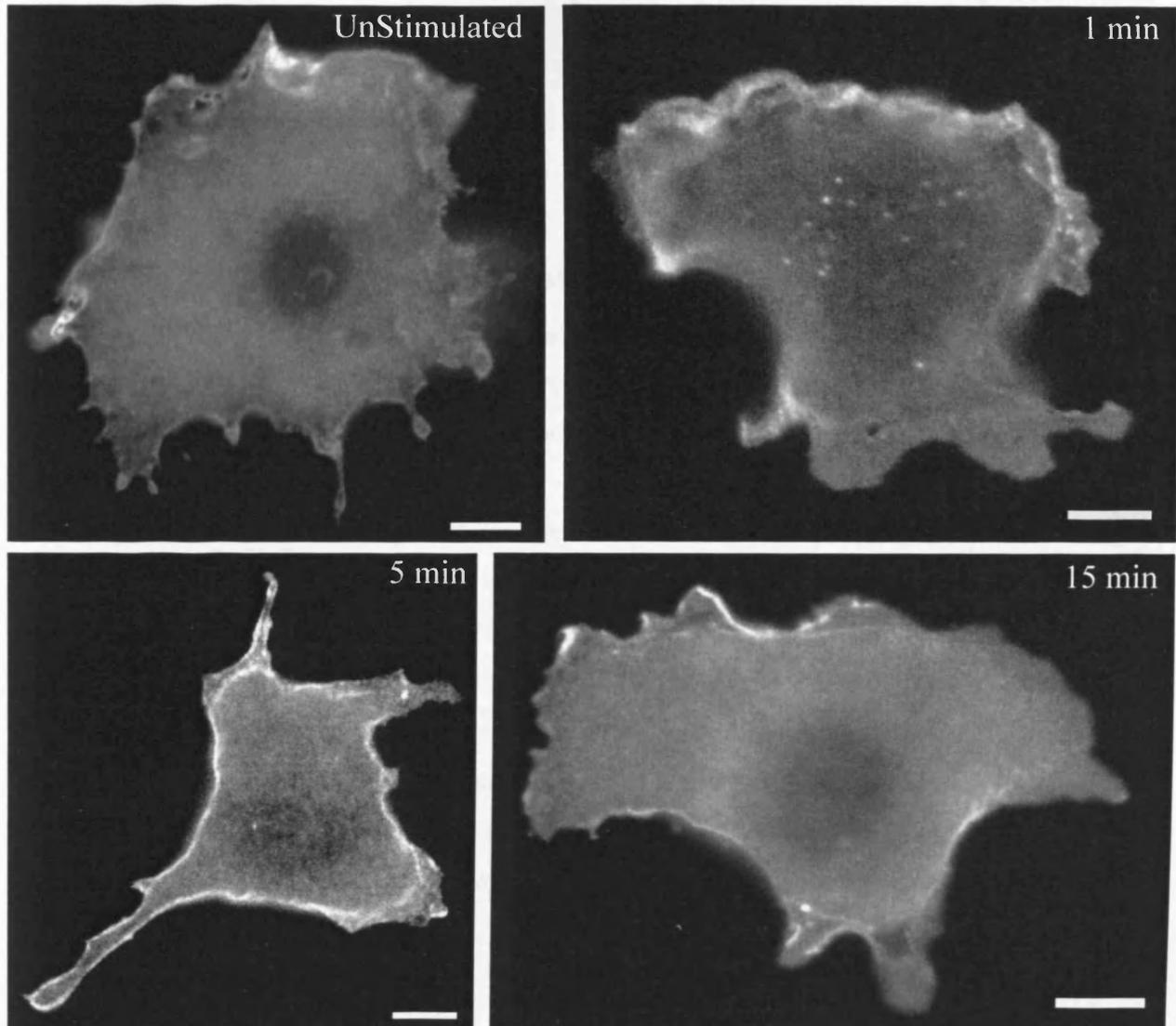


Figure 4.10 - DsRedII-Grp-1 re-localises to the plasma membrane in response to PtdIns (3,4,5) P_3 production. PAE cells stably expressing VEGF receptor-2 were transiently transfected with DsRedII-Grp-1. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for the times indicated. These are representative cells from a single experiment. The scale bar represents 10 μ m.

Real time imaging experiments have illustrated changes in Grp-1 localisation, in live PAE cells expressing viral VEGF receptor-2. A QuickTime movie composed of DsredII-Grp-1 images; taken every 5 seconds for 15 minutes, show punctate clusters of Grp1 that appear to shuttle to and from the membrane (Figure 4.11). VEGF was added to the cell media, to a final concentration of 200ng/ml, after 10-15 seconds of the experiment starting. This is a representative cell out of a total of two cells studied.

4.3 - Discussion

PLC γ 1 has been successfully used here to show that VEGF receptor-2 expressed in PAE cells is able to couple to downstream signalling proteins in a similar manner to the endogenous receptor in HUVE cells. The close correlation between co-immunoprecipitation and co-localisation data provides us with confidence that PAE cells expressing VEGF receptor-2 is a valid cell line in which to visualise fluorescently tagged signalling proteins. Co-immunofluorescence data (Figure 4.2) illustrates that co-localisation is usually most striking in areas where the receptor is more concentrated; membrane ruffles are therefore common sites for co-localisation. In areas in which there are high concentrations of protein co-localisation can therefore be used to compliment co-immunoprecipitation data showing a positive protein-receptor interaction. The co-immunoprecipitation (Figure 4.6) and co-localisation (Figure 4.8) data of p85 and VEGF receptor-2 however has highlighted that some associations shown by co-immunoprecipitation are not always detectable in fluorescence microscopy and that it is difficult to detect associations where only a small percentage of one component re-localises.

PI3K signalling is required in a fundamental cell survival mechanism by which VEGF activates anti-apoptotic signals in endothelial cells. VEGF inhibits apoptosis in HUVE cells in part by activating anti-apoptotic kinase Akt via a PI3K-dependent pathway (Glikli et al., 2002). In addition adhesion to the extracellular matrix induces the PI3K-dependent activation of Akt and over-expression of a constitutively active PI3K or Akt mutant inhibits detachment-induced apoptosis of epithelial cells (Khwaja et al., 1997). PI3K signalling has also been implicated in endothelial cell migration, a process that requires the turnover of focal adhesions and the reorganisation of the actin cytoskeleton. Signalling downstream of VEGF receptors activates the 125 P-FAK/paxillin pathway (Abedi and Zachary, 1997; Takahashi et al., 1999a), which is critical in mediating the turnover of focal adhesions, and the p38 MAP

kinase pathway (Masson-Gadais et al., 2003), which has been implicated in the reorganisation of the actin cytoskeleton (Rousseau et al., 2000; Rousseau et al., 1997). The importance of FAK for cell migration is evident by the impairment of this process in fibroblasts from FAK knockout embryos (Ilic et al., 1995), and the promotion of the migration of Chinese hamster ovary cells, over-expressing FAK, on fibronectin (Cary et al., 1996; Reiske et al., 1999). The PI3K inhibitors wortmannin and LY294002, considerably reduce VEGF-induced migration of PAE cells expressing VEGF receptor-2 suggesting that migration of these cells is dependent on PI3K (Qi and Claesson-Welsh, 2001). In PDGF-stimulated PAE cells expressing the PDGF β receptor, and insulin stimulated Swiss 3T3 cells, PI3K is critical for p^{125} FAK activation. In VEGF stimulated PAE cells expressing VEGF receptor-2 however this situation is reversed and FAK activation is independent of PI3K but PI3K activity is dependent on p^{125} FAK.

The migration of PAE cells induced by the stimulation of VEGF receptor-2 is dependent on both p^{125} FAK and PI3K (Qi and Claesson-Welsh, 2001). VEGF stimulates the phosphorylation of p^{125} FAK and consequently the phosphorylation and activation of paxillin. Immunofluorescence experiments have shown localisation of both p^{125} FAK and paxillin to endothelial focal adhesions and furthermore, VEGF stimulation of HUVE cells stimulates either the formation of new focal adhesions or an increased recruitment of these proteins to existing ones. p^{125} FAK activation is accompanied by an increased association with Grb2, Shc and Src in cardiac myocytes and PI3K in PAE cells expressing VEGF receptor-2. It is likely that p^{125} FAK associates with VEGF receptor-2, either directly or indirectly, however Qi et al report that there is no direct interaction between p85 and VEGF receptor-2 despite VEGF-induced complex formation between p^{125} FAK and PI3K. Holmqvist et al propose that an adapter protein Shb associates directly with VEGF receptor-2 via its SH2 domain and with p^{125} FAK via its PTB domain (Holmqvist et al., 2004). Phosphorylation of Shb and p^{125} FAK is mediated by VEGF activated Src, which can also associate with VEGF receptor-2. Reducing the expression of Shb by siRNA results in decreased VEGF-stimulated p^{125} FAK phosphorylation and PI3K activity. Shb is shown to be important for the formation of stress fibres and focal adhesions and endothelial cell migration in response to VEGF.

Cross-talk between integrin signalling and VEGF-signalling is evident in endothelial cells and phosphorylation of p^{125} FAK by an $\alpha v\beta 3$ -VEGF receptor-2 complex has also been demonstrated (Masson-Gadais et al., 2003). This provides a link between integrin and VEGF

receptor activity that could be mediated at focal adhesions and is substantiated by previous work showing co-immunoprecipitation of VEGF receptor-2 with the β 3 subunit of integrins and subsequent activation of the PI3K pathway (Soldi et al., 1999). Although an initial experiment, detailed later in this study, shows no evidence of co-localisation between β 3 and VEGF receptor-2 in HUVE cells a more rigorous study may elucidate how these two pathways integrate PI3K signalling to regulate endothelial cell migration.

Here we show a striking localisation of GFP-p85 at focal adhesions (Figure 4.7) confirmed by p85 co-localisation with paxillin, a key component of focal adhesions and therefore a good marker for these structures. In PAE cells expressing VEGF receptor-2, although different cells contained a variable number of focal adhesions, we could not relate this to the degree of VEGF stimulation. This is in contrast to results published by Abedi et al who show an increase in p^{125} FAK and paxillin localisation to these structures in HUVE cells (Abedi and Zachary, 1997; Takahashi et al., 1999a). A possibility for this is that the greater expression level of VEGF receptor-2 or GFP-p85 in these PAE cells compared to HUVE cells affects the number of focal adhesions in basal and stimulated cells. Using immunofluorescence to stain p^{125} FAK in focal adhesions Abedi et al show that unstimulated HUVE cells contain very few of these structures. Stimulation with VEGF, at a lower dose than used here but for longer time periods, results in an increase in focal adhesion staining. This latter difference between our study and that of Abedi et al could also explain the difference in the number of focal adhesions in unstimulated and VEGF-stimulated cells. In addition HUVE cells express VEGF receptor-1 whereas PAE cells do not. VEGF receptor-1 specific signalling may therefore be required for a VEGF-mediated increase in focal adhesions. We have noted however that the size of focal adhesions appears to increase upon VEGF stimulation. If this is confirmed by quantitative analysis then an increase in contact area between cells and the extracellular matrix is likely to aid the migration of these cells and p85 may have a role in this process.

GFP-p85 and paxillin co-localisation appears to be constitutive (Figure 4.7) and if p85 is also constitutively associated with VEGF receptor-2, as Thakker et al suggests (Thakker et al., 1999) it is possible that PI3K is activated in response to VEGF without a change in the localisation of p85. Figure 4.8 shows the localisation of p85 and VEGF receptor-2 in resting and stimulated cells however co-localisation is not obvious in any of the images. In addition, VEGF receptor-2 does not appear to localise to structure that resemble focal adhesions and, as

mentioned above, we were unable to detect the localisation of VEGF receptor-2 at β 3 integrin-containing focal complexes (Figure 6.11). We have not investigated the possible co-localisation of paxillin and VEGF receptor-2. Our imaging data suggests that VEGF receptor-2 is not concentrated with p85 at focal adhesions. The co-immunoprecipitation experiment however shows that a little GFP-p85 can associate, directly or indirectly, with VEGF receptor-2 (Figure 4.6). To explain the apparent discrepancy it is possible that a fraction of VEGF receptor-2 translocates to focal adhesions in stimulated cells or a proportion of p85 relocates to associate with VEGF receptor-2 in response to receptor activation. If in either case only a small amount of the over-expressed protein re-localises then this may not be detected by our imaging techniques as the remainder of the protein dominates the image.

The localisation of p85 does not enable us to determine whether p85 is associated with the catalytic domain of PI3K, p110 and hence whether PI3K is active. PI3K activation in response to VEGF receptor-2 activation has been studied by the use of dsredII-Grp1 as a marker of the lipid product of PI3K. In Figure 4.9 we show co-localisation of GFP-p85 and dsredII-Grp1 in stimulated PAE/VEGF receptor-2 cells. In these cells GFP-p85 is localised to membrane ruffles, which is inconsistent with previous images (Figures 4.7 and 4.8). We believe that GFP-p85 is commonly localised to focal adhesions however the over-expression of dsredII-Grp1 may be causing an increase in the amount of GFP-p85 protein localised to common regions of the plasma membrane.

The localisation of Grp-1 to the periphery of VEGF-stimulated PAE/VEGF receptor-2 cells is consistent in cells expressing GFP-p85 (Figure 4.9) and in cells expressing only dsredII-Grp-1 (Figure 4.10). The membrane localisation of dsredII-Grp1 in response to VEGF suggests that PI3K is activated in these cells. This observation infers that p85 is involved in production of PtdIns (3,4,5) P₃ by PI3K in response to VEGF stimulation of PAE/VEGF receptor-2 cells. Our real time imaging of Grp-1 also indicates that PI3K is active at the membrane in cells that have been stimulated with VEGF. The shuttling of PtdIns (3,4,5) P₃ back to the membrane may illustrate a recycling mechanism however this requires further investigation. A further control experiment, visualising an unstimulated cell over the same time period, will establish whether the shuttling of PtdIns (3,4,5) P₃ is a consequence of VEGF. The visualisation of activated receptors with DsredII-Grp-1 may illustrate concurrent activation of VEGF receptors and PI3K. Furthermore, the use of additional protein markers may demonstrate a

vesicular localisation for Grp-1 and also provide information necessary to determine the fate of PtdIns (3,4,5) P₃ produced by activated VEGF receptors.

The localisation of p85 to many growth factor receptors (platelet-derived growth factor receptor, hepatocyte growth factor receptor, nerve growth factor receptor and insulin receptors via insulin receptor substrate-1) as well as to distinct compartments, focal adhesions for example, suggests a diverse role for PI3K in cellular signalling. In NIH3T3 cells we have observed GFP-p85 clustering in response to HER4 activation by heregulin. This response is similar to that seen by Gillham et al who show GFP-p85 localised to patches across the cell surface in EGF stimulated NIH3T3 cells expressing a chimeric EGF receptor/c-erbB-3 receptor. The membrane patches observed represents focal complexes, which share many constituents with focal adhesions but are morphologically distinct from them. These complexes are proposed to act as inactive cytoplasmic pools of PI3K that accumulate in response to extracellular signals. Gillham et al suggests that the PI3K at these complexes may be involved in the signal pathways that are responsible for membrane ruffling and cell migration. It is possible that we have also observed the clustering of GFP-p85 at focal complexes in NIH3T3 cells expressing HER4 however further imaging, using markers of these structures, is required to confirm this.

In conclusion we have shown that a small amount of GFP-p85 co-immunoprecipitates with VEGF receptor-2 in VEGF stimulated cells however we have been unable to show co-localisation of the two proteins. These results are therefore more difficult to interpret compared to those obtained for our positive control – the association of PLC γ 1 with VEGF receptor-2. We believe that the fluorescence microscopy method used is not sensitive enough to visualise the co-localisation of proteins where the local concentration of one, or both, components is low. It is possible therefore that a small amount of VEGF receptor-2 is localised in p85-containing focal adhesions or that a small amount of p85 is localised to the same regions of the membrane as VEGF receptor-2. In support of this explanation we have shown that the lipid product of the PI3K reaction is detectable in the membranes of VEGF stimulated cells, suggesting that PI3K is activated in response to VEGF, a process that is likely to involve p85 interacting with VEGF receptor-2.

Chapter 5

Signalling via Shc family adapter proteins

Chapter 5 – Signalling via Shc family adapter proteins

Introduction

The src homology collagen protein, ShcA, is an ubiquitously expressed adapter protein that associates with the Grb2-Sos protein complex to mediate growth factor induced activation of the Ras/MAP kinase pathway. There are three protein isoforms of ShcA, p46, p52 and p66, generated from the same messenger RNA through alternative translation initiation (p46 and p52) or RNA splicing (p66). All ShcA isoforms contain a carboxy-terminal Src homology-2 (SH2) domain and an amino-terminal phosphotyrosine-binding (PTB) domain. In addition, between these two domains, Shc proteins contain a collagen homology-1 (CH1) domain. The CH1 domain is rich in glycine and proline amino acids that have the potential to mediate protein-protein interactions and also contains key tyrosine residues. For p52 ShcA these are at positions 239, 240 and 317. Phosphorylation of these residues is detected as an early event in intracellular signalling resulting from the activation of a number of well-characterised receptor tyrosine kinases. ShcA however has no identifiable catalytic domain and these tyrosines aid ShcA in its role as a scaffolding protein, binding signalling components in a complex with activated receptors. The proposed model for signalling via ShcA, as proposed by Ravichandran, involves a two-step process for the recruitment of ShcA to activated receptors (Ravichandran, 2001). Firstly, a small fraction of ShcA is basally localised to the membrane via interactions of the PTB domain of ShcA and phospholipids. The phosphorylation of receptors in response to stimuli then results in the translocation of ShcA to the receptor where it is phosphorylated. The second step involves the recruitment of the Grb2-Sos complex to ShcA. The localisation of Grb2-Sos to the membrane permits activation of membrane-bound Ras, thereby initiating downstream signalling cascades.

Recently it has been established that Shc is one of a family of Shc proteins that all contain SH2, CH1 and PTB domains (Figure 5.1) (Kojima et al., 2001; Nakamura et al., 1998; O'Bryan et al., 1996; Pelicci et al., 1996). ShcB/Sck is expressed in liver, pancreas and prostate and ShcC/N-Shc family members are primarily expressed in the brain. Sck has also been identified in neuronal Schwann cells and in endothelial cells i.e. HUVE and PAE cells (Ratcliffe et al., 2002). The SH2 and PTB domains are highly conserved among the Shc family members however the CH1 domain is more diverse. Despite this all members possess two Grb2 binding sites (discussed in detail later) and one adaptin binding site in their CH1

domains. The adaptin binding site RDLFDMKPFE, found at amino acids 346-355 in the CH1 domain of ShcA, is semi-conserved in Sck and N-Shc. Adaptins are components of the plasma membrane-coated pit adapter complex, AP2, and results from a study by Sakaguchi et al suggest that EGF stimulates ShcA-AP2 complex formation (Sakaguchi et al., 2001). The coupling of activated EGF receptors to the AP2 complex promotes ligand induced receptor endocytosis.

The p66 isoform of ShcA, Sck and N-Shc family members also contain an additional domain at their amino-terminus, called the CH2 domain. In p66 ShcA this domain contains a serine residue at position 36, which is phosphorylated in response to oxidative stress. Although this residue is not conserved in Sck and N-Shc, the CH2 domain of N-Shc contains many other serine residues that are potential phosphorylation sites. Also, throughout the Sck protein, several tyrosine residues represent potential phosphorylation sites for the initiation of downstream signalling cascades and also the CH2 domain of Sck contains two PXPXXP motifs that may bind to SH3 domains in other signalling proteins.

5.1.1 *Phosphorylation of Shc family proteins*

The CH1 domain shows the least amount of sequence homology amongst the Shc family of proteins. This region however contains conserved tyrosine residues that are phosphorylated in response to the activation of growth factor receptors. Grb2 has been show to bind to both sites surrounding tyrosine residues 239/240 and 317 in p52 ShcA as they represent a Grb2-SH2 domain consensus binding-site (van der Geer et al., 1996; Walk et al., 1998). The motif, YVNV at tyrosine 317 in p52 ShcA is similar to YVNT found at the same relative position in Sck, and likewise the YYND motif in ShcA is similar to YYNS found in Sck (Figure 5.1).

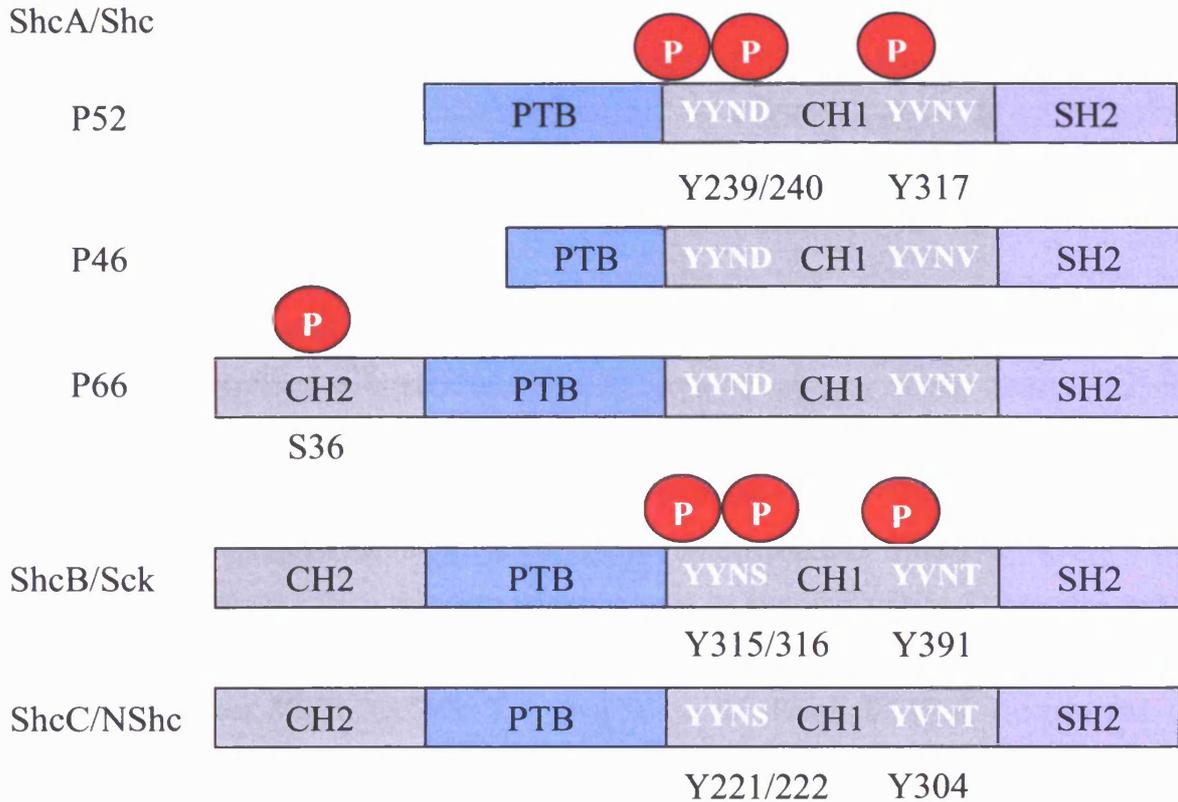


Figure 5.1 Schematic organisation of the Shc family of adapter proteins.

The PTB and SH2 domains of the Shc family share the greatest amount of sequence homology and bind to phospho-tyrosine containing sequences. Within the CH1 domain, of all family members, three tyrosine phosphorylation sites have been identified that conform to Grb2 binding sites. The binding of Grb2 to phospho-tyrosine 317 of ShcA has been shown to activate the MAP kinase pathway. The phosphorylation of tyrosines 239 and 240 can lead to MAP kinase activation and may also result in the activation of c-Myc. The P46 isoform of ShcA lacks the first 46 amino acids of the PTB domain and P66Shc, Sck and N-Shc possess an additional CH2 domain. P66Shc contains a serine phosphorylation site at position 36 that has been implicated in oxidative stress signalling. The serine at position 36 of ShcA is not conserved in ShcB/Sck or ShcC/NShc although ShcC/NShc contains multiple serine residues in this domain.

5.1.2 *ShcA and VEGF signalling*

The interaction of ShcA with many activated receptor tyrosine kinases is mediated through its PTB domain. Upon stimulation of the EGF receptor for example ShcA becomes physically associated with phospho-tyrosine residues 1148 and 1173 of the receptor via its PTB domain (Sakaguchi et al., 1998). The PTB-binding motif of ShcA is not present at all in VEGF receptor-2 and, although it is present in VEGF receptor-1, phosphorylation of this tyrosine residue has not been shown in response to VEGF. Tyrosine 1175 in VEGF receptor-2 however is a major autophosphorylation site and it is contained within a Shc SH2 domain

consensus binding sequence (YIVL). The C-terminal SH2 domain of PLC γ for example, has been shown to associate with this site (Cunningham et al., 1997; Takahashi et al., 2001). The ShcA SH2 domain however binds very weakly to VEGF receptor-2 in GST-SH2 co-precipitation experiments or yeast 2-hybrid studies (Warner et al., 2000).

Although ShcA is a common adapter protein that associates with, and becomes activated by, a large number of activated growth factor receptors, Warner et al show that a very small amount is found in complexes with activated VEGF receptor-2 (Warner et al., 2000). Kroll and Waltenberger report that VEGF-induced stimulation of VEGF receptor-2 in PAE cells results in some receptor-association and phosphorylation of the 46- and 52-kDa isoforms of ShcA as well as weaker phosphorylation of the 66-kDa isoform (Kroll and Waltenberger, 1997). The Grb2-Sos complex is able to associate with ShcA via its phosphotyrosine 317 residue and, as this group show activation of MAP kinase by the phosphorylation of myelin basic protein, they propose that VEGF receptor-2 is coupled to the Ras-Raf-1-ERK cascade and the transmission of this mitogenic signal results in proliferation of PAE cells expressing VEGF receptor-2. The role of ShcA in VEGF receptor-2 signalling is therefore controversial and warrants further investigation.

With regard to the involvement of ShcA in VEGF receptor-1 signalling, reports are also inconsistent. VEGF stimulation of VEGF receptor-1 causes a slight increase in tyrosine phosphorylation of ShcA p52 and ShcA p66 however this does not result in activation of MAP kinase or a mitotic response (Kroll and Waltenberger, 1997). Seetharam et al. also reported very weak tyrosine phosphorylation of ShcA in VEGF receptor-1 expressing NIH3T3 cells and endothelial cells (Seetharam et al., 1995). In agreement with this observation, despite strong binding of VEGF to VEGF receptor-1 in fibroblasts over-expressing this receptor, VEGF had no growth stimulatory effect, MAP kinase was not activated and the induction of early genes, i.e. *c-fos* and *c-myc*, was poor. In addition ShcA could not be detected in complexes with VEGF receptor-1 in HEK-293 cells expressing both proteins (Warner et al., 2000). Despite this latter observation VEGF receptor-1 can interact with the SH2 domain of ShcA in transformed yeast. Whatever role Shc plays in VEGF receptor signalling, it is less significant than its role in EGF receptor signalling.

5.1.3 *Sck and VEGF signalling*

ShcB/Sck was first identified as an EST clone with DNA sequence similarity to ShcA. Screening brain cDNA libraries and genomic libraries for homologies of ShcA identified partial cDNAs of Sck (Nakamura et al., 1998; O'Bryan et al., 1996; Pelicci et al., 1996). Two groups, using the yeast two-hybrid assay, independently identified the interaction of the SH2 domain of Sck with VEGF receptor-2 (Igarashi et al., 1998; Warner et al., 2000). Using mouse VEGF receptor-2 (KDR) Prigent et al. screened a mouse embryo cell-derived cDNA library to identify VEGF receptor-2 interacting proteins (Warner et al., 2000). After screening 100 000 clones, only the cDNA of Sck was shown to interact strongly with the cytoplasmic domain of VEGF receptor-2.

VEGF receptor-2 is believed to be the receptor primarily responsible for the mitogenic effects of VEGF in endothelial cells. However NIH3T3 fibroblasts over-expressing VEGF receptor-2 are weakly mitogenic compared with the response in rat sinusoidal endothelial cells (Takahashi and Shibuya, 1997). In these fibroblasts the MAP kinase pathway was activated at a slower rate and occurred via PLC γ and PKC activation instead of via Ras. Sck may therefore be an example of a protein expressed in endothelial cells, but not in all cell types, which is involved in a novel mechanism to couple activated VEGF receptor-2 to the Ras-MAP kinase pathway. This hypothesis is substantiated by the observation that the SH2 domain of Sck, associates with VEGF receptor-2 to a greater extent than its ubiquitous relative, ShcA (Warner et al., 2000).

Interactions between Sck and the VEGF receptors are likely to be mediated via its SH2 domain and, as for other Shc-SH2 binding motifs, the hydrophobic leucine residue at the +3 position relative to the phosphotyrosine is essential for binding. The SH2 domain of Sck has been shown to bind to VEGF receptor-1 and that this interaction is stronger than that of ShcA (Warner et al., 2000). The SH2 domain of Sck also associates with VEGF receptor-2 in transformed yeast, whereas the SH2 domain of ShcA did not. Similarly GST-SH2 (Sck) bound to tyrosine 1175 of VEGF receptor-2, contained within a YIVL motif; however a GST fusion of the ShcA SH2 domain did not. In HEK-293 cells, transfected with HA-tagged VEGF receptor-2, co-immunoprecipitation showed that a small amount of ShcA associates with the receptor but this did not increase upon receptor stimulation. In contrast, a clear ligand-induced association of an N-terminally truncated Sck protein with VEGF receptor-2 was detected in HEK-293 cells co-transfected with HA-VEGF receptor-2 and a His-tagged

EST-Sck construct encoding a partial Sck protein sequence. This truncated protein lacks part of the PTB domain and the entire CH2 domain and so for this study our first aim was to generate a full length construct with epitope tags to permit the isolation and visualisation of this protein. DNA constructs were generated to express GFP or Flag-tagged full-length Sck proteins.

Although Warner et al. showed that part of Sck associates with VEGF receptor-2, the expression of Sck in endothelial cells was not detected until later. Using RT-PCR and RNase protection analysis Ratcliffe et al. have now shown the expression of Sck mRNA in HUVE cells (Ratcliffe et al., 2002). In addition by producing an antibody to the CH1 domain of Sck they have detected the protein in HUVE and PAE cells by western blotting and in endothelial cells from the vascular beds of mice by immunohistochemistry. With an antibody to Sck, this group were then able to show that Sck is phosphorylated in response to VEGF and associates with phospho-tyrosine 1175 of VEGF receptor-2. Using recombinant SH2 domains of Sck and ShcA, expressed as GST fusion proteins, this group also confirmed that it is the SH2 domains of these proteins that bind to phospho-tyrosine 1175 of VEGF receptor-2. This is a novel feature of Shc family protein interactions as previous reports have shown that it is the PTB domain of these proteins that associate with phosphotyrosine residues in activated receptors.

5.2 - Results

5.2.1 *Cloning of Flag-tagged and GFP-tagged Sck*

Sck was first identified as an EST clone in 1994, and by screening brain cDNA or genomic libraries partial cDNAs were identified (Kavanaugh and Williams, 1994; Nakamura et al., 1998; O'Bryan et al., 1996; Pelicci et al., 1996). From these clones most of the DNA sequence for Sck was determined, including sequence encoding the intact PTB, CH1 and SH2 domains, however the 5' end was missing. The full protein sequence for mouse Sck was reported in 2001 but the DNA sequence was not (Kojima et al., 2001). A partial human cDNA clone, derived from an EST clone, has previously been cloned into a vector containing dsred. This included 1404 base pairs of the human Sck sequence up to the stop codon.

The full-length human Sck DNA sequence was determined as follows. The 5' 100 bps of a partial DNA sequence was used in a BLAST search to identify matches in genomic libraries.

A human chromosome clone (R34739) was found that appeared to contain the complete 5' end of Sck. From the known partial DNA sequences, combined with the genomic DNA sequence, the human protein sequence for Sck was deduced (Figure 5.2). The human and mouse protein sequences were then compared (Figure 5.3). From this we were confident that we had obtained the full DNA sequence for Sck.

Signalling via Shc family adapter proteins

p66ShcA	MNLLPPKPKYNPLRNESLSSMEEGASGSTPPEELPSPPASSLGPILPPLPGDDSPTTLCS	60
Sck	-----MTQGPGRAPPAPPAPPEPEAPTTFCALLPRMPQWKFAAPGGF	43
	: : * : : ** . . . : : ** : * . . .	
p66ShcA	FFPRMSNLRLANPAGGRPGSK--GEPGRAADDGEGIVGAAMPDSGPLLLQDMNKLSGGG	118
Sck	LGRGPAAARAAGASGGADPQPEPAGPGVPALAAAVLGACEPRCAAPCPLPALSRCRGAG	103
	: : * * . : ** . . * * . . . : : ** * . . . : * : : * *	
p66ShcA	GRRTRVEGGQLGG-----EEWTRHGSFVNKPTRGWLHPNDKVM	172
Sck	SRGSRGGRGAAGSGDAAAAAEWIRKGSFIHKPAHGWLHPDARV	163
	. * : * * * . * * * * . : * : * * * * . : * * * * * . : * * * * * . : * * * * *	
p66ShcA	LQSMRALDFNTRTQVTREAISLVCEAVPGAKGATRRRKPCSRPLSSILGRSNLKFAGMPI	232
Sck	LRSMRSLDFNTRTQVTREAINRLHEAVPGVRGWSKKKAPN-KALASVLGKSNLRFAGMSI	222
	* . * * * . * * * * * * * * * * . : * * * * * . : * : : * . : * : * * * * * . * * * * * *	
p66ShcA	TLTVSTSSLNLMAADCKQIIANHHMQSISFASGGDPDTAEYVAYVAKDPVNQRACHILEC	292
Sck	SIHISTDGLSLPSISSVPATRQVIANHHMFASGGDTDMTDYVAYVAKDPINQRACHILEC	282
	: : * * . * * * . . : * : * * * * * * * * * * * * . : * * * * * * * * * * * * *	
p66ShcA	PEGLAQDVISTIGQAFELRFKQYLRNPPKLVTPHDRMAGFDGSAWDEEEEEPPDHQ	352
Sck	CEGLAQSIISTVIGQAFELRFKQYLHSPPKVALPPERLAGPEESAWGDEEDS-LEHNYNS	341
	* * * * * . : * * * * * * * * * * * * . : * * * * * . : * * * * * . : * * * * * . : * * * * *	
p66ShcA	FPGKEPPLGGVDMRLREG----AAPGAARPTAPNAQTPSHLGATLPVGQPVGGDPEVRK	408
Sck	IPGKEPPLGGLVDSRLALTQPCALTALDQGPSPLRDACSLPWDVGSTGTAPPGDGYVQA	401
	: * * * * * * * * * * * * * * . : * : . . : : * . . . * . * * * * :	
p66ShcA	QMPPPPPCPGRELFDPSYVNVQNLDKARQAVGGAGPPNPAING-----SAPRDL	458
Sck	DARGPPDHEEHLVNTQGLDAPEPEDSPKKDLFDMRPFEDALKLHECSVAAGVTAAPLPL	461
	: * * : : . : * : : : . : * : * : : . : * : * : : . : * * *	
p66ShcA	FDMKPFEDALRVPPPQSVSMAEQLRGEF	518
Sck	EDQWSPPTRRAPVAP----TEEQLRQEP	517
	* * : * * * * * * * * * * * * * * . : * * * * * * * * * * * * *	
p66ShcA	QYVLTGLQSGQPKHLLLDPEGVVVRTKDRFESVSHLISYHMDNHLPIISAGSELCLQQP	578
Sck	QYVLTGMHAGQPKHLLLDPEGVVVRTKDVLFESISHLIDHHLQNGQPIVAESELHLRGV	577
	* * * * * . : *	
p66ShcA	VERKL	583
Sck	VSREP	582
	* . * :	

Figure 5.2 - An alignment of the protein sequences for p66ShcA and Sck/ShcB.

The PTB and SH2 domains of P66ShcA and Sck are shown by the shaded sequences. The Grb2 binding sites are in colour and the tyrosine residues within these sites are phosphorylated by the activation of receptor tyrosine kinases. The red and green sequences correspond to the Grb2 binding site at phosphotyrosines 239/ 240 and phosphotyrosine 317 of p52ShcA respectively.

Mori et al reported that the full-length Sck sequence could not be amplified from cDNA by PCR because the 5' end of DNA sequence is extremely GC-rich (Kojima et al., 2001). We therefore adapted the following cloning strategy to amplify the full Sck DNA sequence (Figure 5.4) and, by using the gateway cloning system, added a GFP- or FLAG- tag to Sck (Figure 5.5).

Primers were designed to span the missing 5' end of Sck from the human cosmid clone and the 3' end from a partial DNA construct of Sck made in our lab by Dr S Prigent. In doing this we ensured that the forward primer for the 5' fragment and the reverse primer for the 3' fragment, of Sck contained *attB* sequences for recombination of full length Sck into a gateway donor vector. We also ensured that the PCR products of the 5' and 3' fragments of Sck contained a region of overlap. For the amplification of GC rich DNA sequences we added 5 % v/v DMSO and Betaine, at a final concentration of 1M, to the PCR reaction mix. The PCR products resulting from the amplification of the Sck sequences were 422 bases and 1423 bases as shown in Figure 5.4B These DNA fragments were extracted from a 1% agarose/TBE gel and purified for subsequent steps.

The Sck sequence contains a unique BspMII restriction enzyme site that is present in the overlapping region of the two PCR fragments. Both PCR products were digested with BspMII and the small, surplus DNA fragments were removed by purification on Quickspin Qiagen columns. The two digested fragments that make up Sck were ligated together to construct a DNA fragment of 1799 bps. This was used as a template in another PCR reaction to generate a significant amount of full-length cDNA (Figure 5.4C).

The DNA fragment that we believed contained the full-length Sck sequence flanked by *attB* sites was subcloned into the pDONR™201 vector (Figure 5.5). At this stage diagnostic restriction digests and DNA sequencing confirmed that the vector contained the correct, full-length Sck, DNA sequence. The pDONR™201 vector containing Sck was then recombined with vectors containing either GFP or Flag to create N-terminally fused Sck constructs.

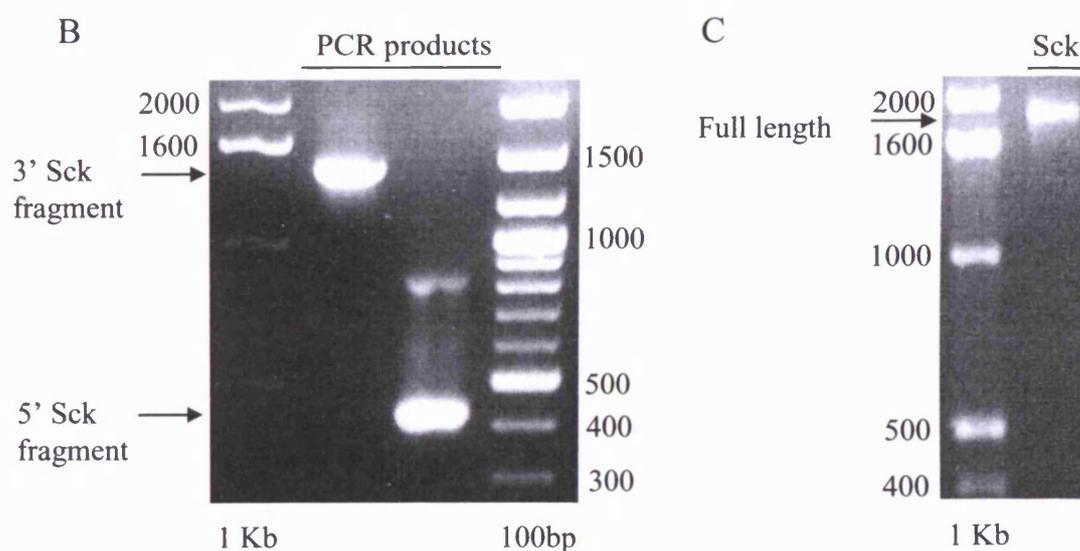
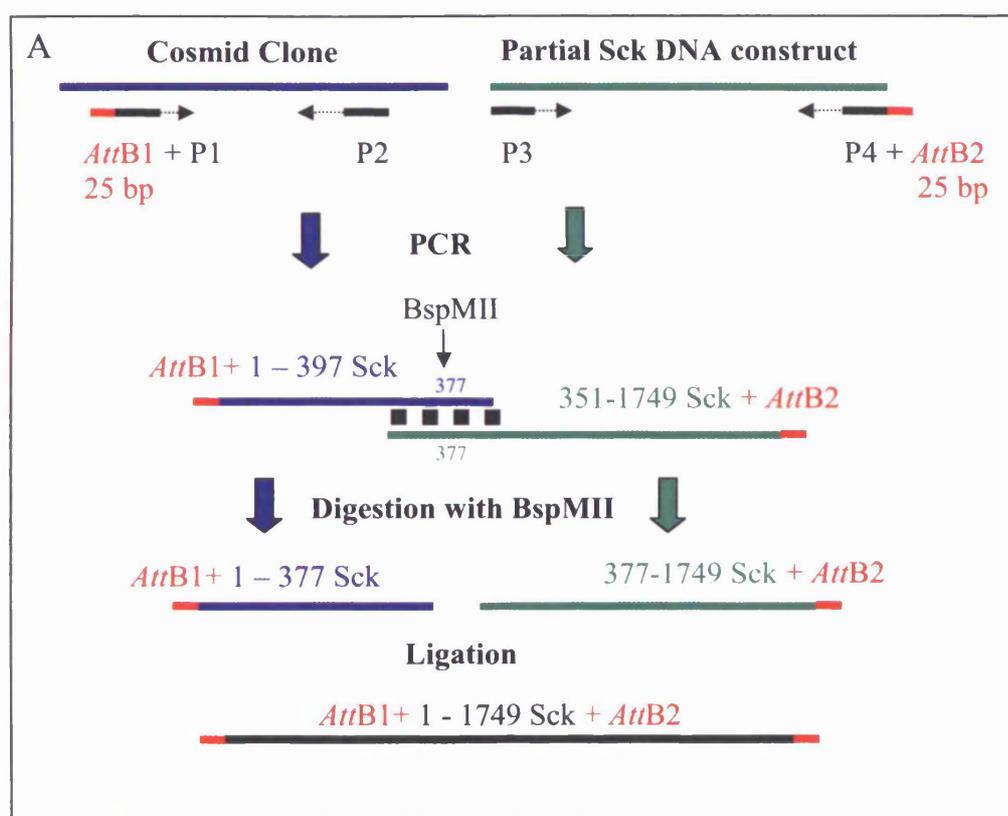


Figure 5.4 – Cloning full length Sck. (A) A schematic of the first part of the cloning strategy used to clone full length Sck into gateway vectors. Two DNA fragments of Sck are amplified from templates using oligonucleotide primers and PCR (B). Primers P1 and P4 include the AttB sites required for cloning into gateway vectors. A unique restriction site is located in the overlap region of the two fragments. Both fragments are cut with the restriction enzyme that recognises this site and the two fragments are ligated together. The full length Sck DNA sequence, which includes the AttB sites at each end, is generated (C).

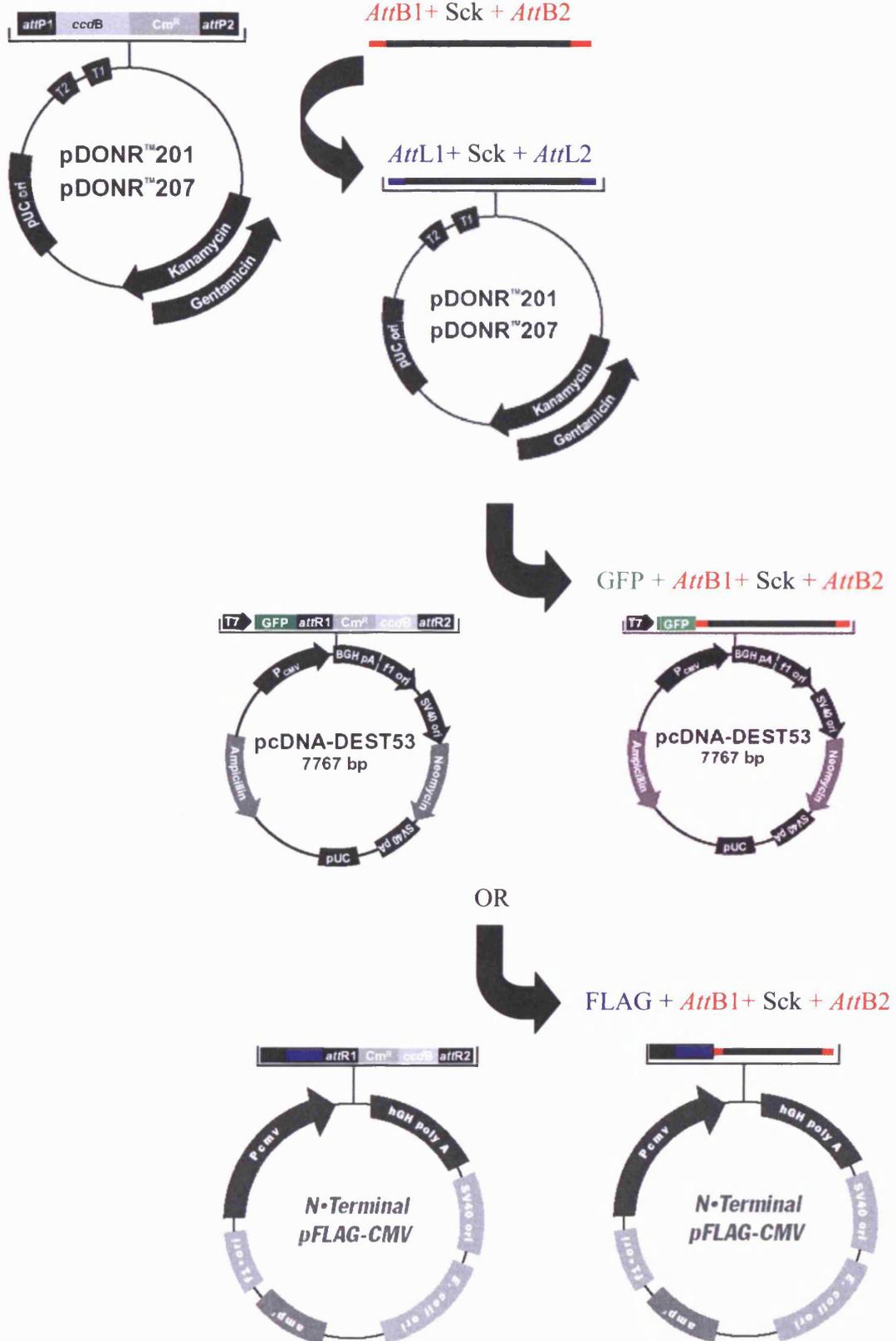


Figure 5.5 - Cloning full length Sck into gateway vectors. A schematic of the second part of the cloning strategy, used to clone full length Sck into gateway vectors. Full length Sck is recombined into pDONR-201 and then recombined further into either pcDNA-DEST53 or N-terminal pFLAG-CMV, which adds an N-terminal GFP or Flag tag to full length Sck.

5.2.2 *Flag-Sck but not Flag-ShcA associates with VEGF receptor-2*

The PTB and SH2 domains of ShcA and Sck are highly homologous however a previous report has suggested that there is a stronger association between the SH2 domain of Sck and VEGF receptors 1 and 2 compared that of the SH2 domain of ShcA. PAE cells expressing VEGF receptor-2 were therefore transfected with Flag-tagged full length p52 ShcA or full length Sck (human (Roberts) sequence in Figure 5.3) and ShcA and Sck were immunoprecipitated using a flag antibody. Western blots of these immunoprecipitations were probed with antibodies recognising VEGF receptor-2, phosphotyrosine, Flag and Shc. Figure 5.6A shows that the Flag antibody successfully precipitates both proteins and that phospho-VEGF receptor-2 associates with Flag-Sck but not Flag-ShcA in VEGF stimulated cells. Unfortunately the amount of precipitated ShcA and Sck could not be compared directly by analysis using a Flag antibody as the heavy chain of the Flag antibody used in the precipitation migrates with Flag-p52 ShcA. Whole cell lysate western blots however show that the expression of Flag-ShcA is actually greater than that of Flag-Sck hence the Flag immunoprecipitates are likely to contain more ShcA than Sck (Figure 5.6B). In addition western blots of whole cell lysates show that phosphorylation of VEGF receptor-2 is similar in cells expressing ShcA and Sck. We have only done this experiment using p52 Shc, however in the future it would be interesting to compare the association of p46 and p66 Shc with VEGF receptor-2 to that of Sck with VEGF receptor-2.

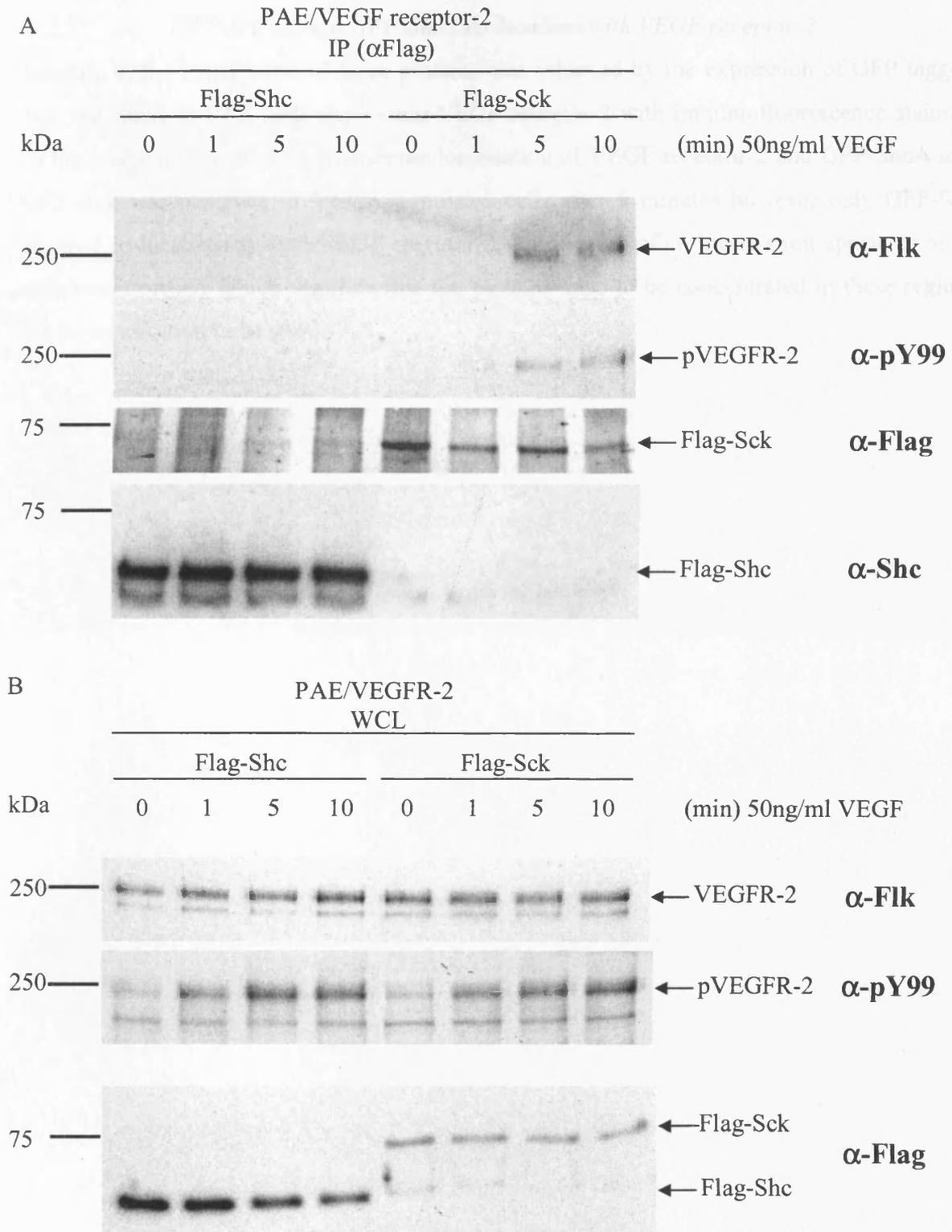
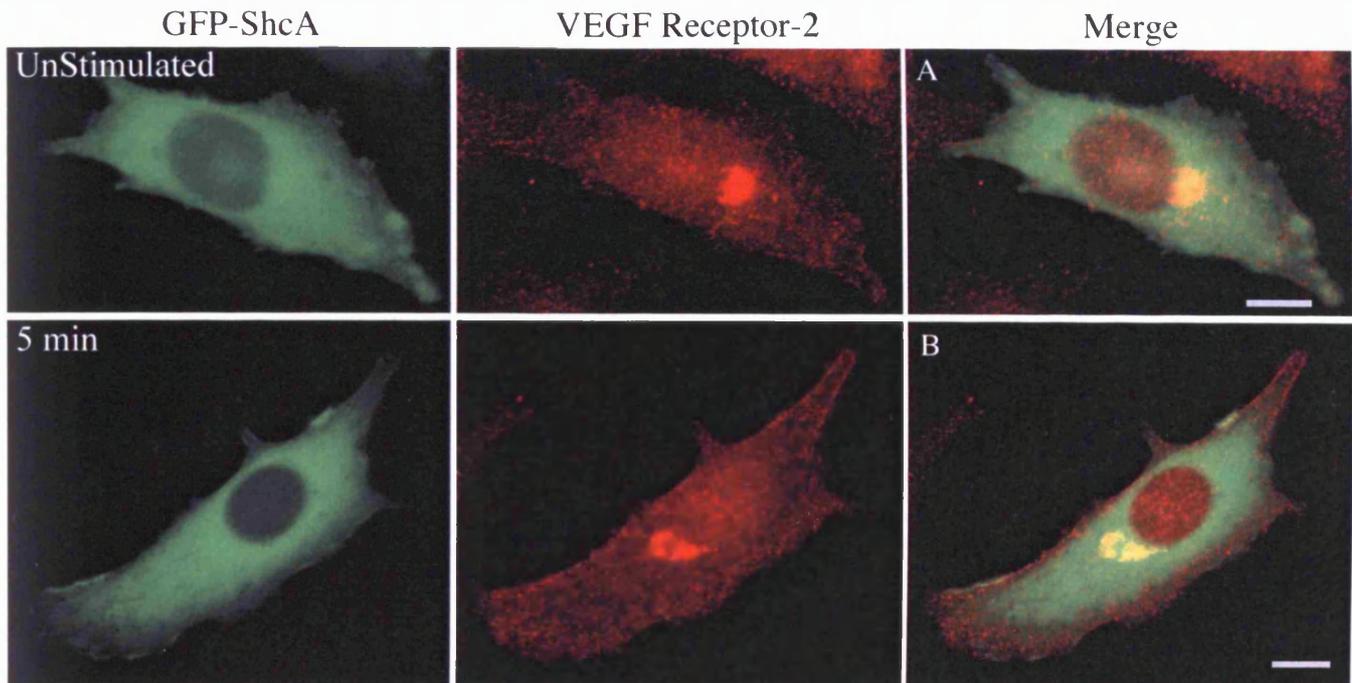


Figure 5.6 – Phospho-VEGF receptor-2 immunoprecipitates with Sck but not with ShcA. Immunoprecipitation of Flag-tagged proteins from the lysates of PAE/VEGF receptor-2 cells, transfected with either Flag-ShcA or Flag-Sck, show that phospho-VEGF receptor-2 associates with Flag-Sck, but not Flag-ShcA (A). Whole cell lysates (B) show that VEGF receptor-2 and Flag-tagged proteins were expressed in all samples and that VEGF receptor was phosphorylated in response to VEGF. These are representative blots from three independent experiments

5.2.3 *GFP-Sck but not GFP-ShcA co-localises with VEGF receptor-2*

Imaging of the localisation of these proteins was achieved by the expression of GFP tagged Sck and ShcA in PAE cells expressing VEGF receptor-2 with immunofluorescence staining of the receptor (Figure 5.7). Membrane localisation of VEGF receptor-2 and GFP-ShcA and GFP-Sck was observed in VEGF stimulated cells after 5 minutes however only GFP-Sck showed co-localisation with VEGF receptor-2. These areas of co-localisation appear to be in membrane ruffles, which suggests that the proteins have to be concentrated in these regions for co-localisation to be identified.

PAE/VEGF Receptor-2 + GFP-ShcA



PAE/VEGF Receptor-2 + GFP-Sck

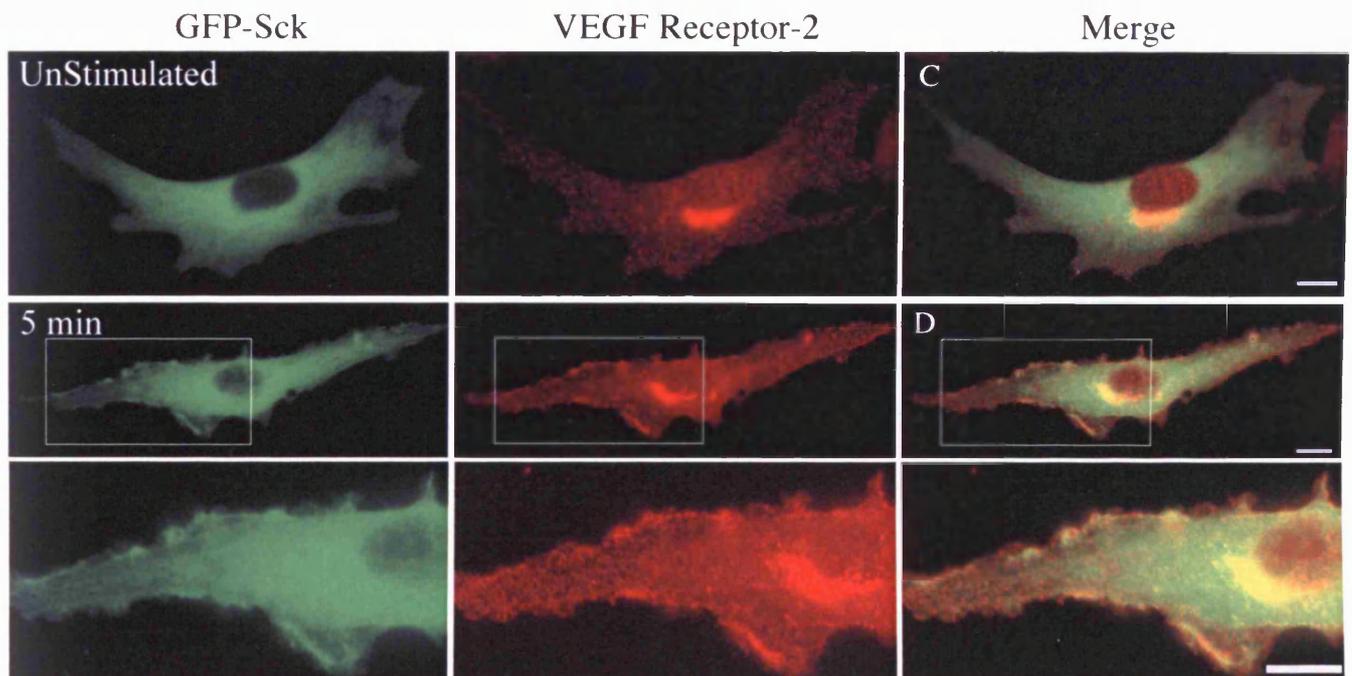


Figure 5.7 - A proportion of GFP-Sck, but not GFP-ShcA, co-localises with VEGF Receptor-2 in VEGF stimulated PAE/VEGF Receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were transiently transfected with either GFP-ShcA (A and B) or GFP-Sck (C and D). Images shown are of cells that were serum starved (Unstimulated, A and C) or stimulated with 50 ng/ml VEGF for 5 minutes (B and D). VEGF Receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to Rhodamine Red. These are representative cells from a single experiment. The scale bar represents 10 μm.

5.2.4 *ShcA and Sck show different phosphorylation profiles following VEGF stimulation of VEGF receptor-2*

Independent studies show that both ShcA and Sck can be phosphorylated in response to VEGF however the extent of phosphorylation of ShcA and Sck has not been compared directly. In addition phosphorylation of the different sites, within Sck, has not previously been examined. We confirm phosphorylation of Shc and Sck using GFP-tagged ShcA and Sck proteins and western blots of unstimulated and stimulated cell lysates probed with phosphotyrosine antibodies. Antibodies to phosphotyrosine residue 317 and to phosphotyrosine residues 239 and 240 of ShcA are now available and as the motifs surrounding these residues are similar in ShcA and Sck the antibodies can be used to detect phosphotyrosine 391 and phosphotyrosines 315 and 316 of Sck respectively. Western blots using these antibodies show that GFP-Sck but not GFP-ShcA is phosphorylated at tyrosines 315/316, which corresponds to tyrosines 239/240 of ShcA (Figure 5.8). The phosphorylation profile of ShcA however shows that it is preferentially phosphorylated at tyrosine 317 and this level of phosphorylation is much greater than that seen for phosphotyrosine 391 of GFP-Sck. By re-probing the western blot with a phosphotyrosine antibody we observe that there is no apparent difference in the amount of total phosphorylation of the two proteins. This blot also shows a similar amount of phosphorylation of VEGF receptor-2. In addition re-probing the blot for GFP or VEGF receptor-2 shows that ShcA and Sck are expressed at similar levels and the receptor is expressed in all cell lysates.

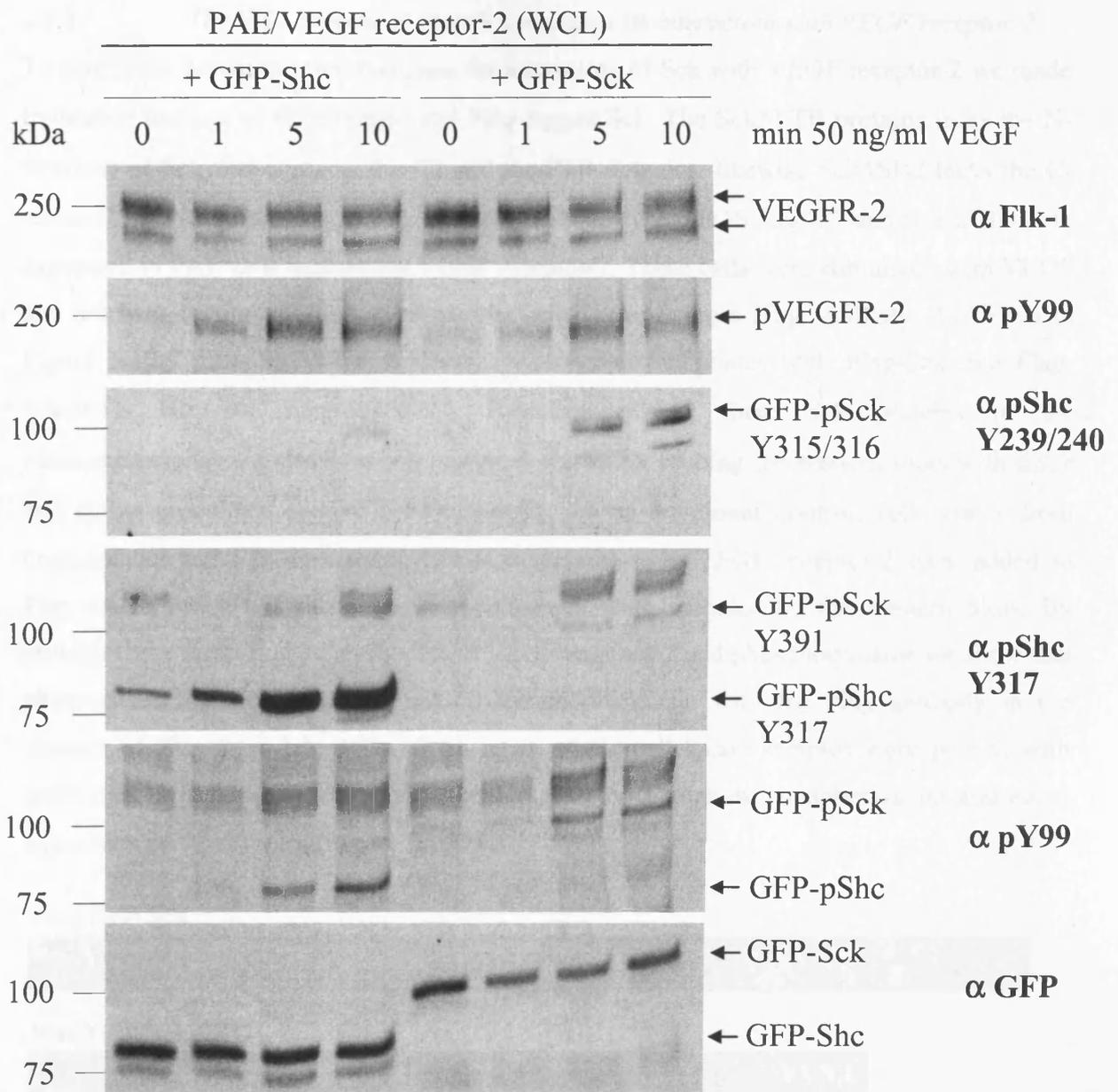
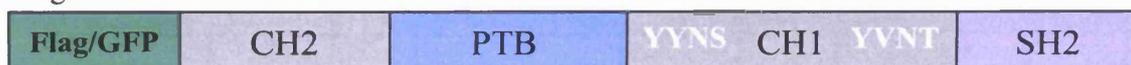


Figure 5.8 – GFP-ShcA and GFP-Sck show different phosphorylation profiles in response to VEGF stimulation. Western blotting of whole cell lysates, from cells expressing VEGF receptor-2, and either GFP-ShcA or GFP-Sck using ShcA phospho-specific antibodies, shows that ShcA is not phosphorylated at tyrosine residues 239 and 240 in response to VEGF but shows a large increase in phosphorylation at tyrosine residue 317. GFP-Sck however is phosphorylated at tyrosines 315 and 316, and at tyrosine 391, in response to stimulation of VEGF receptor-2 with VEGF. The overall phosphorylation levels of GFP-ShcA and GFP-Sck are similar, as is the level of expression of the two proteins. Control blots of total VEGF receptor-2 and phospho-VEGF receptor show that the profile of receptor activation was similar in cells expressing GFP-ShcA and GFP-Sck. These are representative blots from three independent experiments.

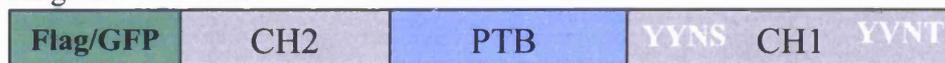
5.2.5 *The SH2 domain of Flag-Sck mediates its interaction with VEGF receptor-2*

To clarify the domain(s) that mediates the interaction of Sck with VEGF receptor-2 we made truncation mutants of GFP-tagged and Flag-tagged Sck. The Sck Δ PTB proteins lacks the N-terminus of Sck that contains the C2 and the PTB domains, likewise Sck Δ SH2 lacks the C-terminal SH2 domain (Figure 5.9). Flag tagged wild-type Sck, Sck Δ PTB and Sck Δ SH2 were expressed in PAE cells expressing VEGF receptor-2. These cells were stimulated with VEGF and Sck was immunoprecipitated from the cell lysates using a Flag antibody. As shown in Figure 5.10A phospho-VEGF receptor-2 co-immunoprecipitates with Flag-Sck and Flag-Sck Δ PTB but not Flag-Sck Δ SH2. Detection of all three Sck proteins in the immunoprecipitates and whole cell lysates is shown by probing the western blots with α Sck and α Flag antibodies (Figure 5.10A and B). As an additional control, cell lysates from unstimulated and VEGF-stimulated cells expressing only VEGF receptor-2 were added to Flag-coated beads and these immunoprecipitates were included on the western blots. By probing these samples with antibodies to VEGF receptor-2 and phosphotyrosine we show that phospho-VEGF receptor-2 does not co-immunoprecipitate with the Flag antibody in the absence of Flag-tagged proteins. Once again whole cell lysate samples were probed with antibodies to phosphotyrosine and to VEGF receptor-2 to show phosphorylation and equal expression of the receptor (Figure 5.10B).

Flag/GFP-Sck



Flag/GFP-Sck Δ SH2



Flag/GFP-Sck Δ PTB



Figure 5.9 - A schematic diagram of epitope-tagged wild type Sck and the truncation mutants of Sck. DNA constructs encoding for truncation mutants of Sck were generated using an alternative PCR primer, compatible with the gateway cloning system (Sck Δ PTB), and primers including an engineered stop codon, for use with the site directed mutagenesis system (Sck Δ SH2).

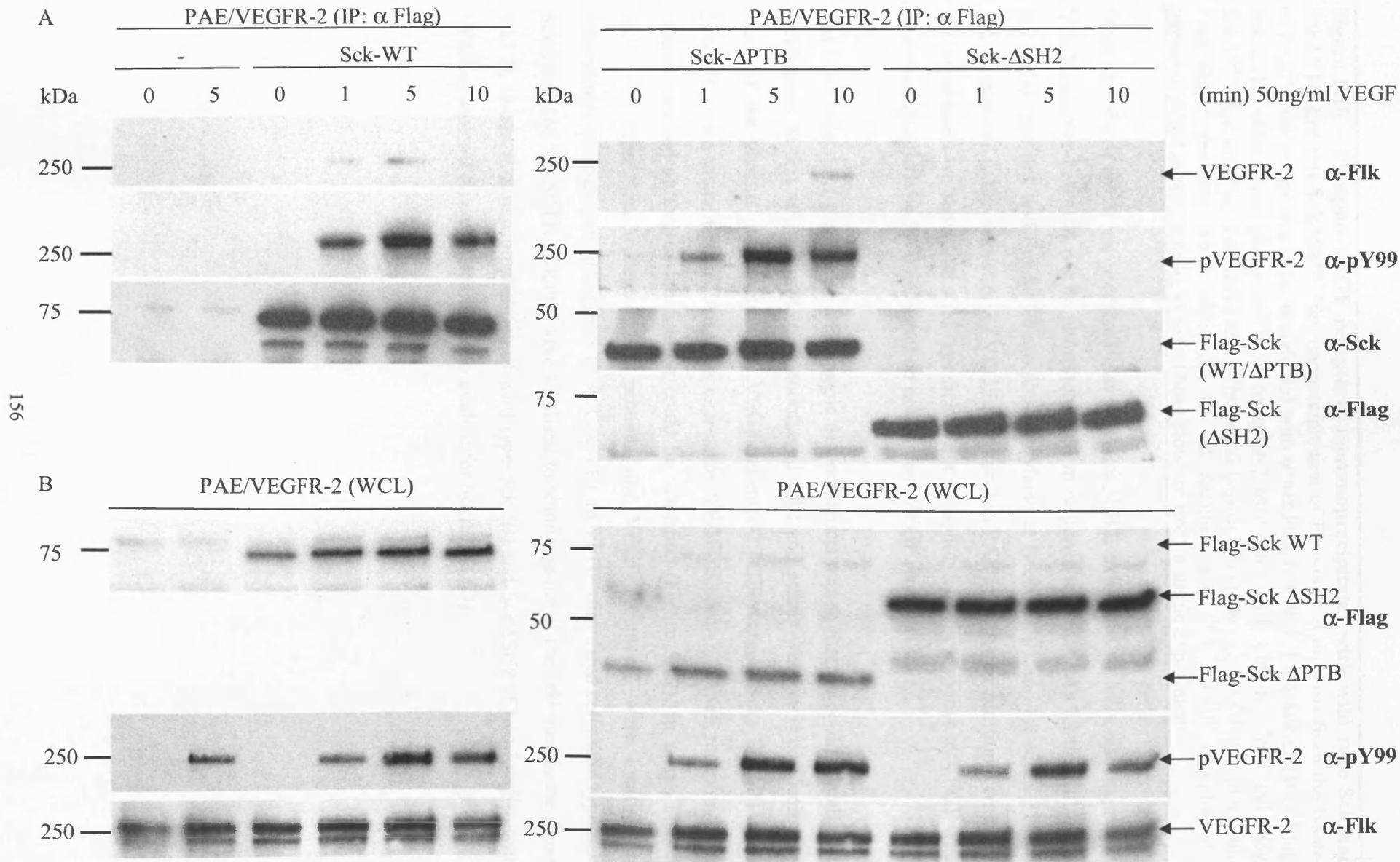


Figure 5.10 – Phospho-VEGF receptor-2 immunoprecipitates with wild type Sck and Sck Δ PTB but not Sck Δ SH2

Figure 5.10 – Phospho-VEGF receptor-2 immunoprecipitates with wild type Sck and Sck Δ PTB but not Sck Δ SH2. Immunoprecipitation of Flag-tagged proteins from the lysates of PAE/VEGF receptor-2 cells, transfected with either Flag-Sck-WT, Flag-Sck Δ PTB or Flag-Sck Δ SH2, shows that phospho-VEGF receptor-2 associates with Flag-Sck-WT and Flag-Sck Δ PTB but not Flag-Sck Δ SH2 (A). Whole cell lysates (B) show that VEGF receptor-2 and Flag-tagged proteins were expressed in all samples and that VEGF receptor was phosphorylated in response to VEGF. These blots are from a single experiment.

From the above result we predicted that GFP-Sck Δ SH2, expressed in cells also expressing VEGF receptor-2, would not be phosphorylated in response to VEGF. As figure 5.11 shows only wild type Sck and Sck Δ PTB are phosphorylated at tyrosine 391 and tyrosines 315 and 316 in response to the activation of VEGF receptor-2 in PAE cells. As an additional control in this experiment we show that wild type Sck is not phosphorylated in response to VEGF when expressed in wild type PAE cells that do not express VEGF receptor-2.

The main reason for generating GFP-tagged Sck constructs was so that we could visualise the localisation of this adapter protein in endothelial cells and assess whether it co-localises to areas of the plasma membrane with VEGF receptor-2. GFP-tagged Sck, Sck Δ PTB and Sck Δ SH2 were visualised in PAE cells expressing VEGF receptor-2. VEGF receptor-2 was immunolabelled so that co-localisation could be identified (Figure 5.12). In serum starved cells wild type Sck, truncated Sck proteins and VEGF receptor-2 were localised to intracellular compartments (Figure 5.12 A, C and E). In VEGF stimulated cells wild type Sck, Sck Δ SH2 and Sck Δ PTB proteins were localised to some regions of the cell membrane figure 5.12 B, D and F. Small proportions of wild type Sck (B) and Sck Δ SH2 (F) co-localise with VEGF receptor-2 however this is not so evident for Sck Δ PTB (D).

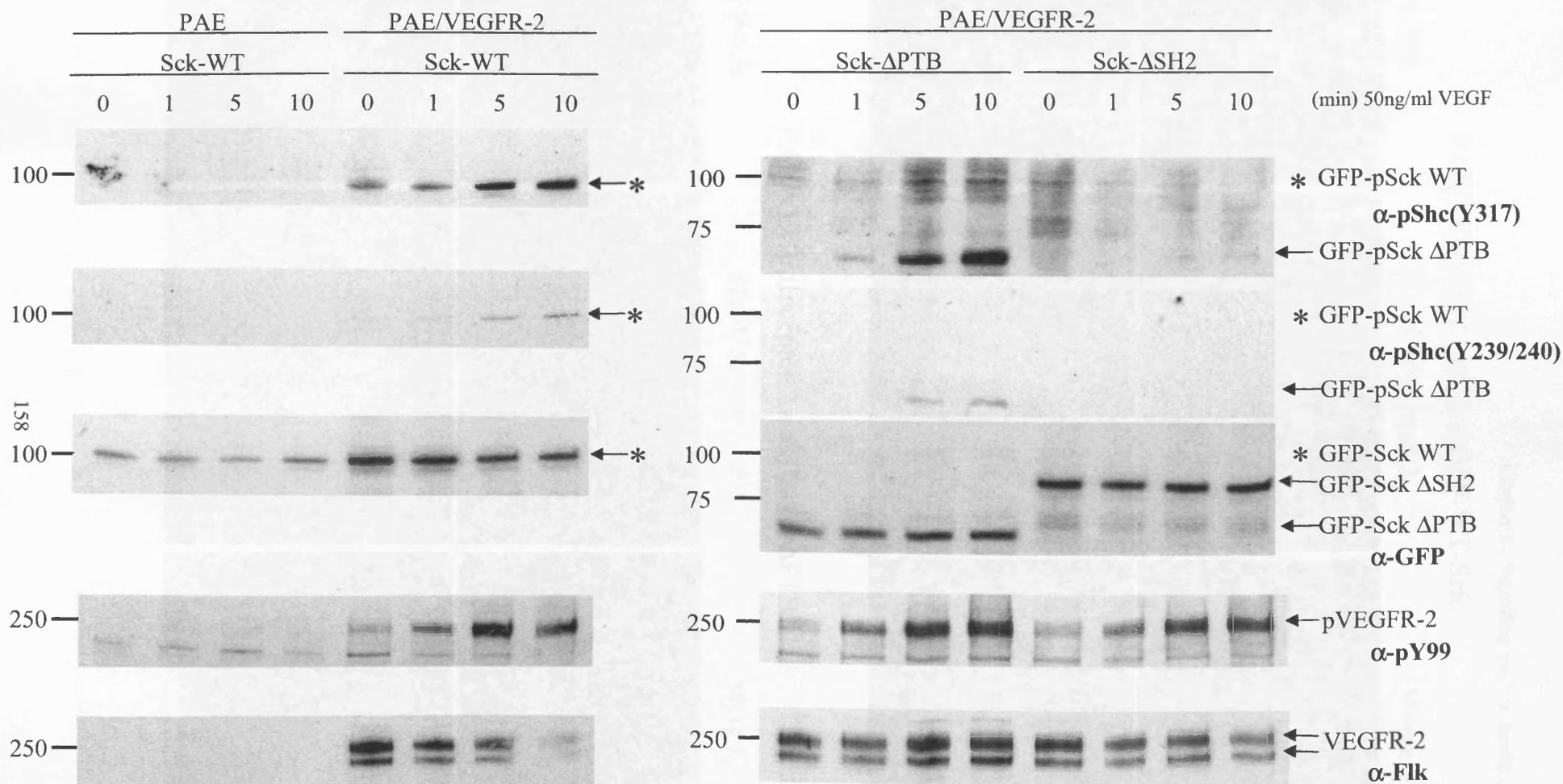
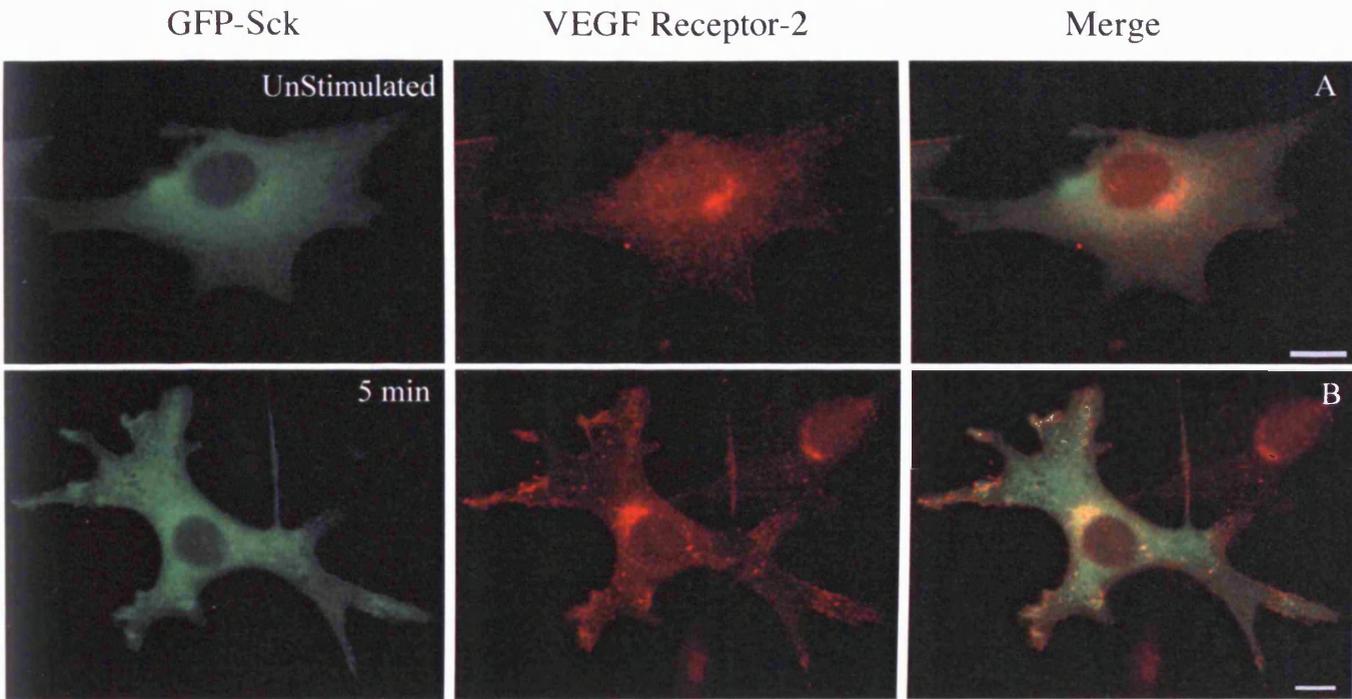
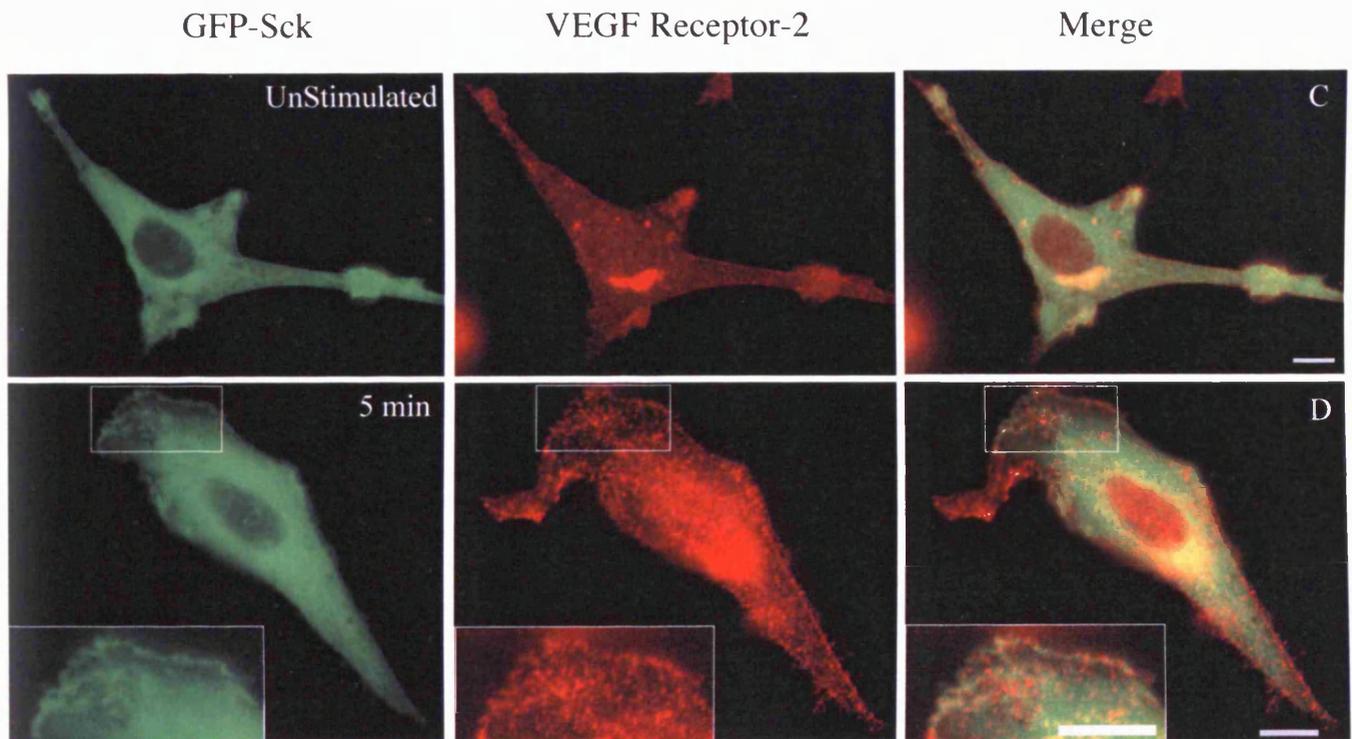


Figure 5.11 - Wild type Sck and Sck Δ PTB are phosphorylated in response to VEGF, but Sck Δ SH2 is not. Whole cell lysate western blots from cells expressing wild type Sck, Sck Δ PTB or Sck Δ SH2 and stimulated with VEGF show that wild type Sck and Sck Δ PTB are phosphorylated at tyrosine 391, equivalent to tyrosine 317 in Shc, and at tyrosines 315/316, equivalent to tyrosines 239/240 in Shc. Sck Δ SH2 however is not phosphorylated at these sites. Western blots probed with GFP show that all three Sck proteins are expressed and blots probed with pY99 and Flk-1 show the levels of phospho-VEGF receptor-2 and total VEGF receptor-2 respectively. These blots are from a single experiment.

PAE/VEGF Receptor-2 + GFP-WT-Sck



PAE/VEGF Receptor-2 + GFP-Sck Δ PTB



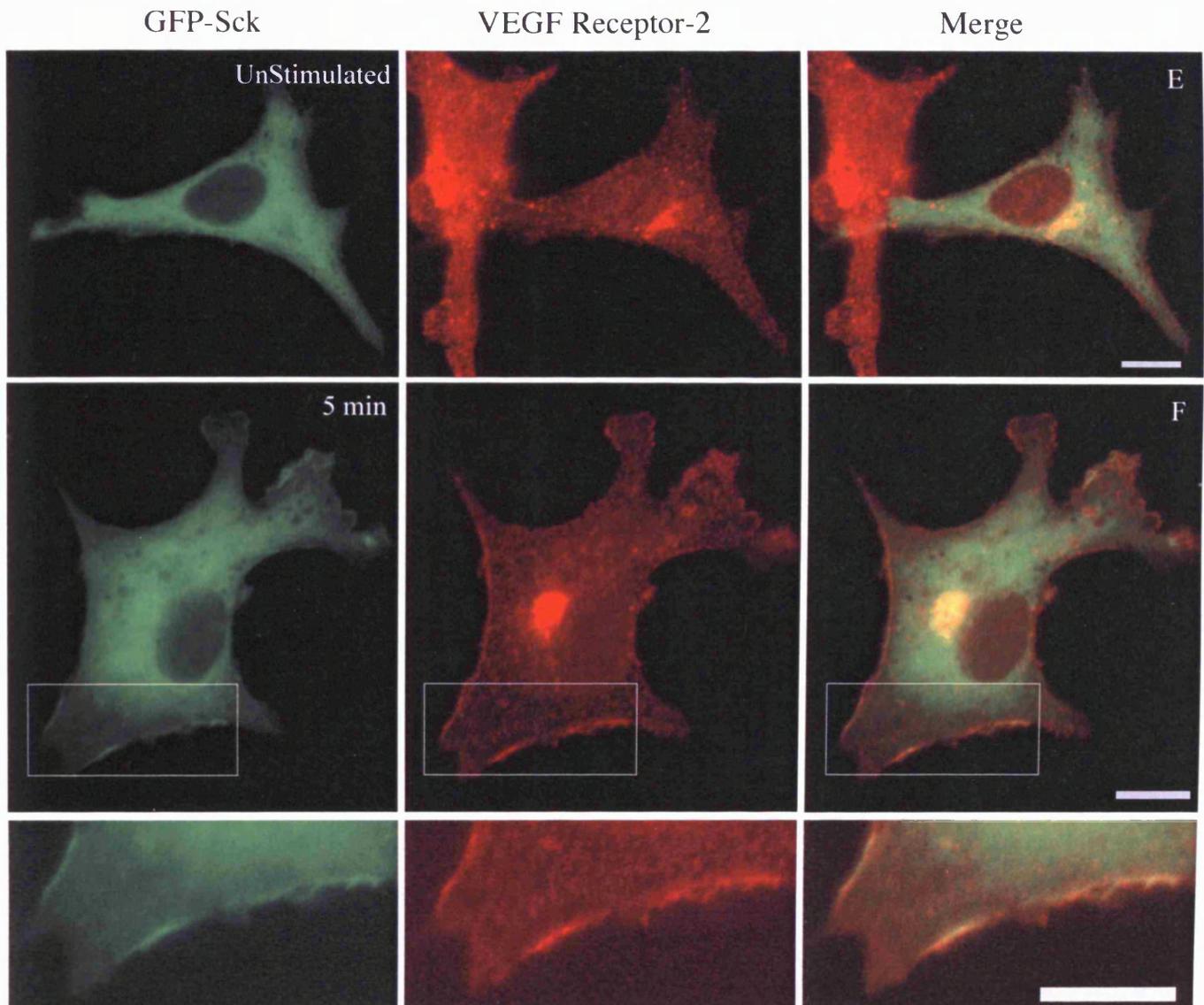
PAE/VEGF Receptor-2 + GFP-Sck Δ SH2

Figure 5.12 - Wild type Sck, Sck Δ SH2 and Sck Δ PTB co-localise with VEGF Receptor-2 at regions of the plasma membrane. PAE cells stably expressing VEGF receptor-2 were transiently transfected with either GFP-Sck (wild type), GFP-Sck Δ PTB or GFP-Sck Δ SH2. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for 5 minutes. VEGF Receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to Rhodamine Red. These are representative cells from a single experiment. The scale bars represent 10 μ m.

5.2.6 *Phosphorylation of tyrosine residue 391 of Sck requires phosphorylation of tyrosine residue 315 and/or tyrosine residue 316*

The identification of phosphotyrosine 391 and phosphotyrosines 315 and 316 of Sck in VEGF stimulated cells prompted us to investigate their role(s) in VEGF-mediated signalling. To further investigate the consequences of phosphorylation at these sites we over-expressed Sck proteins in which either tyrosine residue 391 or tyrosine residues 315 and 316 were mutated to phenylalanine residues. To check that these proteins were not phosphorylated at their mutated sites we stimulated PAE/VEGF receptor-2 cells expressing these proteins with VEGF and ran cell lysates on an SDS-PAGE gel. Western blots probed with the phospho-Shc antibodies are shown in Figure 5.13. From these blots we detected phosphorylation of Sck Y391F at tyrosines 315 and 316 but not at tyrosine 391. For SckY315/316F however we detected no phosphorylation at any of the tyrosine sites. The western blots show that VEGF receptor-2 in these cells was phosphorylated in response to VEGF and that this mutant Sck protein was expressed at similar levels to the wild type and the SckY391F mutant. This result suggests therefore that phosphorylation of tyrosine 391 requires the tyrosine phosphorylation of residues 315 and/or 316.

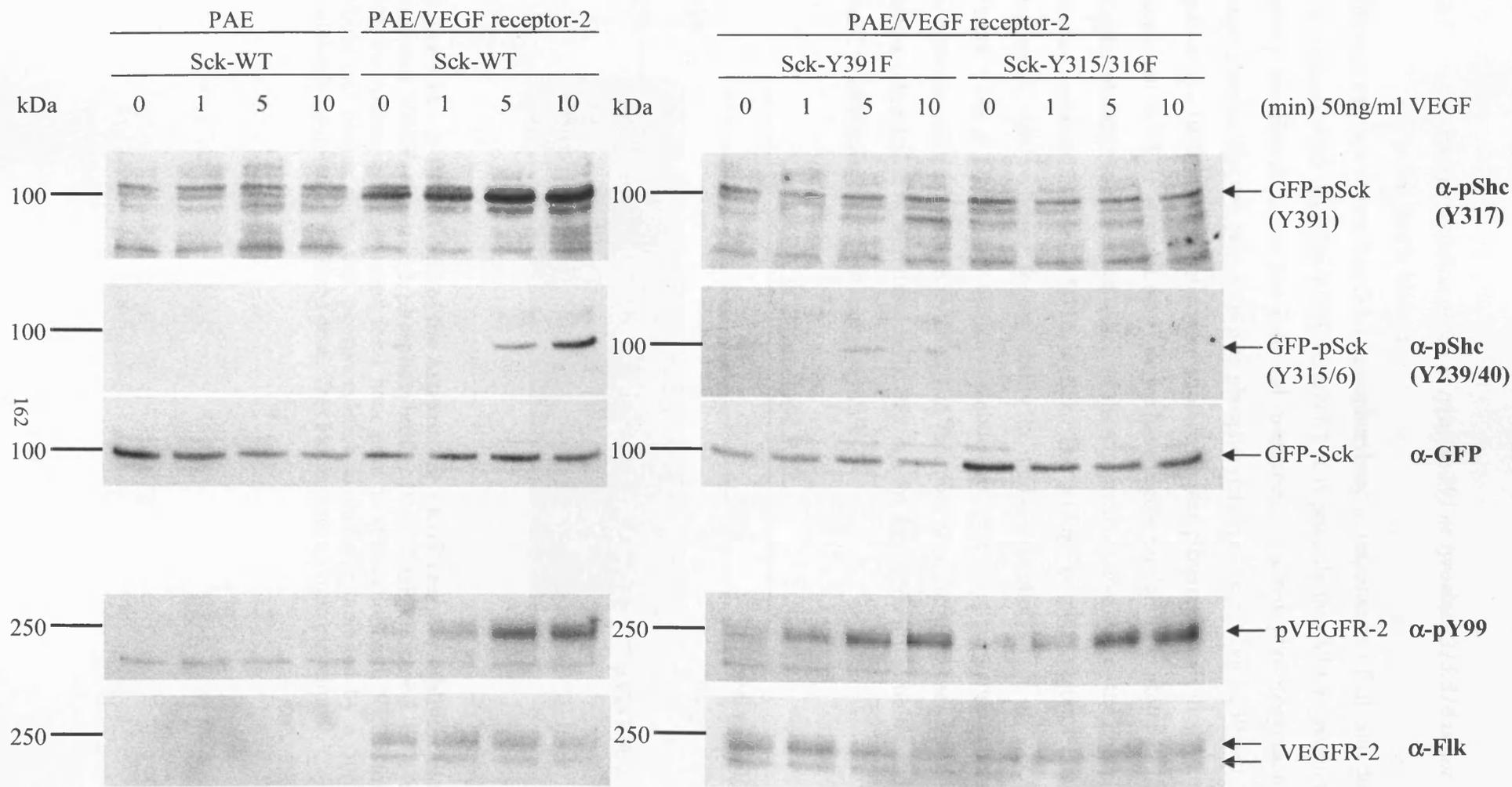


Figure 5.13 - Phosphorylation of tyrosine 391 of Sck requires phosphorylation of tyrosine 315 and/or tyrosine 316. Western blotting of whole cell lysates from cells transfected with GFP-tagged wild type Sck or Sck-Y391F or Sck-Y315/316F and stimulated with VEGF, using phospho-specific Shc antibodies, shows that wild type Sck is phosphorylated at tyrosines 391 and tyrosine 315 and/or 316, and SckY391F is phosphorylated at tyrosine 315 and/or 316 as expected but SckY316/316F is not phosphorylated at all. These blots are representative of two independent experiments.

5.2.7 Phosphorylation of Sck at tyrosine 391 or tyrosines 315/316 is not mediated by Src family kinases

Although we have shown that Sck is phosphorylated in response to VEGF and that it is found in a complex with phospho-VEGF receptor-2 it is possible that VEGF receptor-2 does not directly phosphorylate Sck and potential mediators of indirect phosphorylation are the Src family kinases. Src has been shown to phosphorylate tyrosine residues 239 and 240 of ShcA (Sato et al., 1997) and phosphorylate another adapter protein, Shb (Holmqvist et al., 2004), subsequent to VEGF stimulation. Here we have used a Src family inhibitor PP1 and a VEGF receptor inhibitor to determine which of these proteins is involved in the phosphorylation of tyrosine residues 391 or 315/316 in Sck. The VEGF receptor inhibitor, an Astrazeneca compound, was evaluated for its ability to inhibit phosphorylation of VEGF receptor-2 (Figure 5.14). A 50nM dose partially inhibited receptor phosphorylation whereas a 100nM dose totally inhibited phosphorylation and this dose was therefore used to inhibit receptor autophosphorylation in stimulated cells. PP1 is a Src kinase family inhibitor and was used at 10 μ M to inhibit Src activity in response to VEGF.

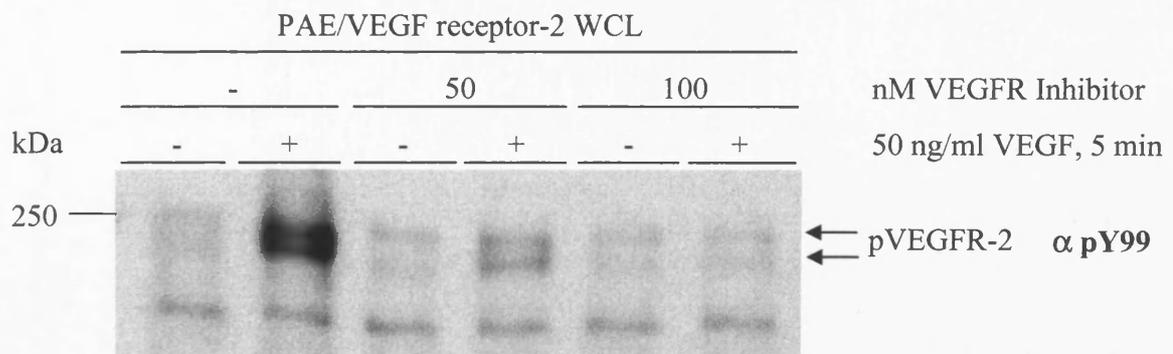
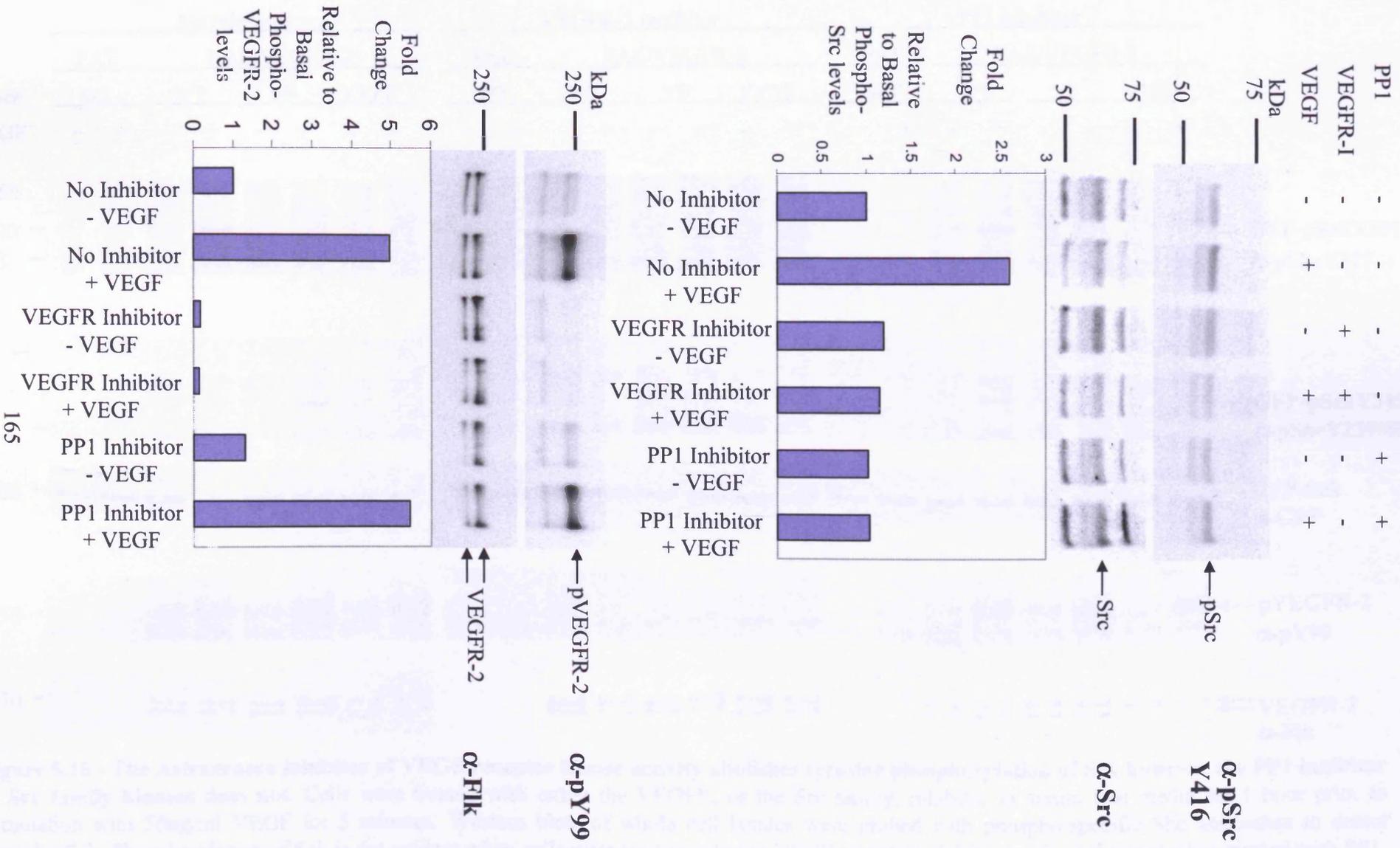


Figure 5.14 – A 100nM dose of the Astrazeneca VEGF receptor inhibitor blocks VEGF-mediated VEGF receptor-2 phosphorylation. PAE/VEGF receptor-2 cells were treated with the Astrazeneca compound for 1 hour prior to stimulation with VEGF for 5 minutes. Whole cell lysates show that phosphorylation is partially inhibited by a 50nM dose but completely inhibited by a 100nM dose. This blot is from a single experiment.

We first show (Figure 5.15) that the VEGF receptor inhibitor is able to inhibit both receptor and Src autophosphorylation and PP1 is able to inhibit VEGF stimulated Src phosphorylation. The Src inhibitor was unable to inhibit basal Src phosphorylation in unstimulated cells however. From these results we were confident that these compounds, used at these concentrations, inhibit the desired kinase and, in the case of the VEGF receptor inhibitor, downstream kinases also. We then treated Sck transfected PAE and PAE/VEGF receptor-2 cells with these inhibitors and stimulated them with 50 ng/ml VEGF for 5 minutes. Control cells were transfected with wild type or mutant Sck and stimulated but not treated with inhibitors. Untreated cells show phosphorylation of tyrosines 391 and 315/316 as seen before (Figure 5.16). Treatment of these cells with the VEGF receptor inhibitor abolished all Sck phosphorylation however treatment with PP1 has no effect on the Sck phosphorylation profile. As expected the PP1 inhibitor did not inhibit VEGF receptor-2 phosphorylation in VEGF stimulated cells.

Figure 5.15 – A 10 μ M dose of PP1 inhibits VEGF-mediated Src phosphorylation and a 100nM dose of the Astrazeneca VEGF receptor inhibitor inhibits basal and VEGF-stimulated VEGF receptor-2 phosphorylation. Cells were treated with these inhibitors in serum free media for 1 hour prior to stimulation with 50ng/ml VEGF for 5 minutes. Western blots of whole cell lysates were probed with a phospho-specific Src antibody, an antibody against Src to detect the total protein levels, a phosphotyrosine antibody and an antibody against VEGF receptor-2. All blots were quantified and the phosphorylated proteins were normalised against the levels of total protein. The graphs illustrate the fold change of phospho-Src and phospho-VEGF receptor-2 relative to basal levels of phosphorylation. These blots are from a single experiment.



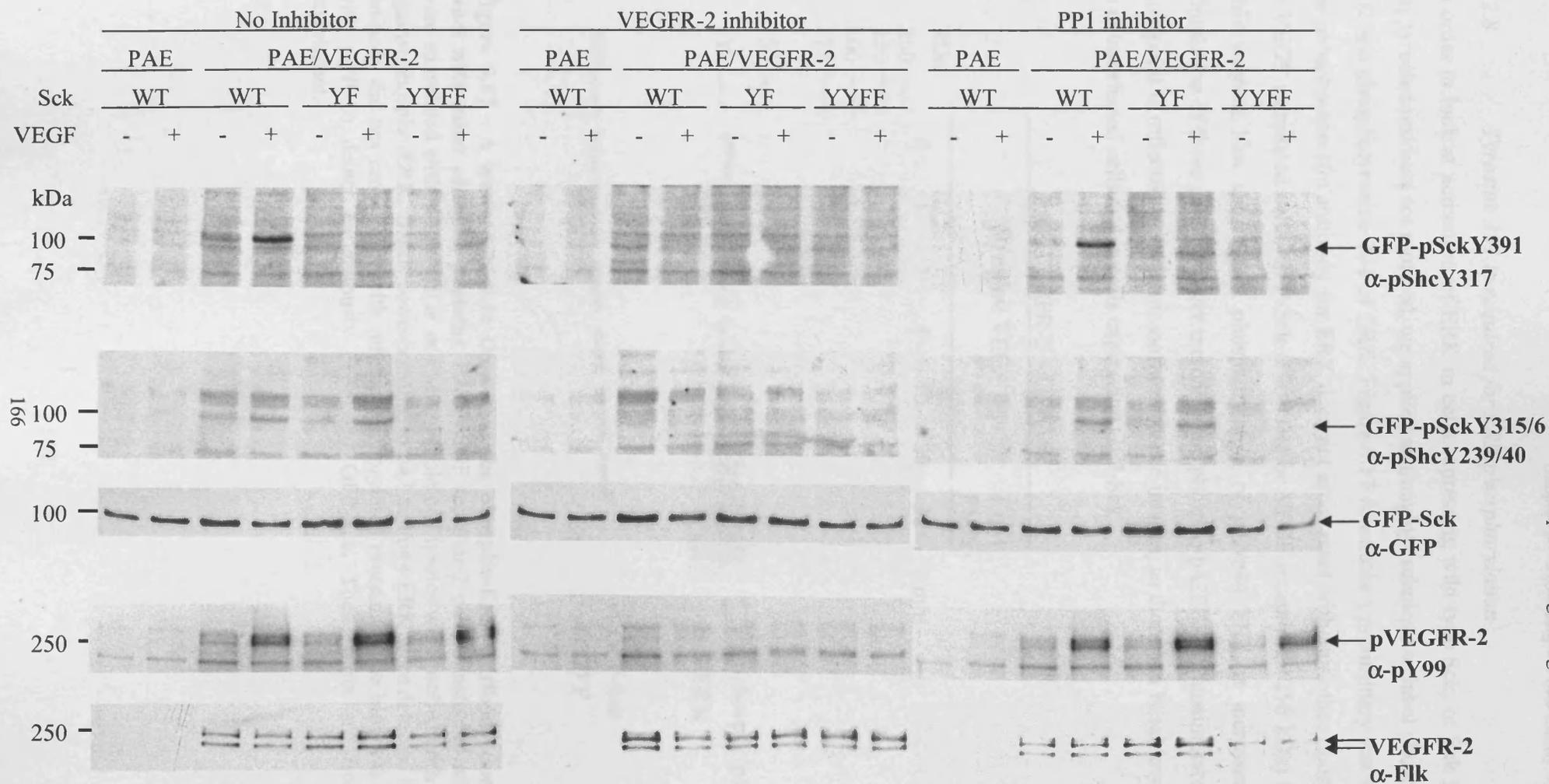


Figure 5.16 - The AstraZeneca inhibitor of VEGF receptor kinase activity abolishes tyrosine phosphorylation of Sck however the PP1 inhibitor of Src family kinases does not. Cells were treated with either the VEGFR, or the Src family, inhibitor in serum free media for 1 hour prior to stimulation with 50ng/ml VEGF for 5 minutes. Western blots of whole cell lysates were probed with phospho-specific Shc antibodies to detect phospho-Sck. Phosphorylation of Sck is not evident when cells were treated with the VEGF receptor inhibitor but is unchanged when treated with PP1. These blots are from a single experiment.

5.2.8 Tyrosine 391 is required for ERK phosphorylation

In order to look at activation of ERK in cells expressing wild type Sck, or Sck in which the key tyrosine residues are mutated, we applied an imaging technique to label phosphothreonine 202 and phosphotyrosine 204 of ERK. Figure 5.17 illustrates a preliminary test showing that the phospho-specific antibody for ERK does not cross react with any other phospho-proteins in VEGF stimulated cells, GFP-Sck (100 kDa) or VEGF receptor-2 (250 kDa) for example. This western blot shows that phosphorylation of p42/p44 ERK is increased by VEGF stimulation of these cells however there is some phospho-p42ERK in unstimulated cells. This later point is reflected in the immunofluorescence images, as there is a basal level of staining in untransfected cells and in cells expressing GFP-Sck.

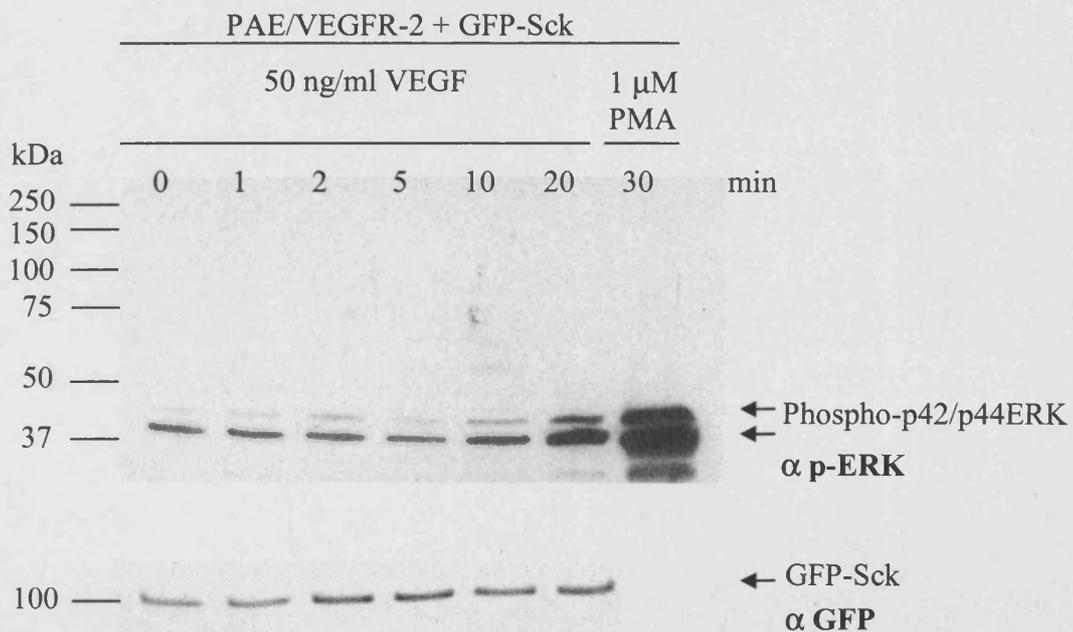


Figure 5.17 - A western blot to show that the phospho-ERK antibody does not cross react with other phospho-proteins. PAE/VEGF receptor-2 cells transfected with GFP-Sck were stimulated with either VEGF or PMA. Whole cell lysates were probed with an antibody against phospho-ERK. Bands corresponding to p42 and p44 ERK were detected however this antibody did not cross react with other phosphorylated proteins. The blot was also probed with GFP to detect exogenously expressed GFP-Sck. These blots are from a single experiment.

The most important observation is that VEGF stimulation results in an increase in phospho-ERK immunofluorescence staining in untransfected cells and cells expressing GFP-Sck (Figure 5.18). Conversely cells expressing GFP-SckY391 (Figure 5.18) do not show an increase in the phosphorylation of ERK despite the fact that untransfected cells do. Control staining shows that at the same exposure and brightness/contrast levels the secondary antibody does not label additional proteins in resting or stimulated cells expressing GFP-Sck. Wild type GFP-Sck is also localised to membrane ruffles, as seen previously, in VEGF stimulated cells.

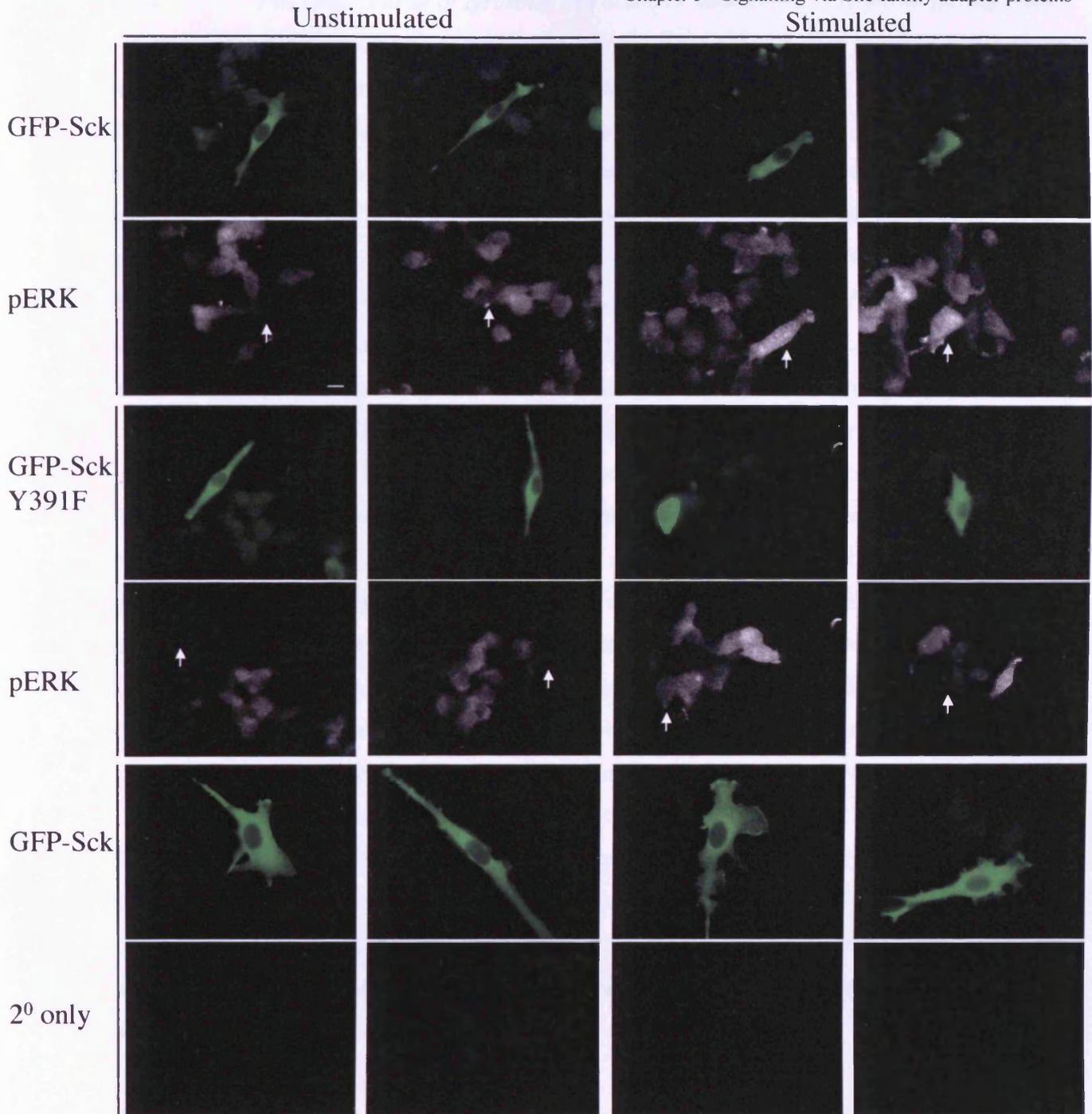


Figure 5.18 - Cells transfected with GFP-tagged wild type Sck and stimulated with VEGF show increased phospho-ERK staining however cells expressing GFP-SckY391F do not. PAE cells stably expressing VEGF receptor-2 were transiently transfected with GFP-Sck or GFP-SckY391F. Images shown are of cells that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for 5 minutes. Phospho-ERK is immunofluorescently labelled using an antibody against phospho-MAPK and a secondary antibody conjugated to Texas Red. All immunofluorescence images were taken at the same exposure and gain times and contrasted and enhanced in the same manner. These are representative cells from a single experiment. The scale bar represents 10 μm .

5.2.9 *Phosphorylation of tyrosines 391 and 315 and/or 316 is required for maximal DNA synthesis*

In order to determine the proportion of proliferating cells in a population the incorporation of 5-Bromo-2'-deoxy-Uridine (BrdU) into newly synthesised DNA can be used to label actively dividing cells. By using a method that involves the immunofluorescence labelling of incorporated BrdU we were able to study populations of PAE cells expressing VEGF receptor-2 and GFP-Sck. Figure 5.19 shows the summary of these results and the analysis of BrdU incorporation into resting and VEGF-stimulated cells expressing VEGF receptor-2 and GFP-Sck. Cells expressing VEGF receptor-2 and wild type Sck or Sck mutants are compared to untransfected PAE cells expressing VEGF receptor-2 only. By studying a population of ~2000 untransfected cells we report that under serum starvation conditions 27% of this population is actively synthesising DNA. The analysis of untransfected cells, stimulated with 50ng/ml VEGF for 24 hours, shows that in a population of ~1500 cells the proportion of cells positive for BrdU is 30%. This proportion is not a significant increase when compared to that of the unstimulated population. In populations of ~100 resting and VEGF stimulated cells expressing GFP-Sck the proportion of cells that incorporated BrdU into newly synthesised DNA is 45% and 56% respectively. The basal level of BrdU positive cells increased by 18% compared to that of untransfected cells. In addition, whereas untransfected cells showed no significant increase in BrdU incorporation upon VEGF stimulation, cells over expressing GFP-Sck show an increase of 11%. In cells expressing GFP-SckY391F the basal level of BrdU incorporation is similar to that seen in untransfected cells however the increase of 10% upon stimulation with VEGF is a similar to that detected in cells expressing wild type Sck. Conversely in cells expressing GFP-SckY315/316F the basal level of BrdU incorporation is similar to that seen in untransfected cells however there is no increase in this upon stimulation with VEGF.

The amount of BrdU incorporation into untransfected cells and cells expressing wild type and mutant Sck under unstimulated and stimulated conditions were subjected to statistical analysis using a two way ANOVA test (Table 5.2). The analysis shows a statistically significant ($p=0.02$) difference in the level of BrdU incorporation between cells expressing endogenous levels of Sck, over-expressing wild type Sck, over-expressing the single tyrosine mutant and over-expressing the double tyrosine mutant, as the calculated F value (20.11) is greater than the critical F value (9.28). The difference between unstimulated and stimulated cells is not statistically significant as the calculated F value (5.75) is less than the critical F value (10.13).

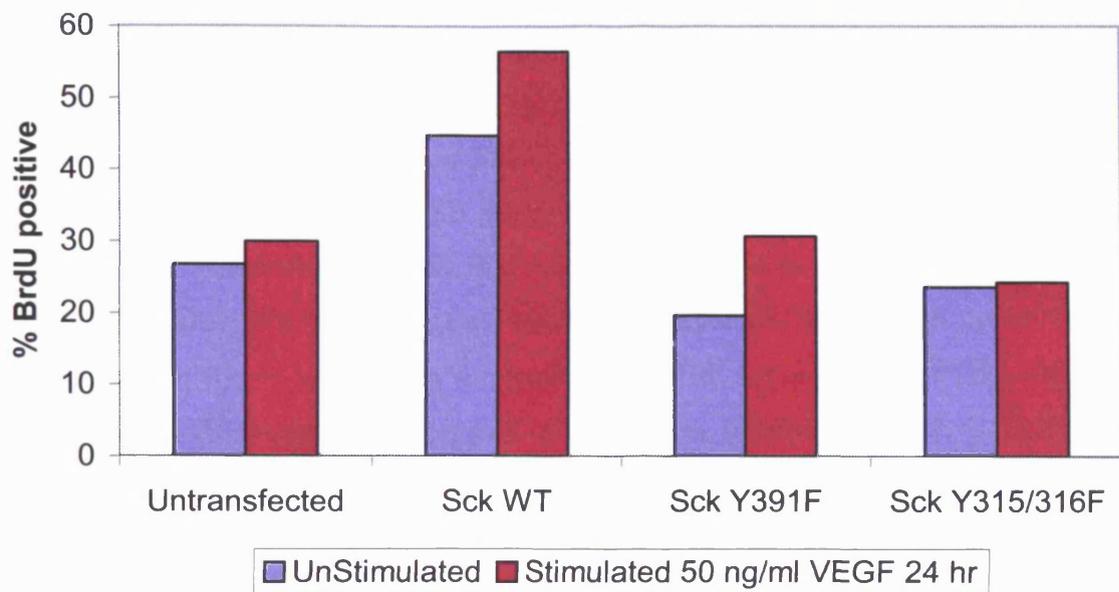


Figure 5.19 - DNA synthesis is increased in cells expressing GFP-tagged wild type Sck but not in cells expressing GFP-tagged SckY391F or SckY315/6F. The incorporation of BrdU into newly synthesised DNA can be detected by immunofluorescence. An antibody against BrdU and, in this case, a secondary antibody conjugated to texas red, labelled untransfected and Sck transfected cells as actively proliferating. Populations of untransfected cells and cells expressing GFP-tagged wild Sck, Sck-Y391F and Sck-Y315/6F were counted and scored as BrdU positive or negative. The numbers of cells counted for each cell population were as follows – Untransfected – 2176 unstimulated and 1479 stimulated, GFP-Sck WT – 103 unstimulated and 101 stimulated, GFP-SckY391F – 102 unstimulated and 101 stimulated, GFP-SckY315/6F – 106 unstimulated and 103 stimulated. The results shown are from a single experiment.

	unstimulated	stimulated
Untransfected	31.14	33.18
wtSck transfected	41.93	48.70
Sck Y391F transfected	26.28	33.64
Sck Y315/316F transfected	29.05	29.52

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	938.84	3	312.95	20.11	0.02	9.28
Columns	89.47	1	89.47	5.75	0.10	10.13
Error	46.68	3	15.56			
Total	1074.99	7				

Table 5.1 – Two way ANOVA summary table. Summary of ANOVA results for the analysis of BrdU incorporation into Sck transfected cells. Percentages given in Figure 5.19 were converted into arcsin values and a two factor, without replication ANOVA test was applied to the table of results. The values were inserted into the table shown. SS, sum of squares; df, degrees of freedom; MS, mean square; F, test statistic; P, probability level; F crit, critical F value.

5.3 - Discussion

In this study we have shown that the ShcA-related protein Sck is able to associate with VEGF receptor-2 whereas ShcA itself cannot. The SH2 domain of Sck mediates this interaction, which is in contrast to the binding of ShcA to activated receptors as its PTB domain that predominantly mediates this. The PTB domain of ShcA also associates with phospholipids and as proposed by Ravichandran this is the first step towards its association with receptors (Ravichandran, 2001). From our imaging studies it is apparent that the PTB domain of Sck may be important for its localisation to membrane ruffles. Although Sck Δ PTB co-localised with phospho-VEGF receptor-2 at regions of the plasma membrane, which is likely to be mediated by the SH2 domain of Sck, the extent of Sck Δ PTB membrane localisation appears to be less than that of Sck Δ SH2. In conjunction with our immunoprecipitation data we hypothesise that the PTB domain of Sck localises the protein to the membrane, in close proximity to VEGF receptor-2, and the SH2 domain of Sck mediate the direct interaction between Sck and the receptor. Despite the inability of ShcA to bind to VEGF receptor-2 it is phosphorylated in response to VEGF stimulation however only at tyrosine 317. The phosphorylation of ShcA at this site may therefore be mediated by a kinase activated downstream of VEGF receptor-2 signalling. The phosphorylation of Sck in response to VEGF is likely to be mediated by the receptor itself and we have shown that the activation of Src family kinases is not involved in Sck phosphorylation. We also show that phosphotyrosine 315 and/or 316 of Sck is necessary for the phosphorylation of tyrosine 391. Expression of SckY391F abolishes ERK phosphorylation however, when tyrosine 391 is mutated, cells are still able to proliferate in response to VEGF. On the other hand, when SckY315/316F is expressed tyrosines 315, 316 and 391 are not phosphorylated and this results in a complete abolishment of cell proliferation in response to VEGF. We propose therefore that phosphorylation of tyrosine 391 in Sck is responsible for downstream phosphorylation of ERK. Tyrosine residues 315 and 316 may be responsible for the increase in the incorporation of BrdU detected in stimulated cells, whereas phosphorylation of tyrosine 391 may be responsible for an increase the basal level of BrdU incorporation. In this study we have only investigated the phosphorylation status of the tyrosine residues in Sck that correspond to the well-characterised Grb2 binding sites in ShcA. It is possible however that one, or more, of the many tyrosine residues found in the Sck protein and/or other amino acids, such as serine or threonine residues, are phosphorylated in response to VEGF and these may initiate downstream signalling or regulate signalling via tyrosines 315, 316 and 391.

The role(s) of Shc-family proteins during development has been studied using transgenic mouse models. ShcA deficient mice are not viable and die due to severe heart defects and improper angiogenesis (Lai and Pawson, 2000). The angiogenic deficiencies observed in ShcA-deficient mice include defective angiogenic remodelling and hence the inability to establish mature blood vessels. Mouse embryonic fibroblasts from ShcA-deficient animals show that these cells have a decreased sensitivity to PDGF-BB- and EGF-induced MAP kinase activation. In addition, analysis of the actin cytoskeleton, focal complex distribution and MAP kinase activity in response to fibronectin show that ShcA is important for the organisation of the cytoskeleton in response to the extracellular matrix. From these observations, and considering that ShcA is primarily expressed in the cardiovascular system during early mouse embryogenesis, it may appear surprising that our results show no interaction between ShcA and VEGF receptor-2. Further studies however have identified a crucial role for ShcA in Met/HGF and ErbB2 receptor signalling to induce the expression of VEGF (Saucier et al., 2004). The precise dosage of VEGF is critical during early development, as shown by the lethal vascular defects in mouse embryos, resulting from the inactivation of a single VEGF allele. Signalling via ERK and PI3K pathways downstream of receptor tyrosine kinases can result in the production of VEGF. The induction of VEGF by the Met/HGF and ErbB2 receptors could therefore use ShcA to activate the MAP kinase and PI3K pathways via the Grb2-Sos-Ras complex and Gab1 respectively. Inhibition of the MAP kinase and PI3K pathways has little effect however on the production of VEGF suggesting that other Shc-mediate pathways may be involved.

Sck deficient animals are viable but show a loss of peptidergic and non-peptidergic nociceptive sensory neurons (Sakai et al., 2000). In the same study mice lacking N-Shc and those lacking Sck and N-Shc were also analysed. Mice lacking both of these Shc proteins showed a significant loss of neurons within the superior cervical ganglia but the loss of N-Shc did not enhance the loss of sensory neurons seen in Sck-deficient mice. Sck is proposed to play a unique role in the survival of sensory neurons and act, together with N-Shc, to support the development/survival of sympathetic neurons. Both proteins are important in mediating survival signals downstream of the Trk receptor tyrosine kinases (Liu and Meakin, 2002). These receptors are normally expressed in the peptidergic and non-peptidergic nociceptive sensory neurons that are missing in Sck null animals. Signalling via TrkA receptors in nerve growth factor (NGF)-stimulated PC12 cells shows that ShcA is used to couple the receptor to MAP kinase and PI3K pathways to mediate cell survival and neurite outgrowth (Obermeier et

al., 1994). Although the ShcA protein is widely expressed, it is present only at low levels in the central nervous system and the expression patterns of Sck and N-Shc are complementary to ShcA (Nakamura et al., 1998; O'Bryan et al., 1996). Sck and N-Shc are likely to function as neuronal adapters instead of ShcA. These adapters show differences in their functions and in their localisation within the brain so are therefore likely to have distinct roles in neuronal signalling. The variation in signalling capabilities of all three Shc proteins is illustrated by the following observations – i) All Shc proteins bind to autophosphorylated EGF receptor, however, Sck binds more efficiently than N-Shc, which in turn was more efficient than ShcA (Nakamura et al., 1998), ii) In NIH3T3 cells expressing the Axl tyrosine kinase, ShcA bound most efficiently, followed by Sck then N-Shc (Zhou et al., 1995a), and iii) Sck bound a Src-phosphorylated protein, pp135 whereas N-Shc was able to do so but to a lesser extent (Nakamura et al., 1998).

Stimulation of neuronal cells with neurotrophins such as NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) activates Trk receptors A, B and C respectively (Barbacid, 1995). These receptors use the Shc family of proteins as adapters to transduce signals through downstream signalling pathways. The Trk receptors play a crucial role in neuronal survival and neurite outgrowth by activating MAP kinase and PI3K pathways (Huang and Reichardt, 2003). As mentioned above ShcA can associate with TrkA but Liu et al propose that Sck and N-Shc, and not ShcA, are the primary adapter proteins in neurons (Liu and Meakin, 2002). In cells such as sympathetic neurons and sensory neurons in the peripheral nervous system where Sck is expressed, but ShcA is not, this appears highly likely. Sck and N-Shc bind to phosphorylated Trk receptors via their PTB domains and consequently the adapters are phosphorylated and they recruit Grb2. The activation of TrkA or TrkC by NGF or NT-3 in cells over expressing Sck and N-Shc therefore results in enhanced MAP kinase activation (Liu and Meakin, 2002). Similarly in mouse cortical neurons Sck and N-Shc interact with Trk receptors and are activated in response to neurotrophic stimulation. This indicates that these adapters are endogenous targets of the Trk receptors in primary cells. All of the Shc proteins associate with the same site in TrkA and TrkB, which suggests that the isoform that is most highly expressed in a particular tissue or at a particular stage in development binds to the receptor. Each Shc protein may therefore have a different role and may mediate unique protein-protein interactions. Immunoprecipitation of Sck and N-Shc has identified phosphorylated proteins in addition to the Trk receptors that associate with the

receptor complex in stimulated cells. These proteins may represent neuron specific downstream targets of Shc adapters.

While VEGF receptor-2 was originally thought to be endothelial cell specific (Ratcliffe et al., 2002) it has recently been identified in neuronal cells (Khaibullina et al., 2004; Nico et al., 2004; Ratcliffe et al., 2002) and plays an important role in neuronal survival (Wick et al., 2002). This has led us to hypothesise that Sck could be used in these cells to mediate VEGF receptor-2 signalling. This was briefly addressed in our lab by Lisa Eaton but will require further study. In the peripheral nervous system VEGF acts as a neurotrophic factor to increase the proliferation of Schwann cells, to enhance cell survival and to stimulate axonal outgrowth (Kutcher et al., 2004). VEGF is expressed in a subset of neurons in the developing and mature central nervous system. As about 90% of cells expressing VEGF also express VEGF receptor-2 and the two proteins co-localise it is probable that VEGF acts in an autocrine manner (Ogunshola et al., 2002). VEGF levels are up regulated in neuronal cultures exposed to hypoxia and additionally in these cells VEGF receptor-2, ERK, p90RSK and Stat3a exhibit a higher level of tyrosine phosphorylation. Immunoprecipitation of VEGF receptor-2 from neurons shows the association of phosphoproteins of 85, 65 and 45 kDa, which the investigators designate as p85, ShcA and Nck. Inhibition of VEGF receptor-2 signalling or sequestration of neuronal VEGF results in an increase in apoptosis of cultured neurons as shown by the TUNEL assay and increased caspase-3 staining. This is due to inhibition of the MEK-ERK-p90RSK pathway that partially mediates survival in cortical neurons. From the results detailed in this study we can speculate that VEGF receptor-2 uses Sck (68 kDa) to couple activated VEGF receptor-2 to this pathway.

Although we have discussed the three Shc family members that are expressed in humans, drosophila only express one Shc protein, in which only tyrosines 239 and 240 are conserved (Luzi et al., 2000). This may highlight that these residues are more significant than tyrosine 317 for downstream signalling. Here we show that Sck, but not ShcA, is phosphorylated at these residues in response to VEGF, which provides further evidence that Sck is the more relevant adapter with respect to initiating VEGF specific signals. With regard to ShcA, Walk et al. have observed subtle, but reproducible differences in the phosphorylation of tyrosines 239/240 versus tyrosine 317 by different kinases (Walk et al., 1998). The phosphorylation state of these two sites may be governed by receptor tyrosine kinases or by other kinases that are activated by a given receptor. In this study Walk et al investigate the consequences of Lck

and Syk family kinase activation following T cell receptor activation, a key event leading to T lymphocyte proliferation. For ShcA they show that the extent and site of phosphorylation is influenced by the specific kinase that is activated. Lck shows a preference for the tyrosine 317 site and phosphorylation of this residue is also dependent on Src kinases. The Syk family of kinases phosphorylate both the tyrosine 239/240 site and the tyrosine 317 site but the latter is phosphorylated with less efficiency. The synergistic activity of these two families of kinases therefore mediates the maximum phosphorylation of ShcA during T cell receptor activation.

Phosphorylation of ShcA ultimately leads to MAP kinase activation via the Grb2-Sos complex and Walk et al propose that tyrosine 239 has a greater role in the recruitment of Sos through Grb2 (Walk et al., 1998). This observation is supported by the work of Harmer et al (Harmer and DeFranco, 1997) however it is in contrast to Gotoh et al (Gotoh et al., 1997) who propose that the binding of the Grb2-Sos complex to tyrosine 317 is the major initiation step in the activation of the MAP kinase pathway. Cells expressing Y317F ShcA do not show activation of MAP kinase as detected by a kinase assay or by western blotting using a phospho-MAP kinase antibody. Wild type ShcA and Y239/240F ShcA however efficiently activate MAP kinase via an association with Grb2. It is possible however that the Grb2-Sos complex, once recruited to Shc family proteins, signals to other pathways that have yet to be identified and/or that different signalling pathways are activated downstream of the two phosphorylation sites identified in these proteins. The phosphorylation of tyrosines 239 and 240 has been shown to result in EGF stimulated mitogenesis of NIH3T3 cells and Gotoh et al propose that this is independent of Ras/MAP kinase activation and may result from a pathway that activates *c-Myc* (Gotoh et al., 1997). In cells expressing wild type or Y317F ShcA, a *c-Myc* RNA message, as detected by northern blotting, is efficiently induced which is saturated by a 0.1ng/ml dose of EGF. This *c-Myc* message is hardly detectable however in cells expressing Y239/240 ShcA and stimulated with the same dose of EGF. Induction of the message is only detected by increasing the concentration of EGF. Shc-mediated signalling may inactivate p53, a repressor of the *c-Myc* gene, to positively regulate *myc* transcription.

ShcA is important for growth factor-induced mitogenesis. This has been demonstrated by the over expression of ShcA-SH2, which acts as a dominant negative peptide and suppresses EGF-induced mitogenesis of fibroblasts (Gotoh et al., 1995), and by the use of antibodies to ShcA, which also suppresses the mitogenesis of fibroblasts in response to EGF, insulin or IGF (Sasaoka et al., 1994). MAP kinase activation, along with *c-Myc* induction and/or another

mitogenic signalling mechanism, are likely to be the routes used to increase growth factor-stimulated cell proliferation. Cells expressing either Y317F ShcA or Y239/240F ShcA are able to proliferate as efficiently as cells expressing the wild type ShcA protein (Gotoh et al., 1997). Cells expressing Y317/239/240F ShcA however show about a 60% reduction in proliferation compared to that of cells expressing wild type ShcA. This suggests that whatever pathways are activated downstream of these two phosphorylation sites they act co-operatively to induce cell proliferation. The possibility that these two pathways involve Ras- and Myc-mediated signalling is supported by the observation that both *v-ras* and *c-myc* oncogenes are required for the transformation of rat embryo fibroblasts (Land et al., 1983).

Studies using a Src family kinase inhibitor to determine the targets of Src in growth factor signalling have shown that Y317F ShcA, but not Y239/240F ShcA or Y317/239/240F ShcA, is phosphorylated by c-Src (Sato et al., 1997). v-Src, a constitutively active form of Src, and the EGF receptor are able to phosphorylate both Y317F and Y239/240F ShcA but not Y317/239/240F ShcA. These mechanisms are likely to be different however as phosphorylation by c-Src requires the co-operative association of the PTB domain of ShcA with PtdIns (4,5) P₂ whereas phosphorylation by v-Src or the EGF receptor does not. This could indicate that PtdIns (4,5) P₂ is a regulator of site-specific phosphorylation of ShcA through a c-Src-mediated system.

In this study we have observed differences in the extent and site of ShcA and Sck phosphorylation in response to VEGF receptor-2 activation. With regard to phosphorylation of Sck we show that VEGF receptor-2 phosphorylates both sites but may prefer the tyrosines equivalent to 239/240 of ShcA. This site is not phosphorylated at all in ShcA in response to VEGF receptor-2 activation. Src has been reported to associate with VEGF receptor-2 and to be activated in response to VEGF stimulation of HUVE cells indicating that ShcA or Sck could be a substrate for Src in stimulated endothelial cells. We show here however that Sck is phosphorylated in response to VEGF receptor-2 activation independently of Src family kinases indicating that the receptor probably phosphorylates Sck directly. In Table 5.1 the consequences of Sck phosphorylation in response to VEGF are summarised.

	Increased tyrosine phosphorylation in response to VEGF?		Increase in phospho-ERK staining on stimulation?	BrdU incorporation?	
	Y391	Y315/6		Basal increase	Stimulated increase
GFP-Sck WT	Yes	Yes	Yes	Yes	Yes
GFP-Sck Y391F	No	Yes	No	No	Yes
GFP-Sck Y315/6F	No	No	-	No	No

Table 5.1 – Summary of observations regarding tyrosine phosphorylation of wild type and mutants Sck proteins and subsequent ERK phosphorylation and BrdU incorporation into newly synthesised DNA.

In cells expressing wild type Sck, activation of VEGF receptor-2 results in tyrosine phosphorylation at both Grb2 binding sites. By comparing untransfected endothelial cells with those expressing wild type Sck we observe that Sck mediates the phosphorylation of ERK in response to VEGF. In addition the over expression of Sck causes an increase in DNA synthesis in resting cells and a further increase upon VEGF stimulation. By investigating the effect of mutating these key tyrosine residues in Sck we conclude that i) Phosphorylation at tyrosines 315 and/or 316 is required for phosphorylation at tyrosine 391, ii) Signalling downstream of tyrosine 391 results in phosphorylation of ERK, which may increase the basal level of DNA synthesis and iii) Phosphotyrosine 315 and/or 316 may mediate an increase in DNA synthesis in response to VEGF stimulation of these cells. Further work will be required to determine the signalling pathway(s) that are initiated following the phosphorylation of tyrosines 315 and 316 and to determine whether Grb2 is involved. As tyrosine 315 is contained within a Grb2-binding motif then this adapter is implicated as a downstream target and also, from studies of the relative position in ShcA, a pathway leading to the induction of *c-Myc* is a strong possibility.

Chapter 6

Down-regulation of VEGF Receptor-2

Chapter 6 – Down-regulation of VEGF receptor-2

6.1 - Introduction

For efficient VEGF signalling the VEGF receptors have to be present on the cell surface, with their extracellular domains exposed to the extracellular environment. The compartmentalisation of receptors can render their extracellular domains inaccessible to external reagents to prevent ligand-receptor interactions. This is one method of desensitising the cell to VEGF and this is completely reversible. VEGF receptor-2 is localised in endothelial caveolae, a major feature in endothelial cells, and association of VEGF receptor-2 with caveolin-1 negatively regulates its activation in unstimulated cells (Labrecque et al., 2003). The inhibitory action of caveolin-1 however is circumvented by receptor phosphorylation and src activation. This results in the rapid dissociation of the receptor-caveolin-1 complex and exit of the receptor from caveolae so that it can initiate signalling.

Ligand mediated endocytosis is likely to be the regulatory mechanism used to permanently reduce the levels of VEGF receptors at the cell-surface of stimulated cells. This mechanism regulates the responsiveness of endothelial cell signalling pathways to continual VEGF activation. In this study we show that VEGF-stimulation of HUVE and PAE cells results in phosphorylation of VEGF receptor-2, which is accompanied by a rapid reduction in the receptor protein level. We have also detected a basal rate of receptor internalisation and observed localisation of VEGF receptor-2 in vesicular structures that contain caveolin-1 and EEA-1, but not markers for late endosomes/lysosomes or focal adhesions.

6.1.1 *Maximal kinase activity is required for internalisation of VEGF receptor-2*

Autophosphorylation of tyrosine residues contained within the catalytic domains of the insulin receptor, the FGF receptor-2 and the c-Met receptor is required for their maximal kinase activity (Longati et al., 1994; Mohammadi et al., 1996; Wilden et al., 1992) and EGF receptor kinase activity is required for maximum rates of receptor internalisation (Wiley et al., 1991). Dougher et al have also reported a link between the phosphorylation level and rate of internalisation of VEGF receptor-2 in BAE cells (Dougher and Terman, 1999). By studying wild type VEGF receptor-2 and a mutant, containing a double tyrosine to phenylalanine mutation at positions 1054 and 1059, they discovered that the phosphorylation level of the mutant receptor was only 10% of the wild type level. These tyrosine residues are

autophosphorylation sites and as they are located within the kinase domain it is not surprising that they appear to be essential for maximal kinase activity of VEGF receptor-2 and hence the phosphorylation of PLC γ , P¹²⁵FAK and paxillin. Internalisation is delayed for the double tyrosine mutant receptor. The rate of receptor internalisation was measured in ¹²⁵I VEGF-stimulated BAE cells, at varying time points, by using an acid wash protocol to selectively remove cell surface ¹²⁵I VEGF. The rate of receptor internalisation in BAE cells and HEK293 cells transfected with native VEGF receptor-2 was compared and were shown to have the same profile of internalisation with 50% of receptor internalised at 25 minutes. The profile for internalisation of the mutant receptor showed a slower rate of internalisation as it took 55 minutes for 50% of this receptor to be internalised. As VEGF was internalised with the mutant receptor this data probably represents the basal rate of receptor turnover i.e. the receptor internalisation that is not dependent on growth factor stimulation.

6.1.2 *Ubiquitination of VEGF receptor-2 by Cbl*

The internalisation of receptors does not necessarily correlate with receptor degradation, and Duval et al. sought to investigate the mechanism for the down-regulation of VEGF receptor-2 (Duval et al., 2003). They noted a significant decrease in VEGF receptor-2 protein levels after 15 minutes of stimulation with 50ng/ml and maximal protein degradation after 30-60 minutes of stimulation. Decreased receptor levels were also evident after treatment with a range of doses of VEGF for 15 minutes. This group attributed the reduction in VEGF receptor-2 protein levels after VEGF stimulation to the ubiquitination of the receptor by Cbl and its consequential degradation. Western blot analysis shows that the activation of VEGF receptor-2 with 10 ng/ml VEGF, to activate the receptor but induce minimal internalisation, results in receptor ubiquitination in response to VEGF. Ubiquitination is classified by the smearing of ubiquitinated proteins on a blot. This was observed for VEGF receptor-2 and followed the kinetics of VEGF-induced tyrosine phosphorylation of the receptor. VEGF stimulation of BAE cells results in rapid and sustained tyrosine phosphorylation of Cbl and promotes its association with activated VEGF receptor-2. Co-immunoprecipitation studies show that VEGF enhances the association of Cbl with VEGF receptor-2. The over-expression of Cbl in COS7 cells, also expressing VEGF receptor-2, shows increased ubiquitination of the receptor and a more rapid rate of receptor degradation. These observations indicate that Cbl associates with, and mediates the ubiquitination of, VEGF receptor-2 resulting in VEGF-dependent receptor degradation. This is further clarified by the over-expression of an ubiquitin ligase-inactive mutant of Cbl that did not enhance receptor ubiquitination or degradation.

In contrast, results obtained by Singh et al show that VEGF receptor-2 mediates the phosphorylation of Cbl but the activation, ubiquitination and down-regulation of the receptor are not affected by c-Cbl activity (Singh et al., 2005). Over-expression of wild type or ligase inactive c-Cbl did not alter the ligand-dependent down-regulation or ubiquitination of a chimeric VEGF receptor-2 compared to that seen with endogenous Cbl proteins. As a positive control for their experiments they show that over-expression of wild type c-Cbl greatly accelerates the rate and extent of mature EGF receptor down-regulation and show evidence of receptor ubiquitination in response to EGF. This group propose that the activation of a non-classical PKC enzyme phosphorylates a serine residue in the C-terminal domain of VEGF receptor-2, which is used, instead of ubiquitin, to mark the receptor for internalisation and proteasomal degradation.

6.1.3 *The endocytic process leads to degradation of ubiquitinated receptors*

The degradation pathway of other receptor tyrosine kinases has been studied in depth and we therefore hypothesised that VEGF receptor-2 trafficking would follow the same route, through endosomal vesicles to the lysosome, leading to degradation. This was tested by the adoption of an imaging approach using protein markers for endosomes and lysosomes. Mono-ubiquitinated receptors are targeted to the endocytic pathway as a prerequisite to their degradation. The Eps15, Hrs and Tsg101 adapter proteins contain an ubiquitin interaction motif (UIM) that binds mono-ubiquitin. UIM motifs are responsible for the trafficking of ubiquitinated receptors from the cell surface to lysosomes and the proteins containing these domains are often referred to as ubiquitin receptors. These receptors are preferentially compartmentalised in endosomal vesicles and may therefore function as specific gating receptors for ubiquitinated cargoes at different stages in the endocytic pathway.

6.1.4 *Clathrin-dependent endocytosis*

The ubiquitin molecules attached to activated receptors act as a signal for their internalisation via clathrin-coated pits. Clathrin coats consist of clathrin and adapter proteins that develop into endosome targeted vesicles. This process requires the formation of clathrin lattices and membrane budding around receptors, modifications to the membrane curvature in preparation for membrane invagination and fission from the plasma membrane resulting in a clathrin-coated vesicle. As Eps15 and epsins contain many protein-protein interaction domains, and

can bind to clathrin and AP2, these proteins have been reported to be likely candidates to link ubiquitinated cargoes to the clathrin machinery at the plasma membrane (reviewed in Dupre et al., 2004; Haglund et al., 2003). It is possible that Eps15 or epsin could interact directly with ubiquitinated cargoes, via UIM motifs, or indirectly via proteins such as Crk (Dupre et al., 2004).

In addition, a second complex aids the internalisation of receptors by modifying the curvature of the membrane. Following the binding of Cbl to an activated receptor a CIN-85 (Cbl-interacting protein of 85 kDa), Endophilin A1 complex is recruited (Dikic, 2003). CIN-85 and Endophilin are found in a constitutive complex mediated by the interaction of a SH3 domain in endophilin and a proline rich region in CIN-85. After sufficient membrane curvature deep invaginated pits are formed. Dynamin, a GTPase that acts with endophilin, assembles itself into a helical collar around the neck of the pit, brings the two opposing membranes together and by membrane fission creates free, coated vesicles. A third protein amphiphysin, functions as a linker between dynamin and clathrin coats.

6.1.5 *Internalisation and endocytosis of ubiquitinated proteins by clathrin-independent pathway*

The EGF receptor can be endocytosed by a clathrin-dependent and independent route (Aguilar and Wendland, 2005). The latter route uses caveolae vesicles, which is likely to be an important mode of endocytosis in endothelial cells as they contain few clathrin-coated pits. There are multiple clathrin-independent endocytosis pathways that generally depend on cholesterol- and caveolin-rich membrane microdomains. These domains provide a platform for signalling complexes and can develop into membrane invaginations called caveolae. The internalisation of receptors into caveolae is directed by ubiquitination of the receptor and association with UIM-containing proteins. The route of VEGF receptor-2 internalisation has not been thoroughly investigated and so the endocytosis of ubiquitinated receptors via caveolae vesicles is a viable option. Again we chose an imaging method to determine whether VEGF receptor-2 is localised in caveolae in resting and stimulated cells.

6.1.6 *The targeting of ubiquitinated receptors to multi-vesicular bodies (MVBs) ensures that they are degraded and not recycled back to the plasma membrane*

Internalised receptors are prevented from being recycled back to the plasma membrane by the inward budding of late endocytic vesicles with MVBs. Ubiquitinated targets are sorted to MVBs through the UIM of Hrs and/or through the ubiquitin E2 variant (UEV) domain of Tsg101. At this stage the ubiquitinated proteins are recognised by the ESCRT machinery and Tsg101, a component of ESCRT-I complex, interacts with cargo that should be processed via the ESCRT II and III complexes for degradation. This is a very important stage as sorting into the MVB commits the cargo to the degradation pathway, prevents recycling and ensures termination of the signal. Finally, the fusion of the outer membrane of the MVB with the lysosomal membrane results in the delivery of the luminal MVB vesicles and their contents to the hydrolytic interior of the lysosome, where they are degraded (reviewed in Haglund et al., 2003; Katzmann et al., 2002).

6.1.7 *VEGF and PlGF promote $\alpha v \beta 3$ integrin recycling which is required for the formation and turnover of focal adhesions, processes that are linked to cell migration.*

The integrin $\alpha v \beta 3$ is expressed in endothelial cells and recycling of this integrin is required for the formation and turnover of focal adhesions (Soldi et al., 1999). The recycling of $\alpha v \beta 3$ occurs via two routes and is dependent on some members of the Rab family of small GTPases. The short loop, a Rab4-dependent pathway involving trafficking from early/sorting endosomes to the plasma membrane, is required for focal adhesion formation and the long loop, a Rab11-dependent pathway involving trafficking from late/recycling endosomes directly back to the plasma membrane, is required for cell migration. The stimulation of HUVE cells with VEGF or PlGF does not regulate the internalisation of $\alpha v \beta 3$ nor does it promote integrin degradation. VEGF, FGF and PlGF however stimulate the recycling of $\alpha v \beta 3$ from early endosomes to the plasma membrane. The consequence of this is an increase in the surface expression of $\alpha v \beta 3$, which drives the formation of $\alpha v \beta 3$ -containing focal adhesions and increases the ability of HUVE cells to migrate towards fibronectin (personal communication with Matthew Jones).

6.2 - Results

6.2.1 *VEGF receptor-2 is degraded in HUVE and PAE cells in response to VEGF stimulation*

As previously observed, the stimulation of endothelial cells with VEGF, results in phosphorylation of VEGF receptor-2 in a time and dose dependent manner. Here we quantify the extent of receptor phosphorylation and the rate of receptor degradation over a time course of VEGF stimulation. Western blots, using antibodies to phosphotyrosine and VEGF receptor-2, show an early increase in receptor phosphorylation, followed by a decrease at later time points, and the continual decrease in total receptor protein levels throughout the phosphorylation changes (Figure 6.1). The receptor-specific antibody used for western blotting (Flk A3, sc-6251) binds to the C-terminus, in the intracellular domain, of VEGF receptor-2 (amino acids 1158-1345 of murine VEGF receptor-2). The peak of receptor phosphorylation is evident in HUVE cells after 1-2 minutes of VEGF stimulation and is increased to 8 times that detected in resting cells. In PAE cells this peak of phosphorylation occurs later, averaging at 10 minutes of VEGF stimulation, however this was variable and was detected between 2 and 10 minutes of VEGF stimulation. The increase in receptor phosphorylation was much greater in PAE cells compared to HUVE cells and an average of a 30-fold increase was detected however again this was variable between experiments. By taking into consideration the decrease in receptor levels, receptor dephosphorylation remains a prominent feature in both cell types.

The down-regulation of VEGF receptor signalling by a permanent mechanism is also evident through the degradation of VEGF receptor-2 in HUVE and PAE cells (Figure 6.1). The quantification of total receptor levels in whole cell lysates shows that total VEGF receptor-2 levels, when normalised to vinculin levels, decrease by 50% in HUVE cells after 20 minutes of VEGF stimulation. In PAE cells, stimulated with VEGF for 20 minutes, 38% of the total receptors remain.

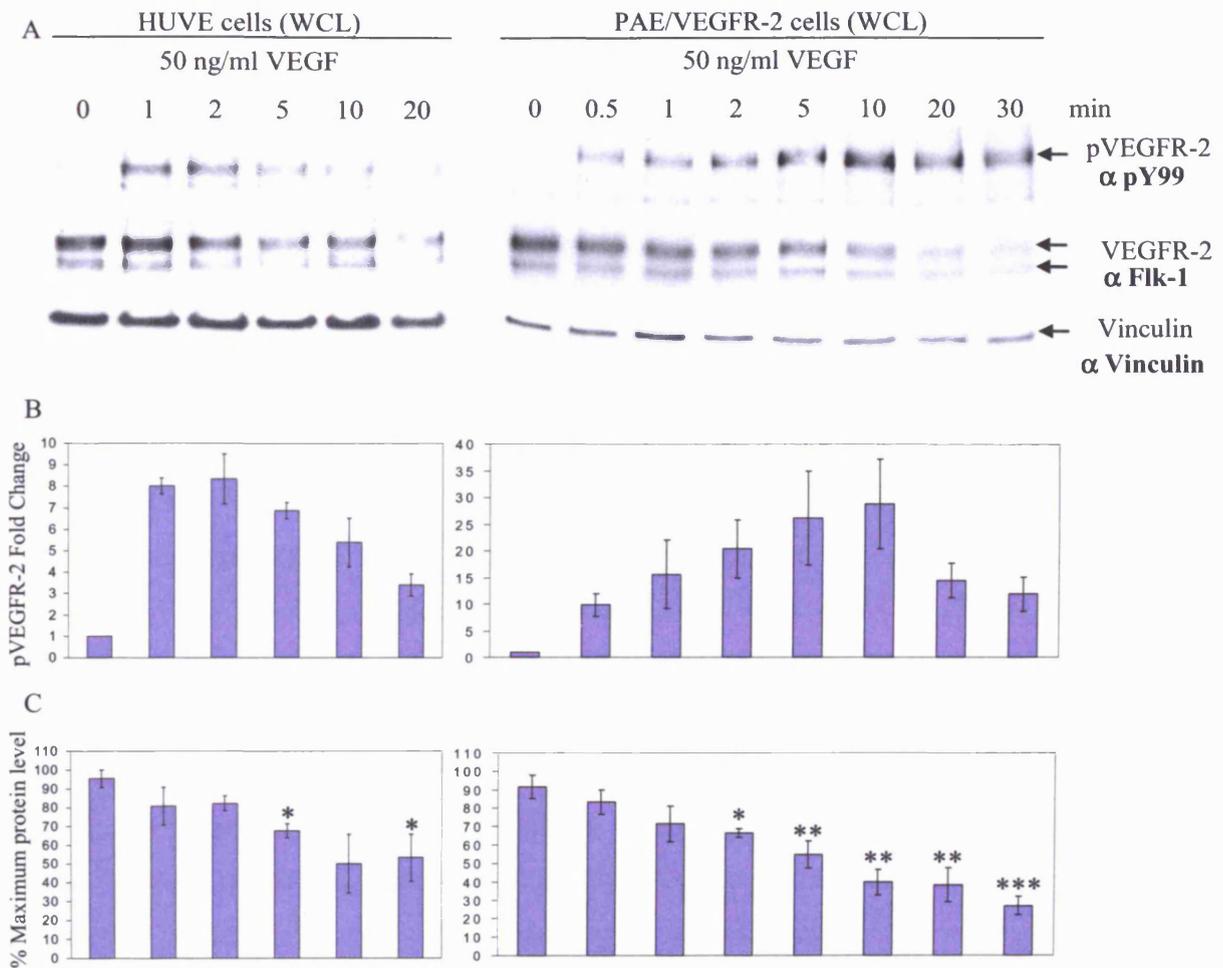


Figure 6.1 – Activation of VEGF receptor-2 in HUVE and PAE/VEGF receptor-2 cells shows a peak in receptor phosphorylation and results in receptor degradation. Western blots of whole cell lysates, from unstimulated and VEGF stimulated HUVE and PAE cells, probed with phosphotyrosine and VEGF receptor-2 antibodies, shows an increase, followed by a decrease, in receptor phosphorylation during which time the receptor is degraded (A). Quantification of western blots is shown, with the normalisation of receptor protein levels to vinculin (C), and the normalisation of phospho-VEGF receptor-2 levels to the level of total receptor (B). Results are expressed as the mean of three independent experiments +/- SEM for HUVE cells and the mean of four independent experiments +/- SEM for PAE cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to unstimulated cells.

An ELISA-based degradation assay has been used to measure the proportion of surface-labelled VEGF receptor-2 that is degraded in HUVE and PAE cells in response to VEGF (Figure 6.2). Firstly all proteins localised at the cell surface are labelled with NHS-S-S-Biotin (step 1). At this stage several plates of cultured cells are designated as controls and they are left on ice while sample cells are treated. The results derived from these control cells represent the total amount of cell surface labelled VEGF receptor-2 and this is assessed for the populations of cells used in each experiment. Additional plates of cultured cells are incubated in serum free media at 37°C for 20 minutes to initiate the internalisation of biotin-labelled receptors (step 2). After this period of incubation, the plates of cultured cells are divided into two. Some cells remain in serum free media for a further 20 minutes and others are stimulated with VEGF for 20 minutes (step 3). Finally, cells from all cultures are lysed (step 4) and the lysates are added to antibody-coated wells of an ELISA plate (step 5). Although many cell surface proteins are biotin-labelled only biotin-labelled VEGF receptor-2 can bind to the anti-VEGF receptor-2 antibodies that coat the well of the ELISA plate. The receptor-specific antibody used in the ELISA assays (Flk A3, sc-6251) binds to the C-terminus, in the intracellular domain, of VEGF receptor-2 (amino acids 1158-1345 of murine VEGF receptor-2). Thorough washing of the ELISA plate ensures that only VEGF receptor-2 is detected. In addition control wells, coated only with IgG, allow for the detection of proteins that have bound non-specifically and an average of these background measurements is subtracted from the results for all other samples. The method, by which biotinylated proteins are detected, involves the addition of streptavidin, conjugated to horseradish peroxidase (HRP), and the development of a coloured compound from an oxidation reaction. This reaction requires hydrogen peroxide (H₂O₂) as an oxygen donor and HRP as a catalyst. The coloured compound is measured spectroscopically.

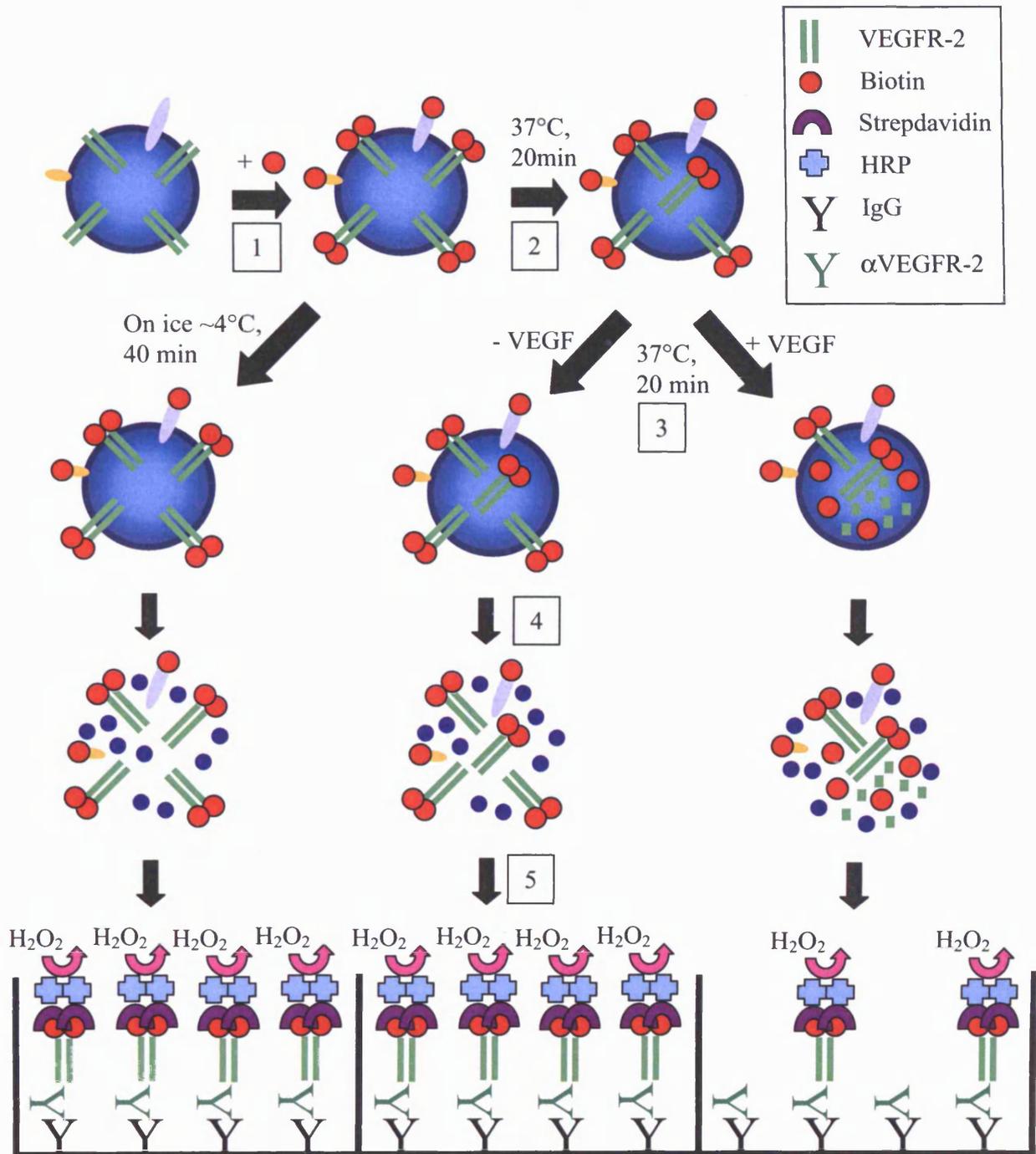


Figure 6.2 - A schematic diagram showing the steps involved in the ELISA-based degradation assay

The down-regulation of cell surface expressed VEGF receptor-2 is confirmed by results from this degradation assay (Figure 6.3). In HUVE and PAE cells, degradation of VEGF receptor-2 was detected in both serum starved and VEGF stimulated cells when compared to those that were untreated. The results presented here represent the percentage of the total cell surface labelled receptors not detected in the ELISA assay i.e. the percentage of receptors degraded during steps 2-3. The proportion of total cell surface labelled receptors degraded under serum starvation conditions was highly variable in HUVE cells, which results in large error bars from the statistical analysis of combined experiments. Analysis of the receptor levels from cells that were stimulated with VEGF shows that a greater proportion of cell surface receptors are degraded in these cells compared to those that were serum starved in the absence of VEGF. The increase in degradation detected between these two cell populations was consistent, ranging from 7-10% and resulting in an average increase of 9%.

In PAE cells, the level of VEGF receptor-2 degradation in cells incubated in serum-free media was more consistent between experiments. As for HUVE cells, VEGF stimulation resulted in an increase in the proportion of receptors degraded when compared to that detected in serum-starved cells. The increase in degradation ranged from 7-22% in three independent experiments, resulting in an average of 13%. In all experiments, for HUVE and PAE cells, a high level of receptor degradation under serum starved conditions correlated to a greater increase in degradation upon stimulation with VEGF.

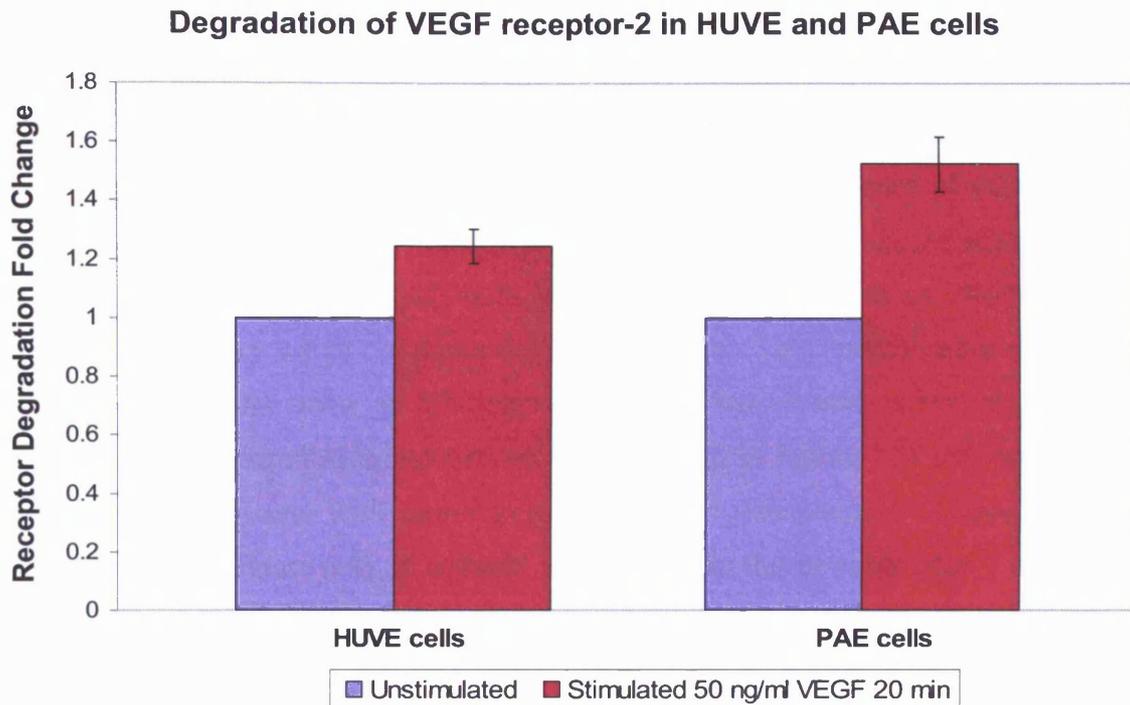


Figure 6.3 – Degradation of VEGF receptor-2 in HUVE and PAE/VEGF receptor-2 cells after stimulation with VEGF for 20 minutes. A decrease in the amount of VEGF receptor-2 detected by the ELISA assay in treated cells, when compared to the total receptor levels detected in untreated cells, is used as a measure of receptor degradation. An increase in receptor degradation, upon stimulation of HUVE and PAE cells with 50 ng/ml VEGF for 20 minutes, relative to normalised levels of degradation in unstimulated cells, is evident. Results from three independent experiments are shown and the mean of these \pm SEM.

The degradation assay, as described above, compares the proportion of surface labelled receptors that are degraded when cells are incubated in serum free media in the absence or presence of VEGF. The proportion of degraded receptors is determined by comparing the level of biotinylated receptors in treated cells with the total amount of surface labelled receptors from untreated cells. By modifying the degradation assay we are able to disregard the receptors that remain at the cell surface and detect only those that are internalised. As in the degradation assay we do not detect degraded receptors. This internalisation assay (Figure 6.4) is principally the same as the degradation assay but includes a few alterations. The internalisation and stimulation incubations (steps 2 and 3 in Figure 6.2) are combined and a time course of treatment with serum free media in the presence and absence of VEGF is adopted (step 2 in Figure 6.4). In addition an extra step in this protocol (step 3 in Figure 6.4) results in the reduction of the disulphide bond in the NHS-S-S-Biotin (Figure 6.4B). The consequence of this is that biotin is cleaved from labelled receptors that remain at the cell surface and hence these receptors are not detected. The compound used to reduce the disulphide bond is MesNa and because it cannot penetrate the cell membrane it is not able to cleave biotin from intracellularly localised biotinylated receptors. This assay therefore detects the proportion of receptors initially localised at the cell surface and re-localised to intracellular compartments during the time course. This assay cannot however determine the proportion of receptors that recycle back to the plasma membrane as MesNa also removes the biotin label for these receptors. Recycled receptors are therefore indistinguishable from those that remain at the cell surface.

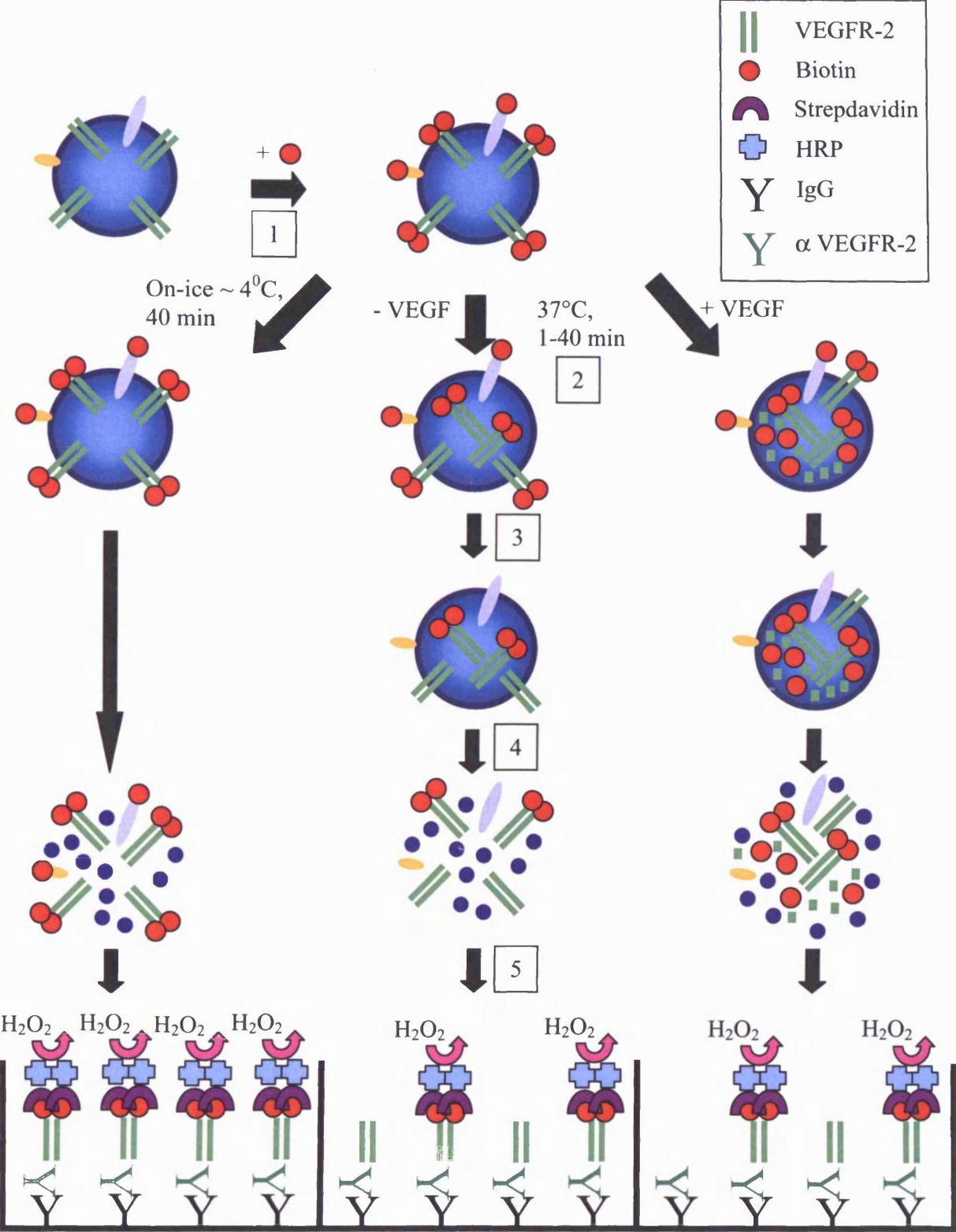
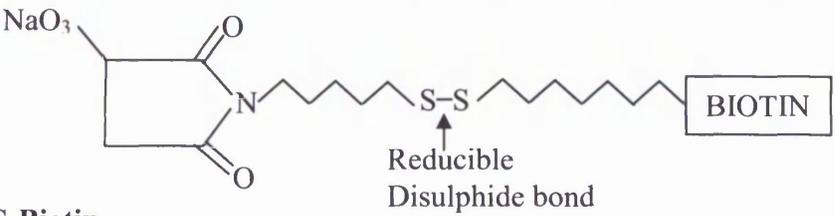


Figure 6.4
A - Schematic diagram
 showing the steps
 involved in the ELISA-
 based internalisation
 assay
B - The structure of NHS-S-S-Biotin



The increase in receptor degradation induced by VEGF suggests that the rate of internalisation of receptors should also increase in response to VEGF. This however was not detected in PAE cells by the above assay (Figure 6.5). The results presented here show the proportion of VEGF receptor at the cell surface, after cells were incubated in serum-free- or VEGF-supplemented media, in relation to the receptor levels in untreated cells. These results indicate that receptors are internalised in the absence of VEGF and stimulation does not appear to increase this. The level of cell surface receptors in cells incubated in serum-free media or stimulated with VEGF for 40 minutes is reduced by about 30% when compared to untreated cells.

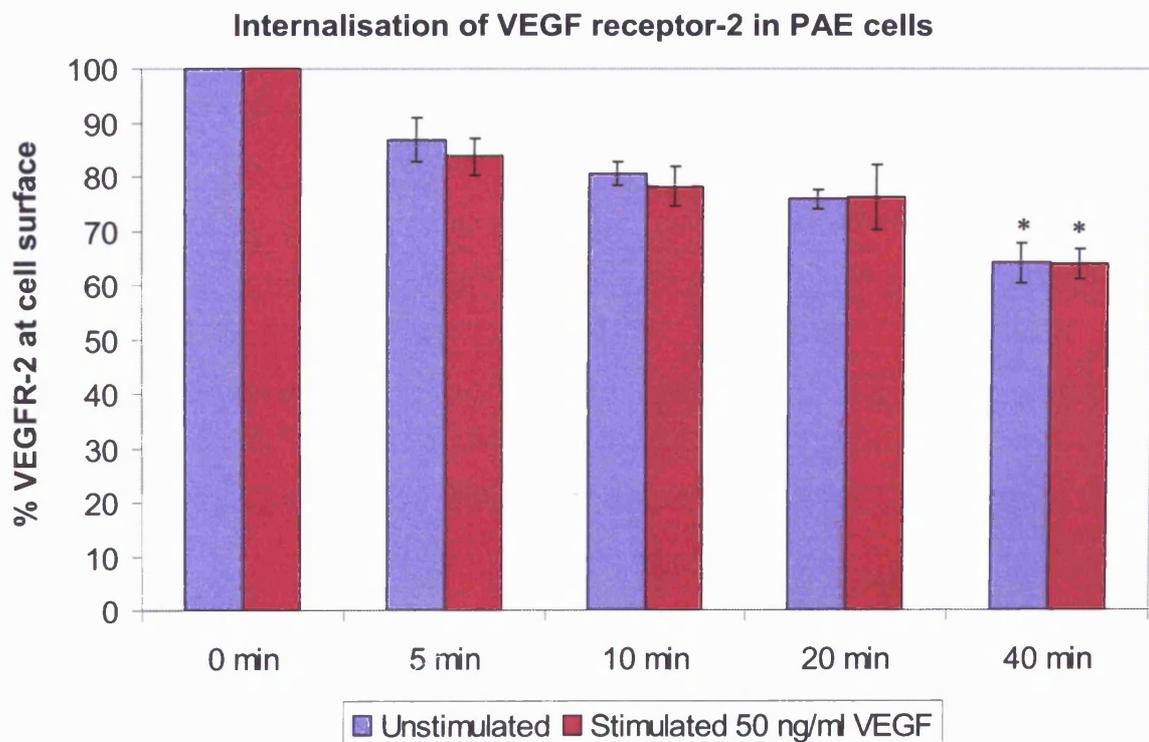


Figure 6.5 – VEGF receptor-2 is internalised in unstimulated and stimulated PAE/VEGF receptor-2 cells. The detection of VEGF receptor-2 in internal pools of PAE/VEGF receptor-2, and the comparison of these levels to the level of total receptor at the cell surface in untreated cells, was used to determine the percentage of VEGF receptor-2 remaining at the cell surface after cells were incubated in serum free media or VEGF supplemented media. Results are expressed as the mean of four independent experiments \pm SEM. * $P < 0.05$ compared to stimulation of cells with VEGF for 5 minutes.

6.2.2 *Immunostaining of VEGF receptor-2 shows localisation of the receptor at the plasma membrane and in intracellular vesicles*

Fluorescence microscopy has been a useful tool in identifying the localisation of VEGF receptors. Immunofluorescence experiments using an antibody against VEGF receptor-2 have shown that in unstimulated HUVE and PAE cells these receptors are localised within membrane and intracellular vesicular structures (Figure 6.6). The receptor-specific antibody used (Flk C1158, sc-504) binds to the C-terminus, in the intracellular domain, of VEGF receptor-2 (amino acids 1158-1345 of murine VEGF receptor-2). By imaging cells that were stimulated with VEGF for 1-5 minutes, fixed and permeabilised we have observed the relocalisation of a portion of these receptors to the membrane. When cells are stimulated for 10 minutes the membrane localisation is no longer obvious and the staining is confined to vesicular structures once again.

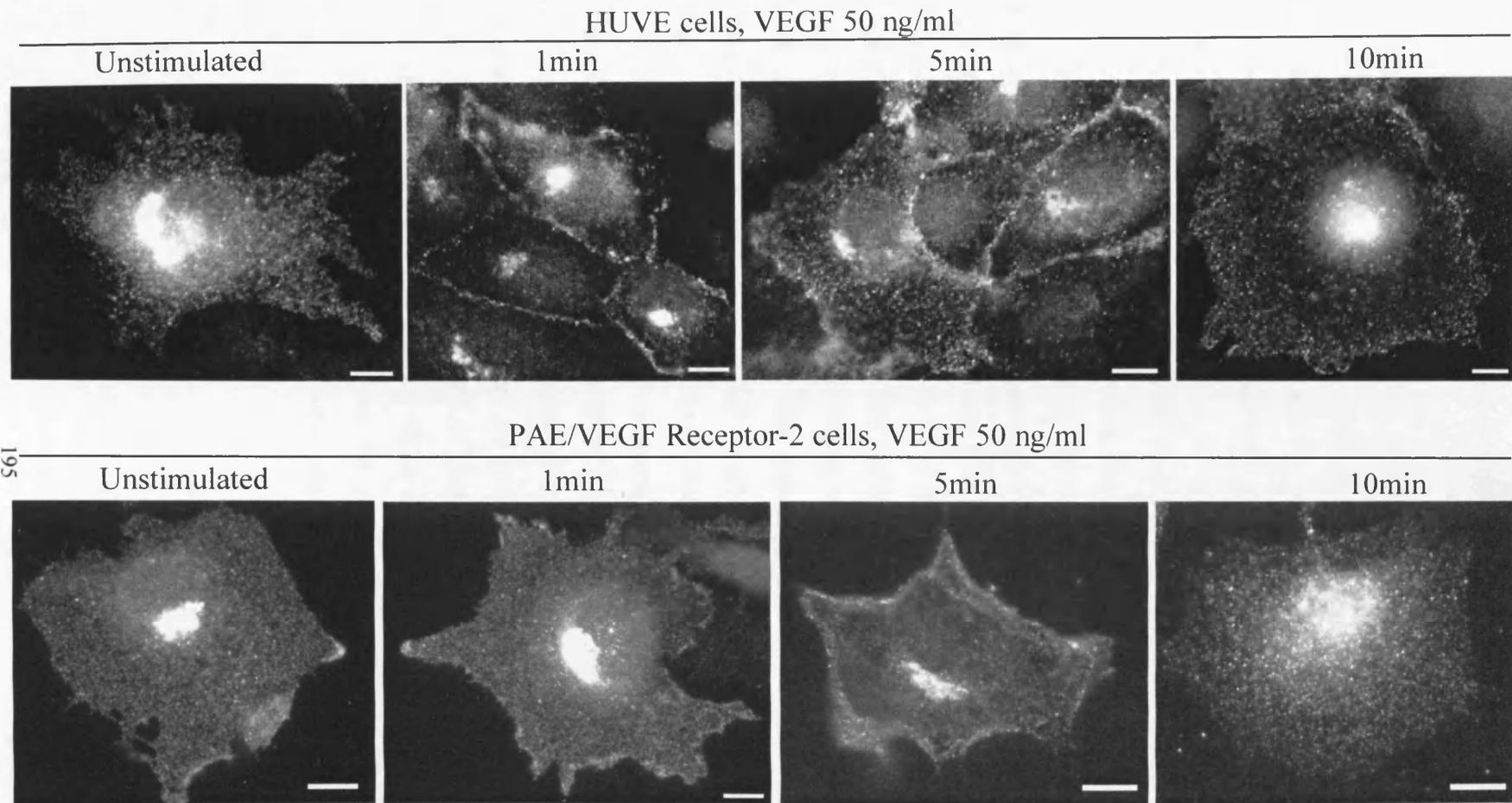


Figure 6.6 - VEGF Receptor-2 localises to regions of the plasma membrane in cells stimulated with VEGF for 1-5 minutes and to vesicle-like structures in cells stimulated for 10 minutes. Images shown are of HUVE cells and PAE cells stably expressing VEGF receptor-2 that were serum starved (Unstimulated) or stimulated with 50 ng/ml VEGF for the times indicated. VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to FITC. These are representative cells from at least three independent experiments. The scale bars represent 10 μ m.

6.2.3 *Immunostaining of VEGF receptor-2 and EEA1 or CD63, markers of early or late endosomes/lysosomes respectively, provides some evidence for VEGF receptor trafficking.*

To further investigate the vesicles in which VEGF receptor-2 is localised we used co-immunofluorescence to determine whether VEGF receptor-2 is trafficked through the endocytic pathway when HUVE cells are stimulated with VEGF. Antibodies to VEGF receptor-2 and a marker of early endosomes (EEA1) shows that the vesicular-like staining of VEGF receptor-2 in unstimulated cells, or in those that have been stimulated for 10 minutes with VEGF, do not co-localise with EEA1 (Figure 6.7 A and B). A significant percentage of the receptor is localised to EEA-labelled vesicles however in cells that have been stimulated with VEGF for 30 or 60 minutes (Figure 6.7 C and D). The staining of these endosomes with receptor and EEA1 antibodies is often unequal so that the vesicles may appear predominantly green or red. Consequently it is difficult to conclude that co-localisation does occur from the merged images alone. By enlarging areas of stimulated cells, and by using arrows to indicate labelled vesicles, we show that VEGF receptor-2 and EEA-1 are obviously co-localised within early endosomes when the expression levels of the proteins are approximately equal. In addition, we have taken images of cells stimulated with VEGF for 30 and 60 minutes and immunostained for EEA-1 and VEGF receptor-2, printed them onto acetate, overlaid the images and counted the number of co-localising “spots”. This method therefore eliminates the problems of disproportionate protein expression levels. A summary of the results is given in Figure 6.8, which shows the percentage of EEA-1 positive spots that co-localise with VEGF receptor-2, and vice versa, in cells stimulated with VEGF for 30 minutes and 60 minutes. In stimulated cells 40-60% of spots that are positive for VEGF receptor-2 are also positive for EEA-1. Likewise approximately 20% of EEA-1 positive early endosomes contained VEGF receptor-2 protein. Statistical analysis of this data using the student’s t test indicates that there is no statistical significance between the results from cells stimulated for 30 minutes compared with cells stimulated for 60 minutes.

Figure 6.7 – VEGF receptor-2 and EEA-1 co-localise in a small number of vesicle-like structures in VEGF stimulated HUVE cells. Images shown are of HUVE cells that were serum starved (Unstimulated - A) and stimulated with 50 ng/ml VEGF for the times indicated (B-D). VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to rhodamine red. EEA-1 is immunofluorescently labelled using an antibody against EEA-1 and a secondary antibody conjugated to FITC. These are representative cells from a single experiment. The scale bar represents 10 μm .

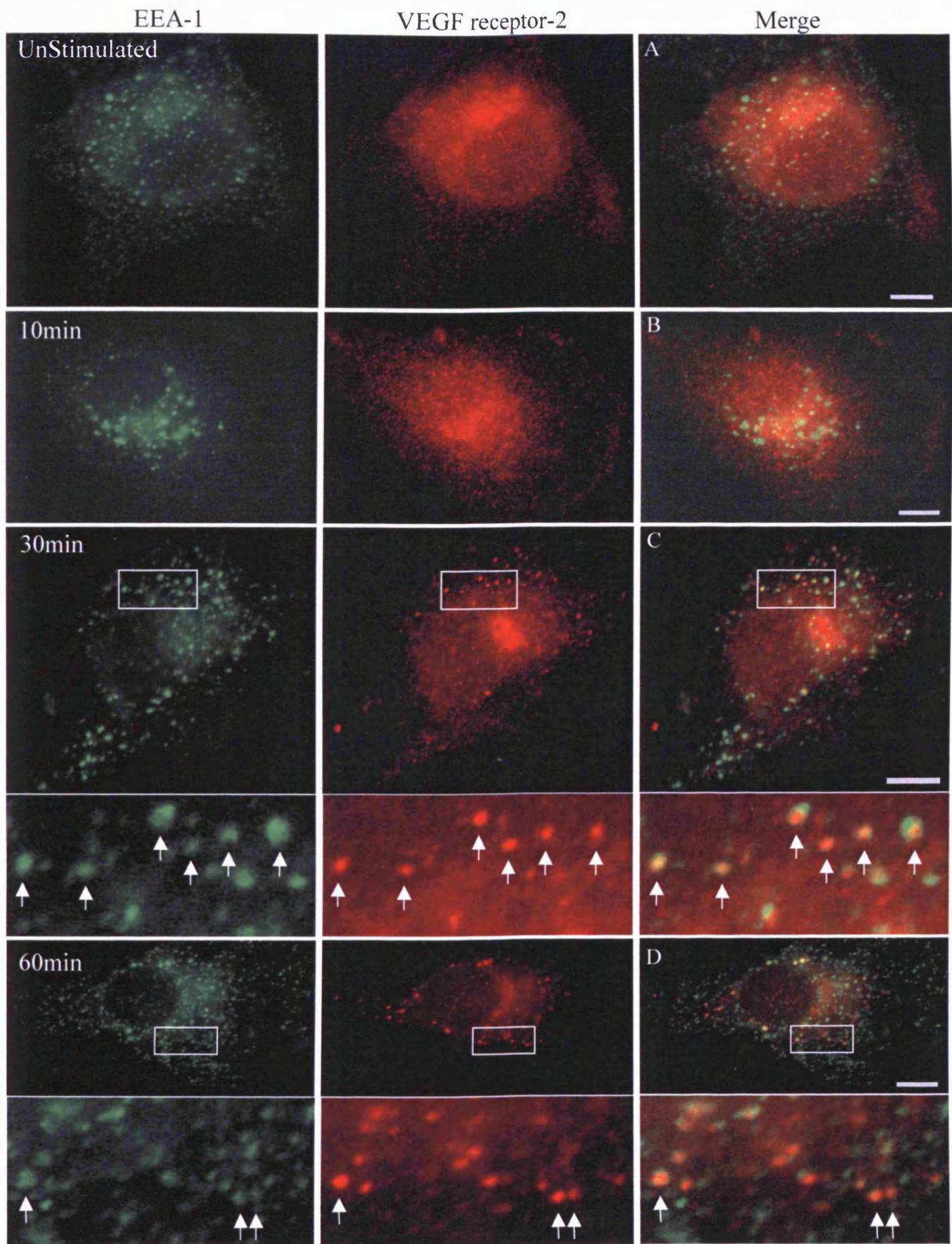


Figure 6.7

Co-localisation of EEA-1 and VEGF receptor-2 in HUVE cells

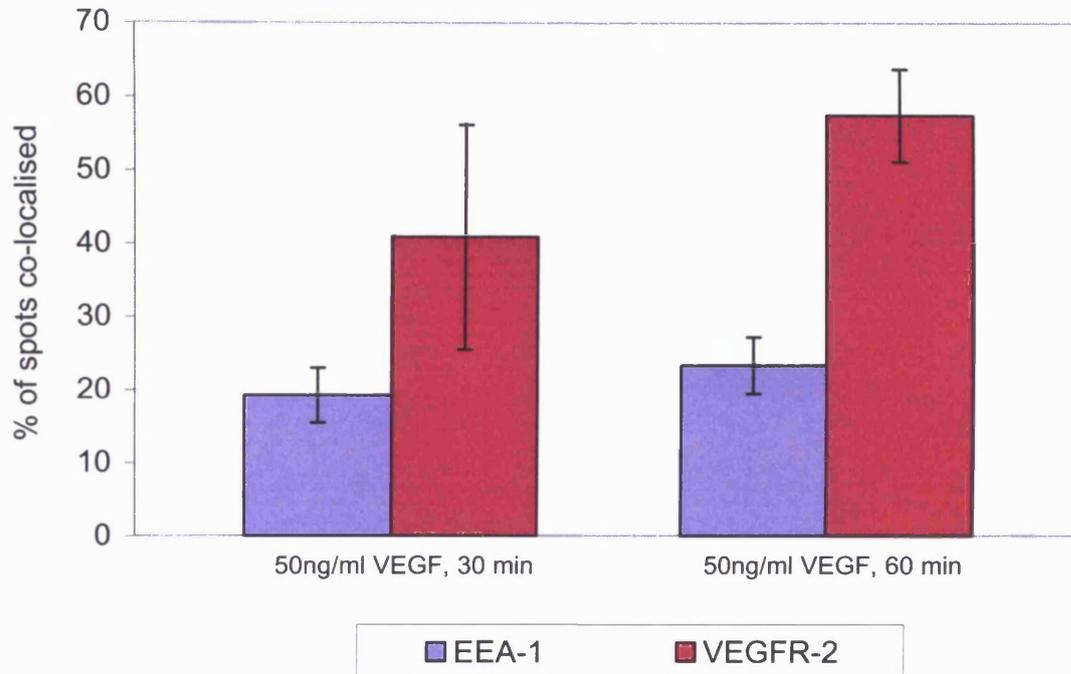


Figure 6.8 - Quantification of fluorescent spots that represent punctate areas of VEGF receptor-2 and EEA-1 proteins. VEGF receptor-2 proteins in HUVE cells were immunolabelled using an antibody to the receptor and a secondary antibody conjugated to a red fluorescent tag and EEA-1 proteins were similarly labelled however the secondary antibody was conjugated to a green fluorescent tag. The images of these cells are shown in Figure 6.7. Here the number of red and green spots in 4 HUVE cells stimulated with VEGF for 30 minutes and 4 HUVE cells stimulated with VEGF for 60 minutes were counted and the number of co-localised spots noted. This graph shows the average percentage of EEA-1 that colocalises with VEGF receptor-2 per cell \pm SEM and vice versa. Number of spots counted EEA-1, 30 min VEGF = 519, VEGFR-2, 30 min VEGF = 380, EEA-1, 60 min VEGF = 668, VEGFR-2, 60 min VEGF = 278.

The possible localisation of VEGF receptor-2 to late endosomal/lysosomal compartments however cannot be confirmed from the images in Figure 6.9. The staining of CD63, a marker for these structures, is not as distinct as that of EEA1. Staining around the nucleus is evident with the receptor and the CD63 antibody however co-localisation in distinct structures is not obvious. The enlarged images of cells stimulated with VEGF for 30 and 60 minutes show that the vesicular-like structures, present in both red and green images, do not co-localise (Figure 6.9 C and D).

Figure 6.9 – VEGF receptor-2 and CD63 do not co-localise in vesicle-like structures in VEGF stimulated HUVE cells. Images shown are of HUVE cells that were serum starved (Unstimulated - A) and stimulated with 50 ng/ml VEGF for the times indicated (B-D). VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to rhodamine red. CD63 is immunofluorescently labelled using an antibody against CD63 and a secondary antibody conjugated to FITC. These are representative cells from a single experiment. The scale bar represents 10 μm .

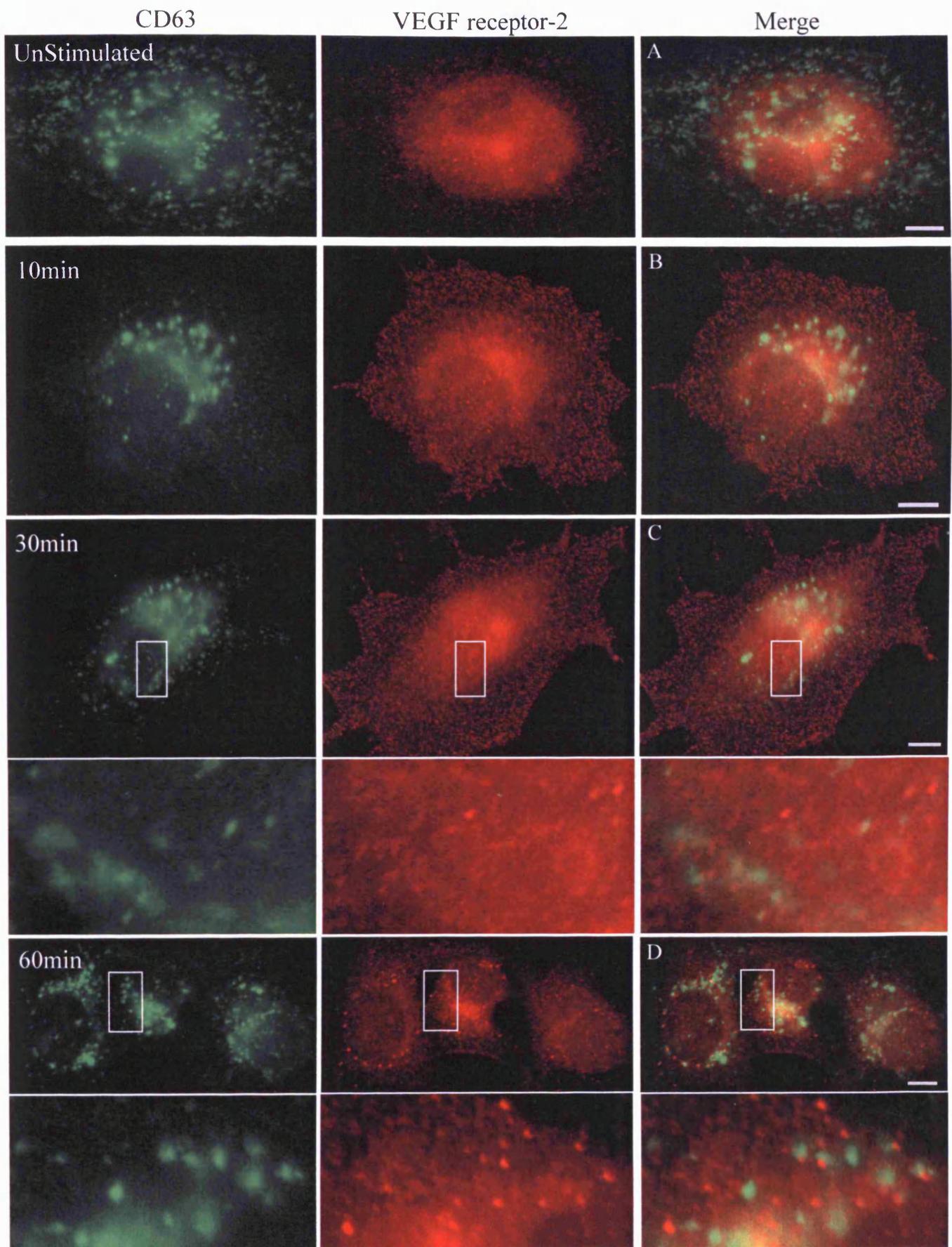


Figure 6.9

6.2.4 *VEGF receptor-2 co-localises with caveolin-1*

Co-staining of VEGF receptor-2 and caveolin-1 show that the receptor co-localises with caveolin-1-containing structures in resting and in stimulated cells (Figure 6.10). There are no obvious differences in the number of vesicles when comparing cells that were not stimulated to those that were stimulated.

Figure 6.10 – VEGF receptor-2 and caveolin-1 co-localise in vesicle-like structures in unstimulated and VEGF stimulated HUVE cells. Images shown are of HUVE cells that were serum starved (Unstimulated) and stimulated with 50 ng/ml VEGF for the times indicated. VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to rhodamine red. Caveolin-1 is immunofluorescently labelled using an antibody against caveolin-1 and a secondary antibody conjugated to FITC. These are representative cells from a single experiment. The scale bar represents 10 μm .

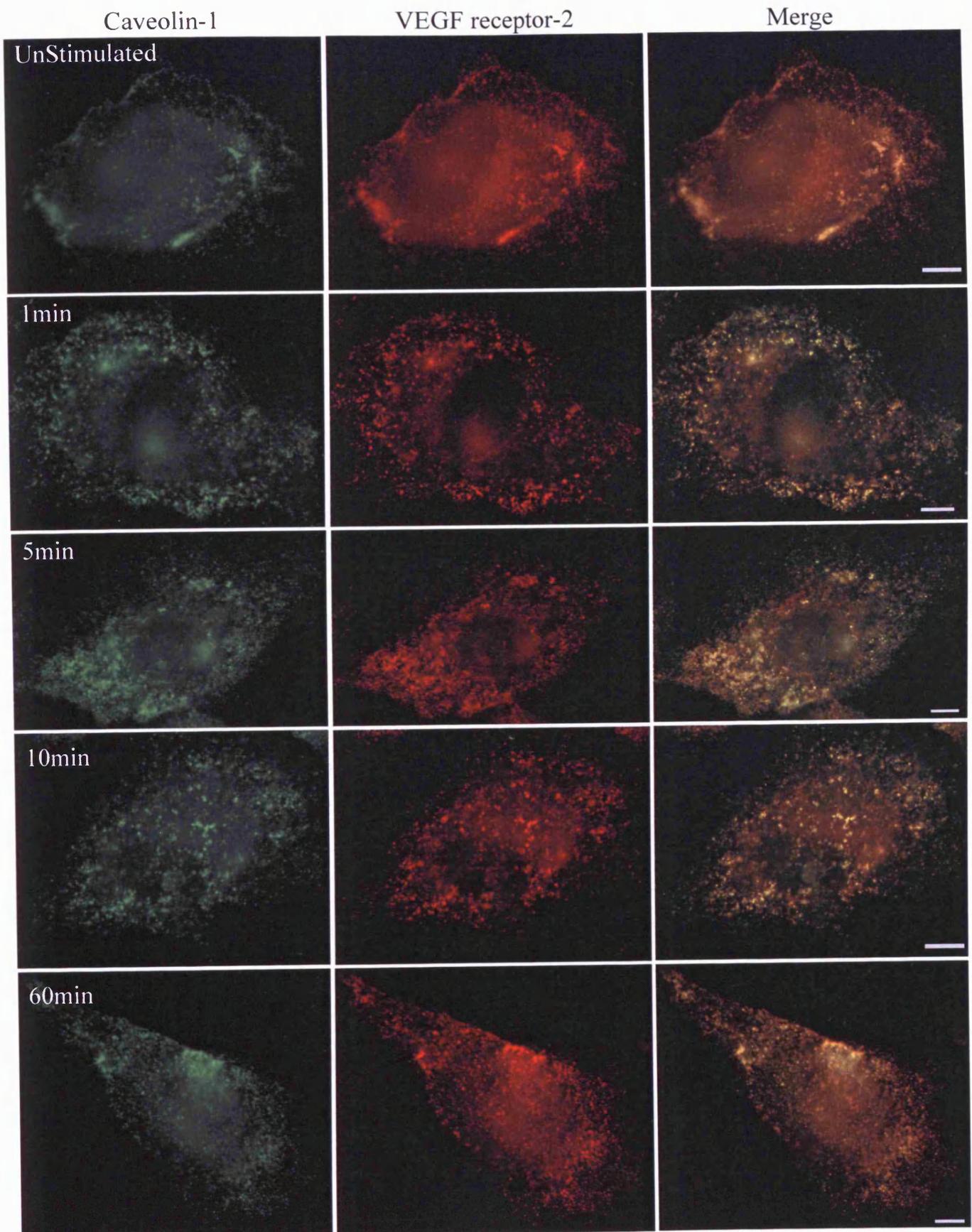


Figure 6.10

6.2.5 *VEGF receptor-2 does not appear to co-localise with β 3 integrin*

Since VEGF receptor-2 and α v β 3 integrin have previously been shown to associate (Soldi et al., 1999), and VEGF increases the recycling of α v β 3 integrin (M. Jones et al., unpublished work), we sought to investigate whether these proteins can co-localise. HUVE cells were stimulated with VEGF over a time course of 60 minutes. As previously described, VEGF receptor-2 shows membrane localisation after 1-5 minutes of VEGF stimulation (Figure 6.11 (ii) and (iii)), and then after 10-60 minutes of stimulation it is localised in punctate structures that resemble vesicles (Figure 6.11 (iv) and (vi)a-(viii)a). An antibody to β 3 integrin shows the most prominent staining in HUVE cells that have been stimulated with VEGF for 60 minutes. The structures in which the β 3 subunit is localised resemble α v β 3-containing focal adhesions (Figure 6.11 (viii)b). The concentration of the β 3 integrin is not sufficient for the detection of any defined structures in unstimulated cells (Figure 6.11 (v)b), nor those stimulated with VEGF for 1- 10 minutes (data not shown). The merged images, of cells in which both VEGF receptor-2 and β 3 integrin can be visualised, do not show any co-localisation of the two proteins ((vi)c-(viii)c). Although both proteins have been shown to localise to early endosomes and to focal regions of the cell membrane this data suggests that they are localised to distinct structures.

Figure 6.11 – VEGF receptor-2 and β 3 integrin localise to different vesicle-like structures and localise to regions of the plasma membrane at different times during VEGF stimulation of HUVE cells. Images shown are of HUVE cells that were serum starved (Unstimulated (i) and (v)) and stimulated with 50 ng/ml VEGF for the times indicated (ii-iv and vi-viii). VEGF receptor-2 is immunofluorescently labelled using an antibody against Flk-1 and a secondary antibody conjugated to FITC ((i)-(iv) and (v)a-(viii)a). β 3 integrin is immunofluorescently labelled using an antibody against CD61 and a secondary antibody conjugated to Texas Red ((v)b-(viii)b). These are representative cells from a single experiment. The scale bar represents 10 μ m.

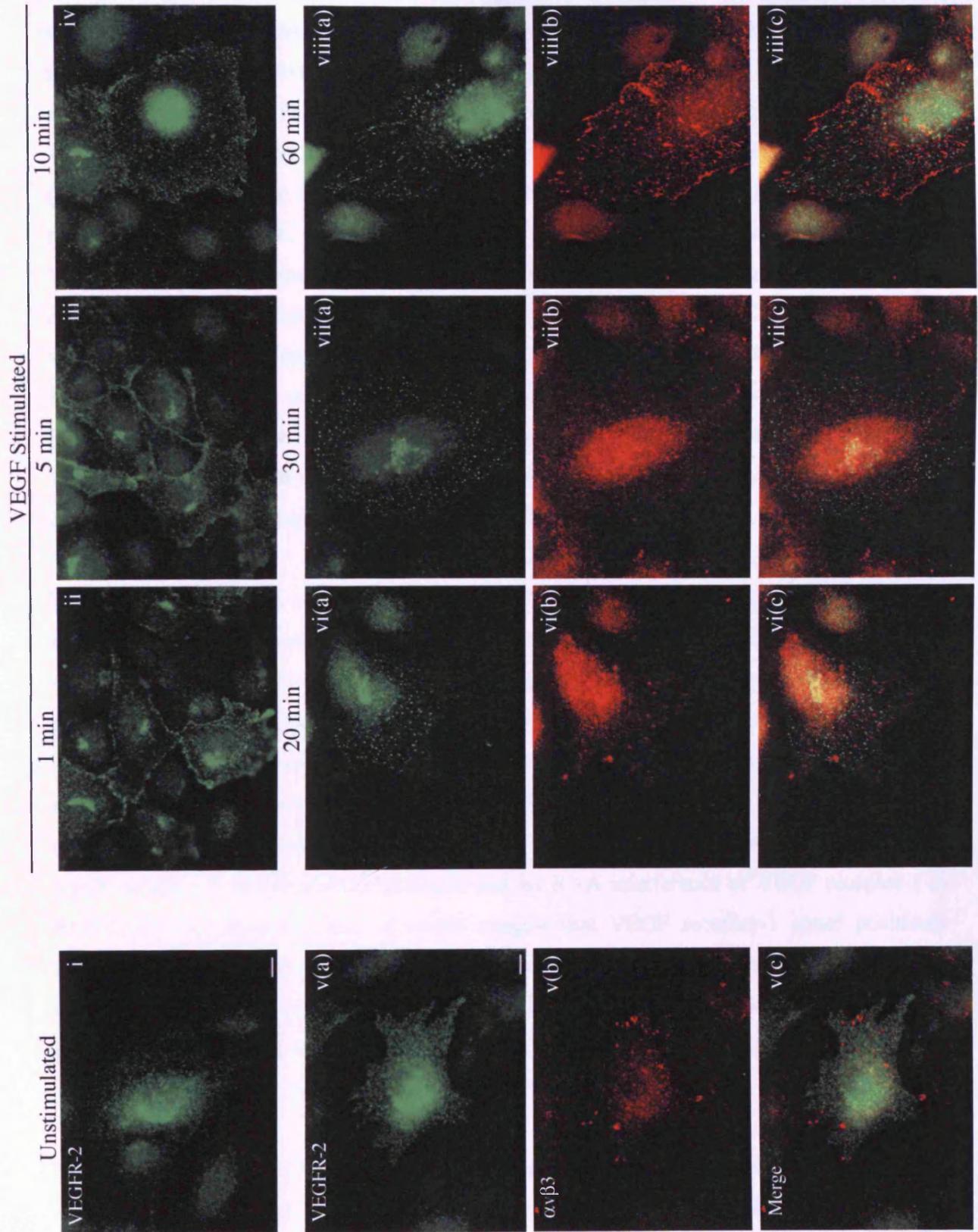


Figure 6.11

6.3 - Discussion

The profile of VEGF receptor activation shows a peak of phosphorylation, which is suggestive of phosphatases acting to temporarily dephosphorylate the receptor and to terminate signalling. This is a rapid event, occurring in HUVE cells after 2 minutes of VEGF stimulation, and HCPTP-A/LMW-PTP has been implicated as a specific phosphatase for VEGF receptor-2 (Huang et al., 1999). From the results obtained in the cell selection studies (Figure 3.1) the decrease in ERK phosphorylation occurs at the same time as the decrease in receptor phosphorylation. This indicates that this is an efficient mechanism for switching off VEGF proliferation signals. There are however specific MAP kinase phosphatases that contribute to the dephosphorylation of ERK (Toledano-Katchalski et al., 2003) and due to the very rapid dephosphorylation of ERK in HUVE, PAE and HEK293 cells it is probable that these phosphatases are active in these VEGF stimulated cells. The possibility that other proteins contribute to the termination of signalling downstream of VEGF receptor-2 is evident from the observation that the phosphorylation of ERK appears to be reduced to its basal level at a faster rate than the dephosphorylation of VEGF receptor-2.

VEGF receptor-2 levels are down regulated in HUVE and PAE cells in response to VEGF stimulation. The increased efficiency of VEGF receptor-2 degradation in PAE cells may be due to the over expression of the receptor in these cells and/or a lower expression level of proteins that inhibit this process. For example, the absence of VEGF receptor-1 in these cells could disturb a regulatory mechanism. HUVE cells express both receptors and these results may indicate that VEGF receptor-1 is responsible for the rapid rate at which maximum receptor phosphorylation is attained and also that VEGF receptor-1 inhibits the degradation of VEGF receptor-2. If this were to be confirmed, by RNA interference of VEGF receptor-1 in HUVE cells for example, then it would suggest that VEGF receptor-1 could positively influence VEGF receptor-2 signalling. In addition expression of VEGF receptor-1 in HUVE cells may promote the recycling of VEGF receptor-2 from early endosomes back to the plasma membrane for reactivation. The recycling assay, using cells in which VEGF receptor-1 is absent, may confirm or contend this possibility.

The relatively small increase in degradation observed in the ELISA-based degradation assay, compared with the larger increases seen with the western blots of total cell lysates, is likely to be due to the proportion of receptors detected by each experiment. In the western blot assay

all receptors are detected however in the ELISA-based assay only those receptors that are exposed to the cell surface are labelled with biotin and hence detected in the assay after VEGF treatment. The degradation assay therefore monitors the changes in cell-surface labelled receptors. From these results one explanation may be that VEGF stimulation of cells promotes the release of VEGF receptors from internal compartments, caveolae for example, and their exposure to the extracellular environment leads to phosphorylation and degradation.

The internalisation assay, like the degradation assay, is also limited in that it does not detect the change in localisation of receptors that are not present on the cell surface at the time of biotin labelling. As the assay detects receptors that are localised to internal compartments, internalised receptors that are degraded or recycled back to the plasma membrane are not detected. A change in the kinetics of receptor degradation and/or recycling in VEGF stimulated cells may therefore mask the true effect of VEGF on the rate of receptor internalisation in this assay. Given that we detected an increased level of receptor degradation in VEGF-stimulated cells a reduction in the amount of receptor at the cell surface might be expected. The results from the internalisation assay suggest however that the level of receptor at the plasma membrane of serum starved and VEGF-stimulated cells is the same. This may indicate that a greater amount of receptor is recycled back to the plasma membrane in stimulated cells compared to those that are serum starved. The use of inhibitors of the lysosomal or proteasomal pathways and of recycling pathways in conjunction with this assay would help to decipher the effects of VEGF on VEGF receptor-2 internalisation.

Both ELISA-based assays assume that biotin labelling of cell surface proteins does not interfere with the binding of VEGF to VEGF receptor-2. The binding of biotin, to the amine group at the amino terminus of proteins, and to the epsilon amine of lysine residues within the protein, could affect the efficiency with which VEGF binds to heparan sulphate, neuropilin-1 or the VEGF receptors. A VEGF binding assay, using radiolabelled VEGF for example, would identify any effects of protein biotinylation.

The ubiquitin ligase Cbl has been strongly implicated in the initialisation of VEGF receptor-2 internalisation and its degradation. Cbl has been shown to associate with VEGF receptor-2 and be phosphorylated in response to VEGF (Duval et al., 2003). Despite this however we were unable to show co-immunoprecipitation of Cbl and VEGF receptor-2 or phosphorylation of Cbl in VEGF stimulated PAE cells expressing VEGF receptor-2. After many attempts we

decided to accept this result and we modified our experiments to determine whether VEGF receptor-2 follows the endocytic route to the lysosome as described for many receptor tyrosine kinases. We felt that this would be a more enlightening line of investigation, as this has not been reported on previously.

Imaging data has shown the localisation of VEGF receptor-2 in vesicles and at the plasma membrane. Immunofluorescence using an antibody to the extracellular domain of VEGF receptor-2 has confirmed that some of these vesicles are present on the cell surface in resting and stimulated cells (data not shown). Unfortunately difficulties in co-staining these cells with an antibody to the extracellular domain of the receptor with a reference marker hindered attempts to quantify the proportion of cell surface localised receptors. The reason for this was due to cross-reactivity between the secondary anti-goat antibody, conjugated to rhodamine red, with anti-mouse and anti-rabbit primary antibodies.

Internalisation of VEGF receptor-2 into vesicles that resemble early endosomes is evident in HUVE and PAE/VEGF receptor-2 cells that have been stimulated with VEGF for 10 minutes (Figures 6.6, 6.7, 6.9, 6.10 and 6.11). In addition, results from an ELISA-based internalisation assay (Figure 6.5) suggest that approximately 20% of the cell surface localised VEGF receptor-2 in PAE/VEGF receptor-2 cells is internalised after 10 minutes of stimulation with VEGF. The accepted route of internalisation, and hence degradation of tyrosine kinase receptors, is via early endosomes, late endosomes and lysosomes. VEGF receptor-2 and EEA-1, a marker for early endosomes, co-localise in cells that have been stimulated with VEGF for as long as 30 and 60 minutes (Figure 6.7 and 6.8). The presence of VEGF receptor-2 in early endosomes suggests that VEGF receptor-2 is internalised in stimulated cells, at least in part, through the conventional endocytic pathway. Results from western blots and an ELISA-based degradation assay suggest however, that some VEGF receptor-2 is degraded in HUVE and PAE/VEGF receptor-2 cells during much shorter time courses of VEGF stimulation. Western blots shown in Figure 6.1 suggest that approximately half of the total VEGF receptor-2 expressed in PAE/VEGF receptor-2 and HUVE cells is degraded within 5 and 10 minutes of VEGF stimulation respectively. Furthermore, the degradation assay, the results of which are shown in Figure 6.3, shows that receptors, initially localised at the plasma membrane, are degraded after 20 minutes of VEGF stimulation. These observations suggest that VEGF receptor-2 may also be internalised and degraded via a route that does not involve early endosomes and that occurs within 10 minutes of receptor stimulation.

Tyrosine kinase receptor degradation has been well studied and trafficking from early endosomes to late endosomes/lysosomes results in lysosomal degradation of activated receptors. The EGF receptor, complexed with EGF, can be seen in early endosomes within 2-5 minutes of EGF-induced receptor internalisation at 37°C and in intermediate and late endosomes within 10-15 minutes (reviewed in Sorokin, 1998). Here we show that VEGF receptor-2 is also rapidly activated and degraded in response to VEGF stimulation however our data does not suggest that rapid degradation occurs via the early endosome and lysosome. VEGF receptor-2 is not localised in early endosomes until 30 minutes after VEGF stimulation. Furthermore, CD63 is a marker for late endosomes/lysosomes and an antibody to this protein has been used to visualise these structures in HUVE cells. Co-staining of VEGF receptor-2 and CD63 does not show convincing co-localisation (Figure 6.9). A large area around the nucleus of HUVE cells is stained with antibodies against VEGF receptor-2 and CD63 however this is a common feature in many of the images shown throughout this study and therefore may not be specific for late endosomes/lysosomes. Punctate staining of VEGF receptor-2 and CD63 is evident also, and although VEGF receptor-2 and CD63 localise to these vesicle-like structures; they do not appear to co-localise. In conclusion, the immunofluorescence staining of VEGF receptor-2 and CD63 suggests that VEGF receptor-2 is not degraded in the lysosome however further studies are required to entirely disregard this common method of degradation. The use of lysosomal inhibitors in the degradation assays detailed here may help to clarify the importance of the lysosome in VEGF receptor-2 degradation.

VEGF receptor-2 co-localises with caveolin-1, a major protein constituent of caveolae. Internalisation of VEGF receptor-2 therefore could occur via a clathrin-independent pathway and as endothelial cells have an abundance of caveolae this mechanism is an attractive alternative. The method of internalisation used by receptors, and other proteins in general, may depend on the relative amounts of caveolae and clathrin-coated pits in different cell types.

Caveolae have been implicated in the internalisation of several proteins and a few examples are given below, some of which are very relevant to endothelial cells:

- The cholera toxin and the Simian Virus 40 use caveolae-mediated endocytosis to enter mammalian cells, they are however likely to be able to use other mechanisms also (reviewed in Smart et al., 1999).
- The transforming growth factor (TGF) β receptor can be internalised into caveosomes, which leads to its degradation via a Smad7 and Smurf2-dependent pathway. The TGF β receptor can alternatively be internalised via clathrin-coated pits, which has the opposite affect and leads to productive signalling in EEA1-positive endosomes (Di Guglielmo et al., 2003).
- Membrane-type 1 matrix metalloproteinase (MT1-MMP) degrades components of the extracellular membrane. This is essential for the process of endothelial cell migration towards a VEGF stimulus by capillary tube formation and invasion of the surrounding fibrin matrix. Internalisation of MT1-MMP regulates its protease activity and can occur via caveolae trafficking and clathrin mediated endocytosis (Remacle et al., 2003) however inhibition of the former mechanism decreases endothelial cell migration and tube formation. Galvez et al show that MT1-MMP is localised in caveolae but not in clathrin vesicles in human endothelial cells. Clusters of MT1-MMP are found with caveolin-1 and $\alpha v\beta 3$ in extensions of migratory cells and the mobilisation of this complex has been proposed to regulate the proteolytic activity of endothelial cells during migration (Galvez et al., 2004).
- Constitutive caveolae endocytosis is an important mechanism for transendothelial transport of albumin and albumin-conjugated nutrients, fatty acids and hormones across the endothelial barrier (Minshall et al., 2003). Albumin uptake into endothelial cells involves the binding of albumin to its receptor gp60, which is localised to caveolar membranes, and results in the activation of Src. The phosphorylation of caveolin-1 and dynamin-2 by Src is required for signalling-mediated fission of caveolae vesicles from the plasma membrane to generate a caveosome (caveolin-1 containing endosomes). The expression of Y14F caveolin-1 or Y597F dynamin-2 abolishes albumin and cholera toxin subunit B endocytosis, expression of dominant negative Src kinase inhibited gp60-stimulated albumin uptake and the phosphatase inhibitors, vanadate and okadaic acid promote the caveolae endocytosis whereas the kinase inhibitors, staurosporine and genistein, repress the process. In addition the internalisation of caveolae requires the involvement of the actin cytoskeleton and

therefore inhibition of actin depolymerisation or stabilisation inhibited SV40 virus entry into cells.

It is therefore feasible that activation of VEGF receptor-2 results in phosphorylation of Src, which facilitates phosphorylation of caveolin-1 and dynamin to mediate the internalisation of VEGF receptors. The results shown here, to some extent, support the possibility of VEGF receptor-2 internalisation into caveosomes. Figure 6.10 shows that VEGF receptor-2 and caveolin-1 co-localise, Figures 6.7 and 6.8 suggest that VEGF receptor-2 is present in a small number of early endosomes and Figure 6.12 illustrates that the fusion of caveolae with early endosomes can occur in the clathrin-independent/caveolar internalisation pathway. The clathrin-dependent and caveolar internalisation pathways converge at early endosomes as Rab5 targets caveolar vesicles to early endosomes where the two vesicles fuse (Pelkmans et al., 2004). Lysosomal degradation of VEGF receptor-2 however still involves trafficking via early and late endosomes, even if internalisation occurs via caveolar vesicles. The identification of the compartment labelled with a question mark in Figure 6.12 may identify an alternative, quicker method of VEGF receptor-2 degradation. This is only a hypothesis as first we must show that VEGF receptor-2 internalises via caveolae in response to activation by VEGF. Although Labrecque et al and Ikeda et al show that VEGF receptor-2 is localised with caveolin-1 in resting cells, Labrecque et al propose that this is to inhibit VEGF receptor-2 activation (Labrecque et al., 2003) and Ikeda et al show that VEGF promotes the release of the receptor from caveolae/lipid rafts and accumulation at focal adhesions (Ikeda et al., 2005). Neither of these studies considers that activated VEGF receptors could be internalised via caveolae.

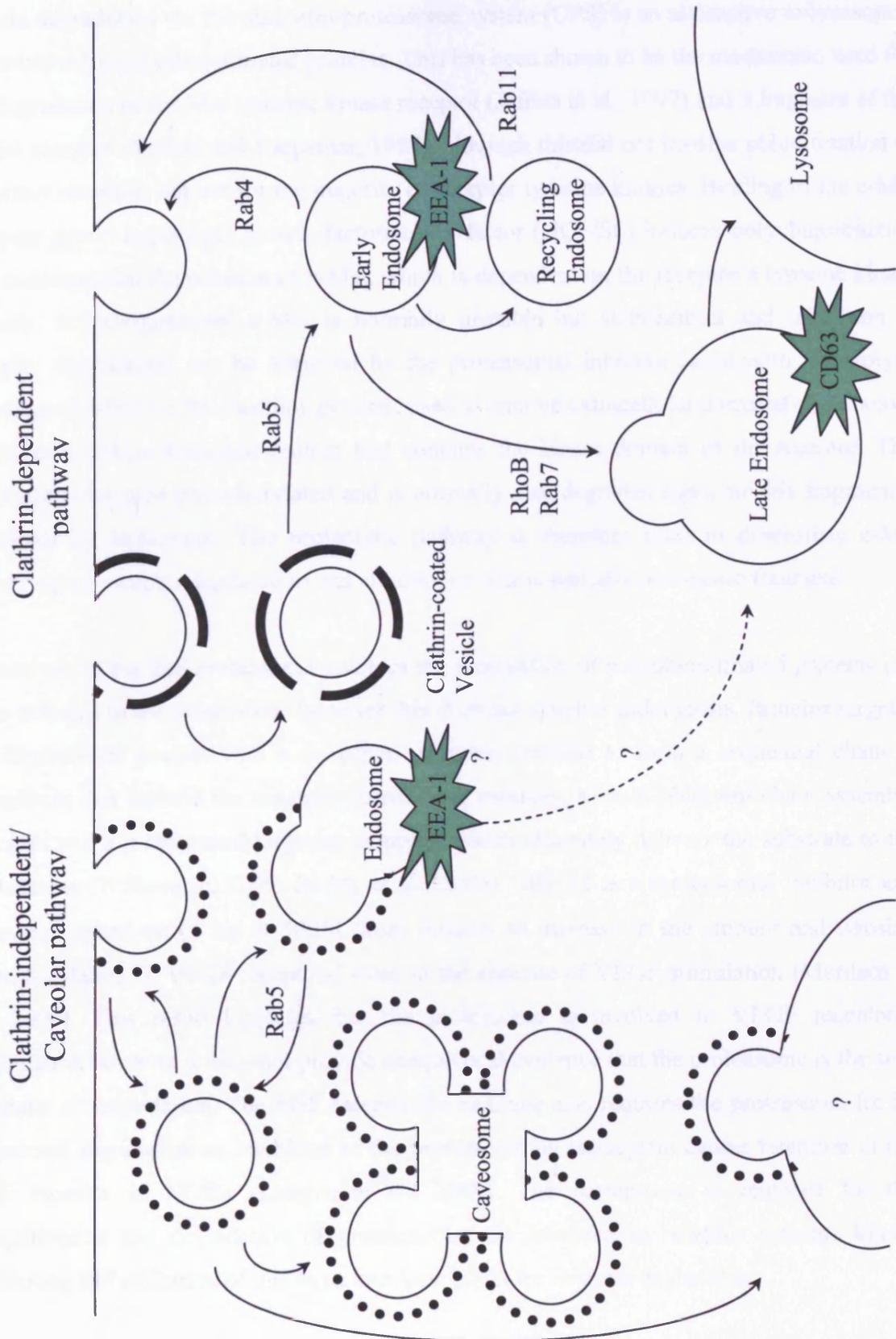


Figure 6.12 - A diagram illustrating two endocytosis pathways including some of the proteins involved in deciding the fate of internalised proteins and two markers for vesicles within the pathway. Part of diagram taken from (Pelkmans et al., 2004).

Protein degradation via the ubiquitin-proteasome system (UPS) is an alternative to lysosomal degradation for polyubiquitinated proteins. This has been shown to be the mechanism used for the degradation of the Met tyrosine kinase receptor (Jeffers et al., 1997) and a fragment of the ErbB4 receptor (Vecchi and Carpenter, 1997), although this did not involve ubiquitination of the intact receptor, but not for the majority of receptor tyrosine kinases. Binding of the c-Met receptor ligand hepatocyte growth factor/scatter factor (HGF/SF) induces polyubiquitination and consequential degradation of c-Met, which is dependent on the receptor's tyrosine kinase activity. Polyubiquitinated c-Met is normally unstable but stabilisation and inhibition of receptor degradation can be achieved by the proteasomal inhibitor lactacystin. Proteolytic cleavage of c-Met by the shedding process, used to remove extracellular domains of receptors, produces a 55kDa truncated protein that contains the kinase domain of the receptor. This fragment is tyrosine phosphorylated and is normally also degraded however this fragment is stabilised by lactacystin. The proteolytic pathway is therefore used to desensitise c-Met signalling by receptor degradation and elimination of a potentially oncogenic fragment.

Proteolysis in the 26S proteasome involves the recognition of polyubiquitinated proteins and their delivery to the proteasome however this does not involve endocytosis. Proteins targeted for degradation interact with a collection of escort proteins to form a sequential chain of complexes that include the substrate recruitment complex, a multi-ubiquitin chain assembly complex and a proteasomal-targeting complex, which ultimately delivers the substrate to the proteasome (Huibregtse, 2005; Richly et al., 2005). MG132 is a proteasomal inhibitor and long-term treatment (4 hrs at 50 μ M dose) induces an increase in the amount and tyrosine phosphorylation of VEGF receptor-2 even in the absence of VEGF stimulation (Murdaca et al., 2004). This result indicates that the proteasome is involved in VEGF receptor-2 degradation however it does not provide unequivocal evidence that the proteasome is the sole mediator of degradation. The EGF receptor for example also requires the proteasome for its lysosomal degradation as inhibition of the proteasome by lactacystin causes retention in the EGF receptor in MVBs (Longva et al., 2002). The proteasome is required for the ubiquitination and degradation of proteins that are involved in receptor tyrosine kinase trafficking and inhibition of this has a knock on effect for receptor degradation.

Singh et al also propose that VEGF receptor-2 could be down regulated via proteasomal degradation and a phosphoserine residue in the C-terminal tail of the receptor, instead of ubiquitin, could be the signal for this (Singh et al., 2005). This group also show that inhibition

of the 26S proteasome blocks down-regulation of VEGF receptor-2 when TPA is used to mimic the VEGF-induced second messenger DAG.

In addition to receptor degradation, receptors can be internalised and recycled back to the plasma membrane. Although we suggest that VEGF may increase the rate of VEGF receptor recycling, in a similar manner to the increased VEGF-stimulated $\alpha\text{v}\beta\text{3}$ integrin recycling, we were unable to detect VEGF receptor-2 with β3 integrin at the cell surface or in endosomes (Figure 6.11). The internalisation of $\alpha\text{v}\beta\text{3}$ integrin and the formation of rab4-positive early endosomes have been shown in NIH3T3 cells (Roberts et al., 2001). In this study β3 integrins were labelled with a specific antibody at 4°C and a raise in temperature initiated internalisation. Incubation of cells at 22°C for 15 minutes, removal of cell surface antibodies and immunofluorescence allowed visualisation of accumulated β3 with rab4 in early endosomes. Here, β3 integrins expressed in HUVE cells were not pre-loaded with antibody, and temperature changes were not employed to drive internalisation; hence our method was not optimised to visualise low concentration of integrins in these intracellular compartments. It is possible that VEGF receptor-2 and β3 integrin are found together in endosomes and that we were unable to visualise this. Alternatively the staining of β3 may highlight endosomal structures however VEGF receptor-2 does not internalise via the same route as integrin recycling.

Studies using inhibitors of VEGF receptors 1 and 2 are currently investigating the role of conventional VEGF signalling in integrin recycling (M. Jones et al., unpublished work). Results to date show that an inhibitor of VEGF receptor-2 inhibits VEGF and PlGF stimulated $\alpha\text{v}\beta\text{3}$ recycling and surface expression whereas a double inhibitor of VEGF receptors 1 and 2 does not and may in fact increase recycling with PlGF stimulation. Both inhibitors however inhibit VEGF and PlGF mediated formation of focal adhesions highlighting the importance of VEGF receptor signalling in this process. Integrin recycling and VEGF receptor signalling may act together to ensure cell survival as well as cell migration. This may be mediated by preventing the accumulation of unligated integrins at the cell surface as they can induce apoptosis by integrin-mediated cell death.

In conclusion we have shown that activation of VEGF receptor-2 results in its internalisation and degradation. From our imaging data VEGF receptor-2 appears to localise to early, but not

late, endosomes and to caveolin-1, which suggests that the internalisation pathway of the receptor could occur via either the caveolar pathway or the clathrin-dependent pathway or via both pathways during different time courses of receptor activation. The co-localisation of VEGF receptor-2 with caveolin-1 could also indicate that these receptors can be sequestered by caveolae. The mechanism for VEGF receptor-2 degradation remains unknown but further investigation is warranted to determine whether VEGF receptor-2 is degraded by the 26S proteasome instead of lysosomes.

Chapter 7

The Role of Nedd4 in the down-regulation of VEGF Receptor-1

Chapter 7 - The Role of Nedd4 in the down-regulation of VEGF Receptor-1

7.1 - Introduction

The degradation of VEGF receptor-1 has not been extensively studied and the difficulties in detecting phosphorylation of endogenous receptors have raised questions regarding the plausibility of this. Studies using over-expression of VEGF receptor-1 have shown maximal phosphorylation and consequential degradation. Previous work in our lab by Dr E Knight has shown association of Nedd4, an ubiquitin ligase involved in the ubiquitination and degradation of proteins, with VEGF receptor-1 in a yeast two-hybrid screen (PhD Thesis, unpublished (Knight, 2001)). This interaction is mediated by WW domains found in Nedd4. WW domains are protein interactions modules that are characterised by the presence of two tryptophan residues separated by 20-22 amino acids and a proline residue at the +2 position with respect to the second tryptophan. The WW domains of Nedd4 interact with proline-rich sequences and binding is independent of phosphotyrosine residues. A conserved region of the C-terminal domain of VEGF receptor-1 has been identified as a potential Nedd4 binding domain. This region contains a PPPDY motif, in which the tyrosine residue is not phosphorylated, suggesting that this interaction could be independent of VEGF receptor-1 kinase activity.

Nedd4 is involved in the degradation of many receptors, for example, the yeast protein Rsp5, of which Nedd4 is the mammalian homologue, is responsible for the degradation of Ste2p, the yeast alpha factor receptor (Dunn and Hicke, 2001), and the drosophila Nedd4 protein binds to, and ubiquitinates, the commissureless protein to regulate the levels of the roundabout receptor (Myat et al., 2002). The WW domains of Nedd4 bind directly to the epithelial sodium channel in mammalian cells, which induces Nedd4-mediated ubiquitination and degradation of the channel (Staub et al., 2000). In addition HECT E3 ligase activity is reported to be involved in mechanisms resulting in the down-regulation of the Notch receptor (Qiu et al., 2000), TGF β type 1 receptor (Kuratomi et al., 2005), EGF receptor (Courbard et al., 2002), VEGF receptor-2 (Murdaca et al., 2004), IGF-1 receptor (Morrione et al., 1999) and the G-protein coupled receptor CXCR4 (Marchese et al., 2003) in mammalian cells. The mechanism of degradation of these receptors however does not involve a direct interaction with Nedd4. In this study we hypothesised that as Nedd4 can interact with VEGF receptor-1

directly it is a potential mediator of VEGF receptor-1 degradation and we therefore sought to confirm its association and effect.

7.1.1 *Nedd4 does not directly ubiquitinate VEGF receptor-2 but participates in its degradation*

In a series of experiments, detailed in this section, Murdaca et al show that although Nedd4 does not directly ubiquitinate VEGF receptor-2 it does participate in its degradation (Murdaca et al., 2004). Nedd4 therefore acts to control VEGF receptor-2 signalling, and down-regulation, however in this case Nedd4 is not responsible for receptor ubiquitination. Over-expression of Grb10, a positive regulator of VEGF receptor-2 signalling, results in an increase in the amount and the level of tyrosine phosphorylation of VEGF receptor-2 even in the absence of VEGF. Grb10 constitutively associates with the ubiquitin ligase Nedd4 and in doing this prevents Nedd4 from influencing the degradation of VEGF receptor-2. A mutant of Grb10, that is unable to bind to Nedd4, does not modify VEGF receptor-2 protein levels. Nedd4 however does not cause receptor degradation by direct ubiquitination even though VEGF receptor-2 was shown to be ubiquitinated in response to VEGF. Wild type Nedd4 promotes VEGF receptor-2 degradation, whereas a ligase deficient mutant does not, suggesting that Nedd4-mediated ubiquitination of other proteins is required for VEGF receptor-2 ubiquitination and degradation. Potential candidate proteins, that are involved in receptor tyrosine kinase endocytosis, and are ubiquitinated by Nedd4, are Eps15 and Hrs (Katz et al., 2002; Polo et al., 2002).

7.1.2 *Down-regulation of VEGF receptor-1 by the CD2AP-Cbl complex*

Kobayashi et al have also published data showing phosphorylation of VEGF receptor-1 and Cbl-regulated endocytosis and degradation (Kobayashi et al., 2004). This work shows the formation of a VEGF receptor-1-CD2AP-Cbl complex and as CD2AP is a CIN85 family member this complex is similar to that used in VEGF receptor-2 degradation. VEGF stimulated phosphorylation of VEGF receptor-1 results in the recruitment of the CD2AP-Cbl complex to tyrosine 1333 of the receptor, a conserved minor phosphorylation site located in the C-terminal tail. This region in VEGF receptor-1, and a region containing tyrosine 1319 of VEGF receptor-2 (also a conserved residue located in its C-terminal tail), shows similarities with the docking site for Cbl in the EGF receptor. Receptor-Cbl complex formation is enhanced by VEGF and results in tyrosine phosphorylation of Cbl and polyubiquitination of VEGF receptor-1. Ubiquitination of the receptor sorts the complex to endocytic vesicles. Cbl

binds to phospho-tyrosine 1333 of VEGF receptor-1 via its SH2 domain. The SH3 domain of CD2AP associates with Cbl in the absence of ligand and its over-expression enhances VEGF-stimulated receptor internalisation and ubiquitination. Poly-ubiquitination of VEGF receptor-1 was only evident when cells were transfected with full-length Cbl; however degradation of VEGF receptor-1 was still detected when tyrosine 1333 was mutated to a phenylalanine indicating that another degradation pathway may exist.

7.1.3 *A drug-activated, chimeric VEGF receptor-1 was used to study down-regulation*

In order to study VEGF receptor-1 degradation we used PAE cells infected with an adenoviral Myc-tagged chimeric receptor, Myc-GyrB-Flt-1. Dr J. Carter generated adenoviruses in order to achieve efficient expression of chimeric VEGF receptors 1 and 2. The infection of cells, and hence the expression of Myc-GyrB-Flt-1, can be easily checked as the viruses also express GFP. Unfortunately the GFP does not label the receptor however so the Myc tag has been used for its detection. These chimeric receptors consist of the cytoplasmic domains of VEGF receptors 1 and 2 (the kinase domains, kinase insert domains and C-terminal tails) fused to a DNA Gyrase dimerisation domain and a Src myristoylation sequence, and are denoted Myc-GyrB-Flt-1 and GyrB-KDR respectively. Previous work involved the determination of optimal expression levels of Myc-GyrB-Flt-1 in immortalised brain endothelial (IBE) cells. This determined the level of receptor required to achieve maximal activation upon stimulation, without ligand independent activation caused by over-expression (J. Carter et al. unpublished work). Coumermycin A1, a drug that induces chimeric receptor dimerisation and activation (Figure 7.1), induces phosphorylation of Myc-GyrB-Flt-1 in a concentration- and time-dependent manner in IBE cells. Maximal activation of Myc-GyrB-Flt-1 leads to the phosphorylation of ERK, Akt, p38 MAPK and PLC γ 1 and can induce migration, as shown by a wound healing assay, and differentiation in a tube formation assay.

Coumermycin A1

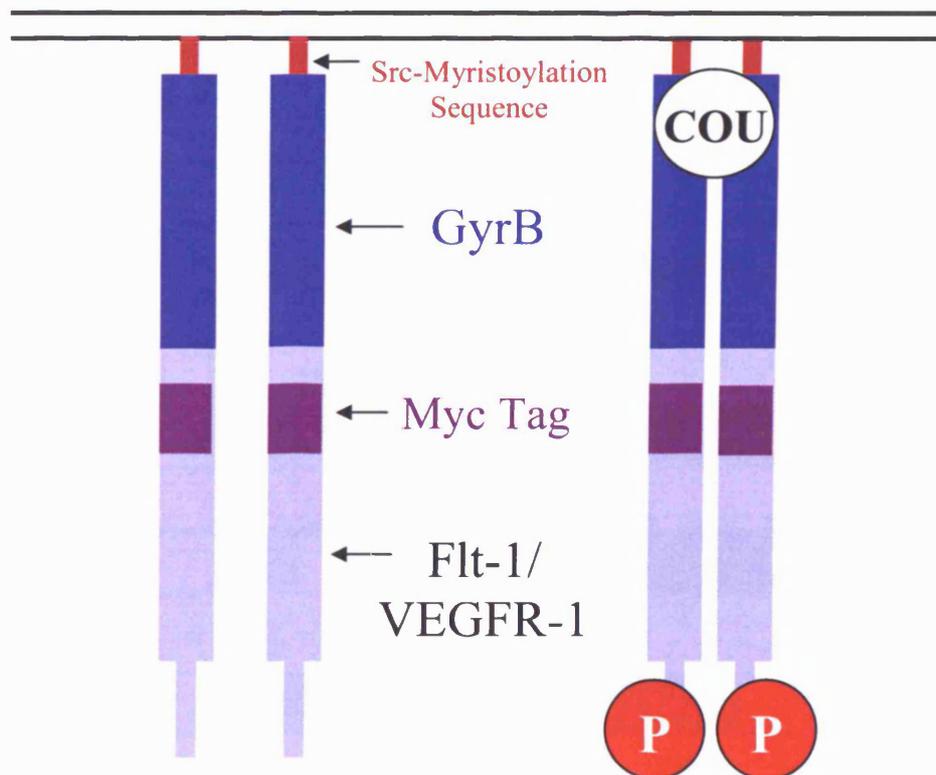
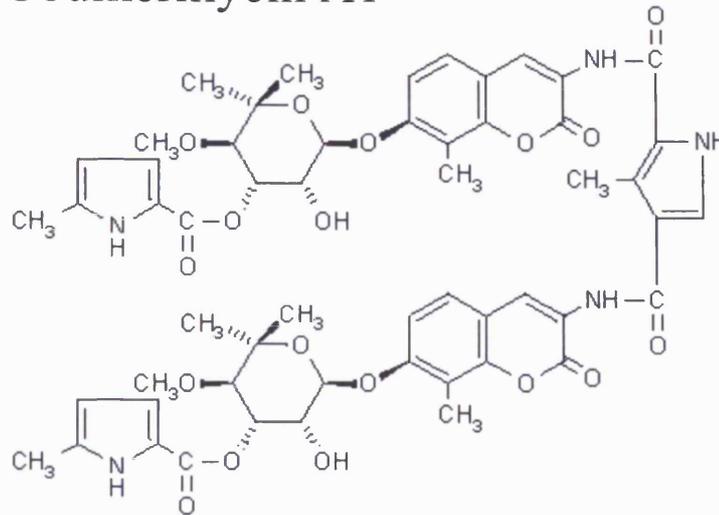


Figure 7.1 A – The structure of coumermycin A1. B - A diagram illustrating activation of the chimeric Myc-tagged Gyr-Flt-1 receptor by coumermycin A1. Coumermycin A1 is a non-mammalian molecule that specifically activates receptors containing the dimerisation domain of the B subunit of DNA gyrase with no possible activation of other receptors (Knight et al., 2000).

Carter et al also show that both GyrB-KDR and Myc-GyrB-Flt-1 receptors are negatively regulated by degradation following treatment of coumermycin A1 (unpublished work). This observation supports previous work demonstrating that a region of the cytoplasmic domain is solely responsible for triggering the down-regulatory response. Both chimeric VEGF receptors demonstrate drug dependent autophosphorylation and show the same phosphorylation profile as receptors in HUVE cells i.e. a bell shaped curve. This suggests that the VEGF receptor-1 chimera can be maximally activated upon dimerisation and any repressor mechanisms are overcome in this system. Maximal kinase activity of this chimeric VEGF receptor-1 facilitates receptor down-regulation, as is the case for VEGF receptor-2. Furthermore if the drug-induced activation of chimeric VEGF receptor-1 results in the maximum phosphorylation of tyrosine residues, those targeted by both VEGF and PlGF for example, then this system may report on the consequences of maximal receptor activation.

As Nedd4 and Cbl have both been implicated in VEGF receptor-1 degradation we examined the association of these proteins with VEGF receptor-1 and receptor levels in cells over-expressing Myc-Gyr-Flt-1. In addition, ligase inactive mutants of Nedd4 and Cbl, Nedd4 C→S, in which the active site cysteine residue is mutated to a serine residue, and Cbl 70Z, in which the ring finger domain is interrupted, were expressed instead of the wild type proteins to determine whether their ligase activity is necessary for their association with VEGF receptor-1.

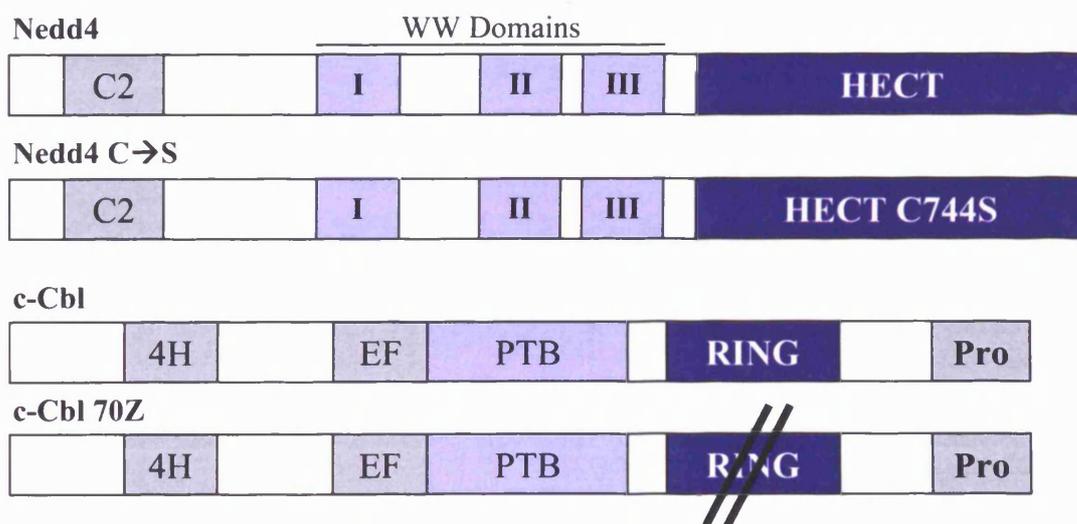


Figure 7.2 – Protein domains of wild type, and ligase inactive, Nedd4 and Cbl proteins. The WW domains in Nedd4 and the PTB domain in Cbl are protein-interaction modules and the HECT domain in Nedd4 and the RING domain in Cbl are E3 ubiquitin ligase domains.

7.2 - Results

7.2.1 *PLC γ 1 associates with phosphorylated Myc-GyrB-Flt-1*

Maximum activation of Myc-GyrB-Flt-1, as shown by western blots of whole cell lysates probed with phosphotyrosine, is achieved by treatment of cells with 1 μ M and 5 μ M of coumermycin for 15 minutes (Figure 7.3B). Exposure of cells expressing GyrB-Flt-1 to shear stress activates the receptor in the absence of coumermycin. In order to prevent this, the cell medium was not changed during the three hours prior to treatment with coumermycin. The SH2 domain of PLC γ 1 is able to bind directly to phosphotyrosine residues in activated VEGF receptor-1. We therefore used this well characterised pathway to further clarify that Myc-GyrB-Flt-1 can associate, in a similar manner to the endogenous receptor, with downstream signalling proteins in PAE cells. As a positive control to show co-immunoprecipitation of Myc-GyrB-Flt-1 we precipitated PLC γ 1 from cells transfected with a DNA construct encoding GFP-PLC γ 1 and infected with virus expressing Myc-GyrB-Flt-1. Figure 7.3A shows that treatment of these cells with 5 μ M coumermycin leads to Myc-GyrB-Flt-1 phosphorylation and association of PLC γ 1 with the activated receptor.

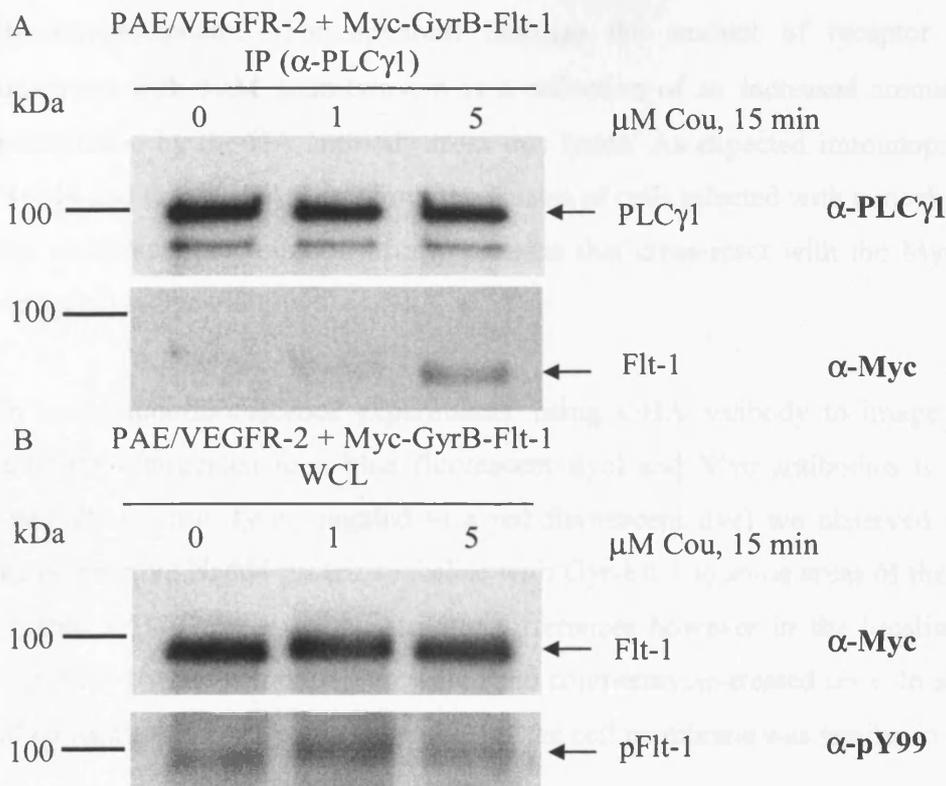


Figure 7.3 – Endogenous PLC γ 1 associates with activated Myc-GyrB-Flt-1 in PAE/VEGF receptor-2 cells. Proteins in immunoprecipitates of PLC γ 1 from PAE cells stably expressing VEGF receptor-2, transiently expressing GFP-PLC γ 1 and infected with virus encoding Myc-tagged GyrB-Flt-1 were separated using SDS-PAGE and detected by western blotting. Panel A shows that PLC γ 1 was precipitated and that Flt-1 co-immunoprecipitated in cells treated with 5 μ M coumermycin. Whole cell lysate samples (Panel B) show the expression of Flt-1 and the phosphorylation status of the receptor. These blots are from a single experiment.

7.2.2 *Nedd4 and Nedd4 C \rightarrow S associate with GyrB-Flt-1 and localise to the plasma membrane*

PAE cells, infected with viruses expressing Myc-GyrB-Flt-1, were transfected with DNA constructs encoding wild type HA-Nedd4 or the ligase mutant, HA-Nedd4 C \rightarrow S. GyrB-Flt-1 was activated by treatment of these cells with 1 μ M and 5 μ M doses of coumermycin for 15 minutes. HA-Nedd4 proteins from cell lysates were immunoprecipitated and the complexes were analysed by western blotting to detect the presence of co-immunoprecipitated GyrB-Flt-1 (Figure 7.4). Wild type Nedd4 was found to associate with GyrB-Flt-1 in resting cells and the amount of associated receptor increased after treatment with 1 μ M coumermycin. Similarly GyrB-Flt-1 associated with Nedd4 C \rightarrow S in untreated cells however we believe that the

activation of the receptor with coumermycin does not increase the amount of receptor co-immunoprecipitated. The apparent increase the amount of receptor precipitated upon treatment with 5 μ M coumermycin is a reflection of an increased amount of Nedd4 C \rightarrow S precipitated by the HA antibody from this lysate. As expected immunoprecipitation of HA-Nedd4 and HA-Nedd4 C \rightarrow S from the lysates of cells infected with a mock virus did not show the co-immunoprecipitation of any proteins that cross-react with the Myc antibody used to detect Myc-Gyr-Flt-1.

In co-immunofluorescence experiments, using a HA antibody to image Nedd4 (secondary antibody conjugated to a blue fluorescent dye) and Myc antibodies to image GyrB-Flt-1 (secondary antibody conjugated to a red fluorescent dye) we observed that wild type and ligase inactive Nedd4 proteins localise with Gyr-Flt-1 to some areas of the plasma membrane (Figure 7.5). There were no striking differences however in the localisation of Nedd4 or GyrB-Flt-1 when comparing untreated and coumermycin-treated cells. In addition the amount of co-localisation of the Nedd4 and Flt at the cell membrane was similar in all cells examined.

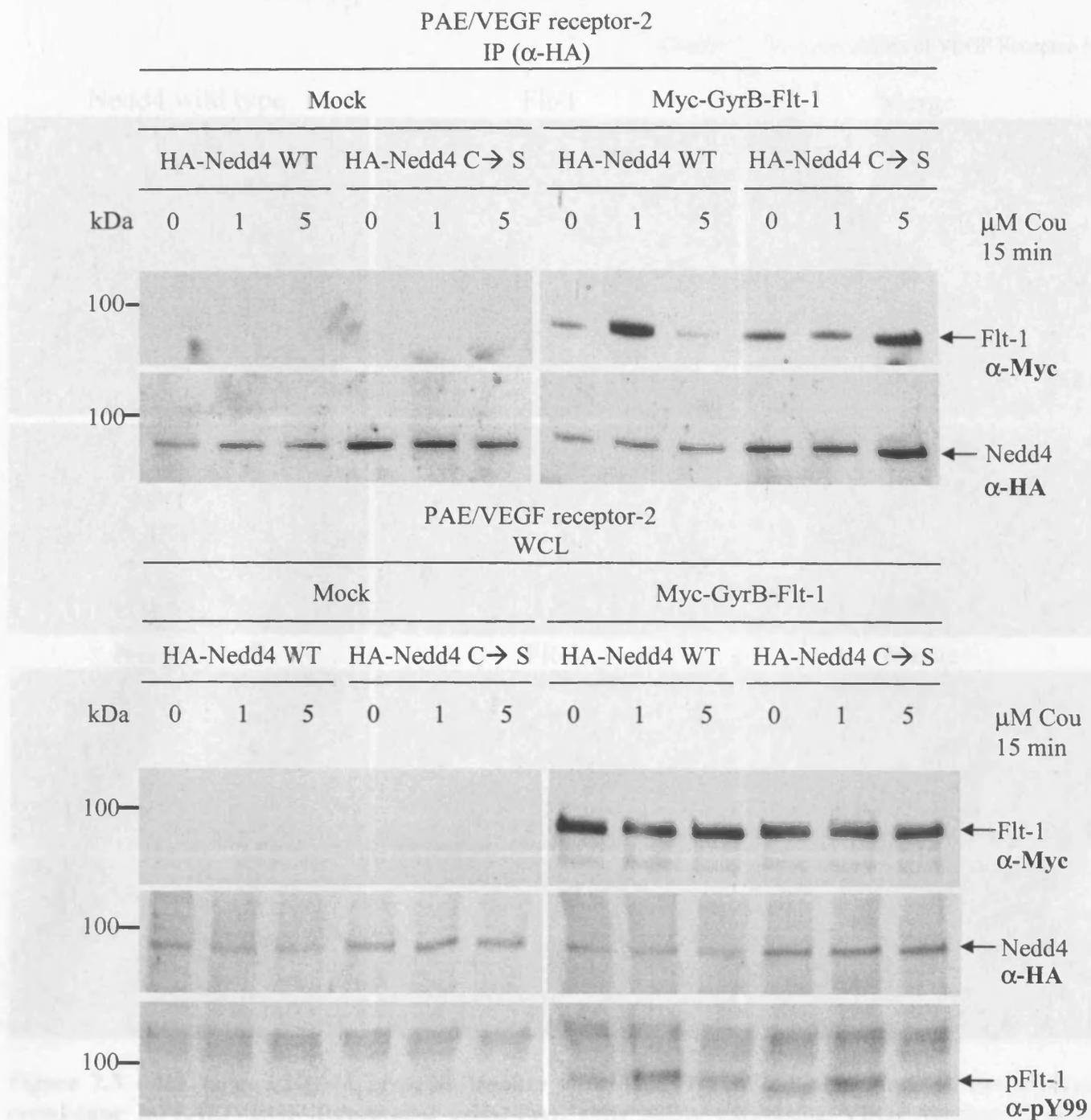


Figure 7.4 – HA-tagged Nedd4 proteins associate with Myc-GyrB-Flt-1 in PAE/VEGF receptor-2 cells. Proteins in immunoprecipitates of HA-Nedd4 from PAE cells stably expressing VEGF receptor-2, transiently expressing Nedd4 proteins and infected with virus encoding Myc-tagged GyrB-Flt-1 were separated using SDS-PAGE and detected by western blotting. Blots of the immunoprecipitated proteins shows that HA-Nedd4 was precipitated and that an increased amount of Flt-1 co-immunoprecipitated in cells treated with coumermycin. Whole cell lysate samples show the expression of Flt-1, the phosphorylation status of the receptor and the relative expression levels of wild-type Nedd4 and Nedd4 C \rightarrow S. These blots are representative of three independent experiments.

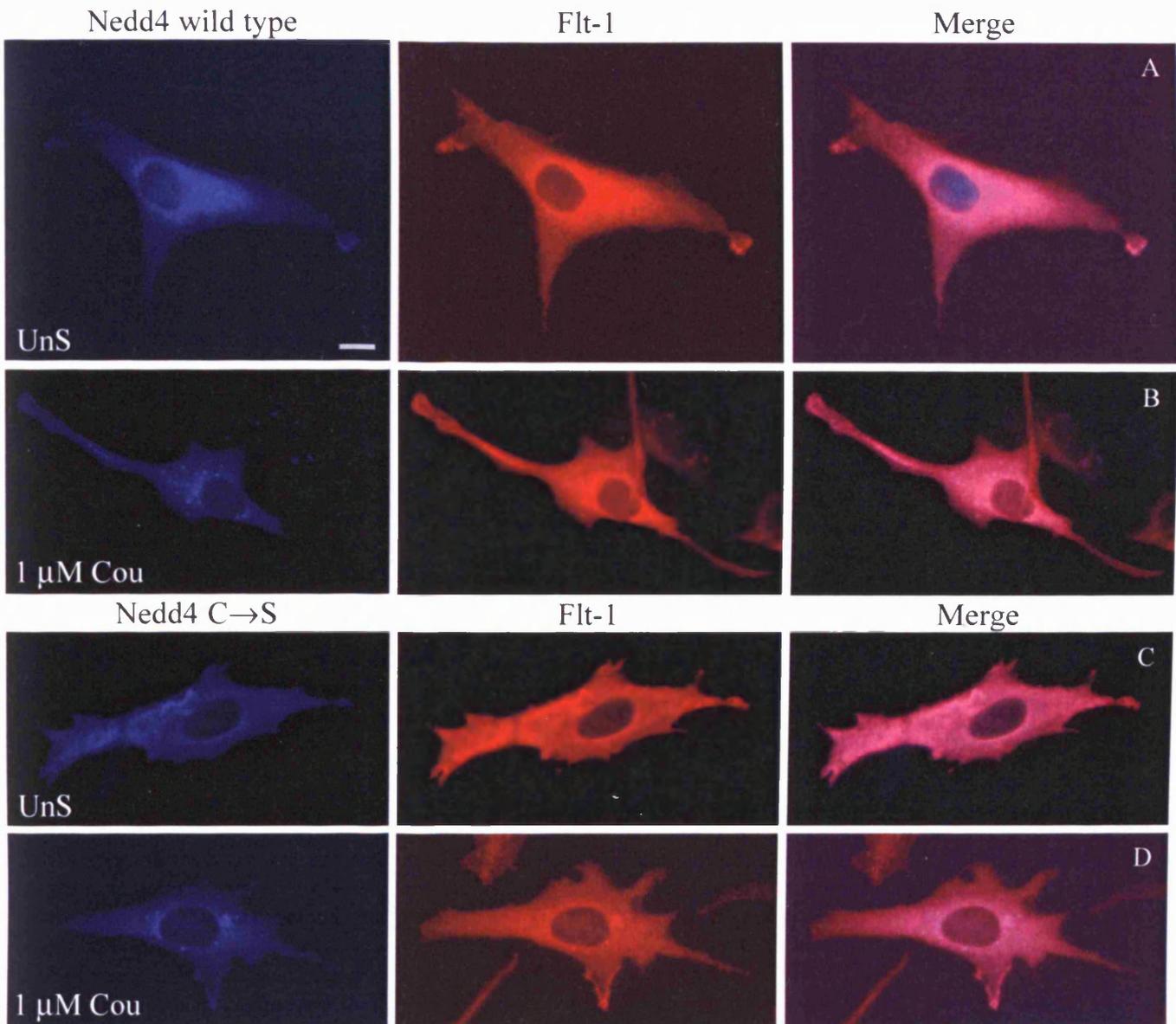


Figure 7.5 - HA-tagged Nedd4 proteins localise with Myc-GyrB-Flt-1 at regions of the plasma membrane in PAE/VEGF Receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were infected with virus encoding Myc-GyrB-Flt-1 and transiently transfected with HA-Nedd4, wild type or the C →S mutant. Images shown are of cells that were serum starved (Unstimulated) or treated with 1 μ M Coumestrol A1 for 15 minutes. Flt-1 is immunofluorescently labelled using an antibody against Myc and a secondary antibody conjugated to Texas Red. Nedd4 is immunofluorescently labelled using an antibody against HA and a secondary antibody conjugated to AMCA. These are representative cells from a single experiment. The scale bar represents 10 μ m.

7.2.3 *Nedd4 and Nedd4 C→S are phosphorylated in resting and stimulated cells*

The phosphorylation of Nedd4 could not be analysed by western blotting of whole cell lysates as the GyrB-Flt-1 protein is of a similar size and the two proteins co-migrate through an SDS-PAGE gel. In order to investigate the phosphorylation status of Nedd4 we immunoprecipitated all tyrosine phosphorylated proteins using a phosphotyrosine antibody. As expected phospho-GyrB-Flt-1 was immunoprecipitated from the lysates of cells treated with 1 μ M coumermycin (Figure 7.6A). In addition Nedd4 proteins were also immunoprecipitated by the phosphotyrosine antibody in lysates from untreated and coumermycin-treated cells. The phosphorylation level of wild type and ligase inactive Nedd4 appears to decrease when Gyr-Flt-1 is activated. The apparent increased phosphorylation status of Nedd4 C→S, when compared to wild type Nedd4, is likely to be due to the greater expression of the Nedd4 C→S protein. In western blots of the whole cell lysate an apparent decrease in receptor protein level is observed upon activation of GyrB-Flt-1 however the expression of ligase inactive Nedd4 does not appear to inhibit this receptor degradation (Figure 7.6B).

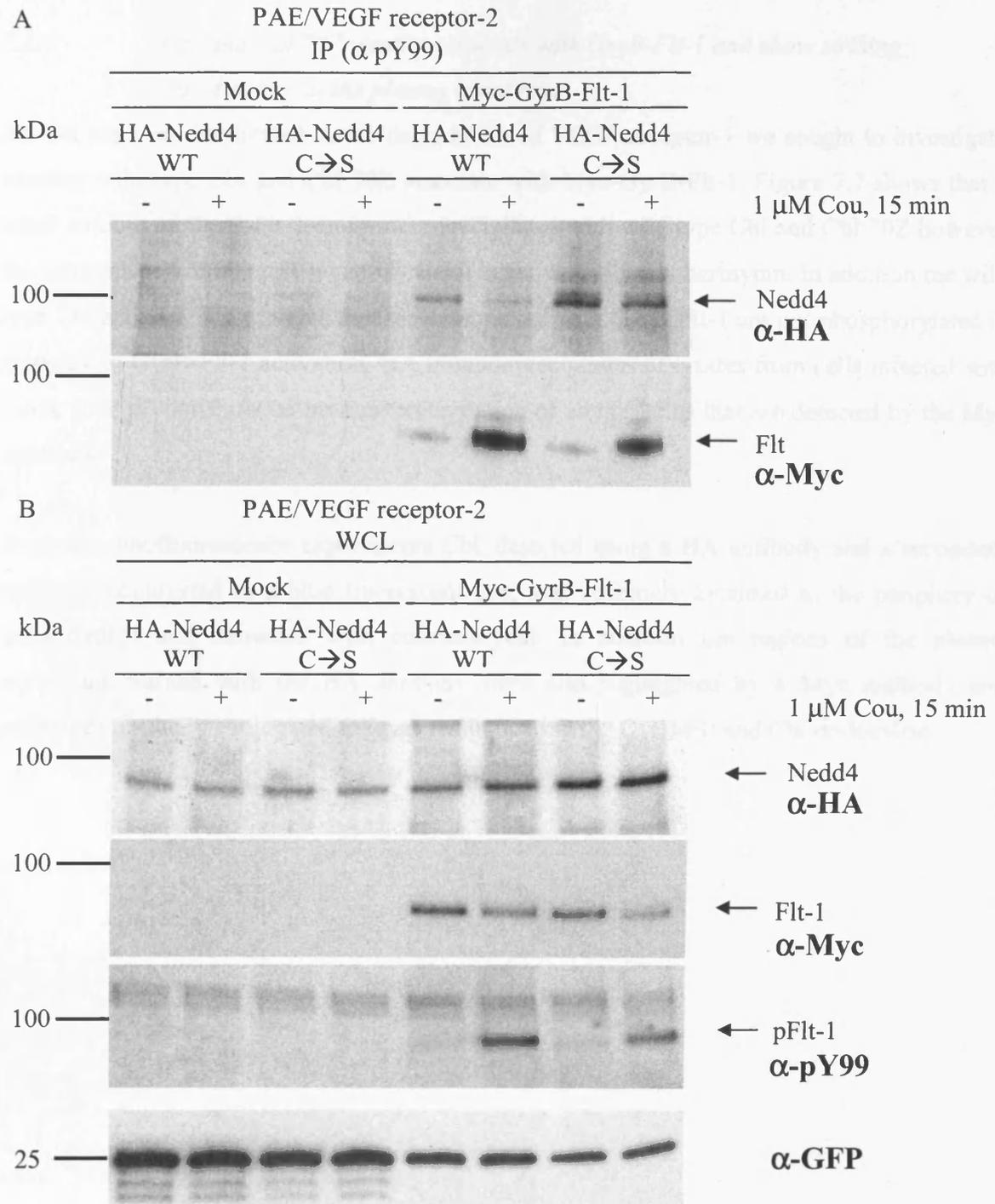


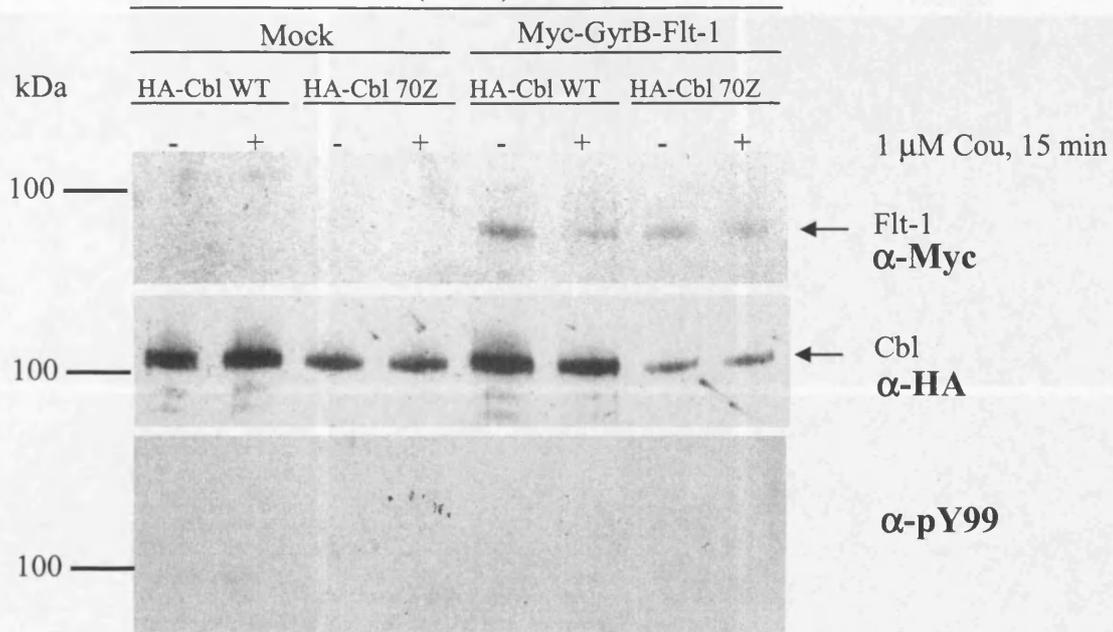
Figure 7.6 – A phosphotyrosine antibody precipitates HA-tagged Nedd4 proteins from lysates of PAE/VEGF receptor-2 cells expressing Myc-GyrB-Flt-1 and wild type or ligase inactive Nedd4. Proteins immunoprecipitated by a phosphotyrosine antibody from PAE cells stably expressing VEGF receptor-2, transiently expressing Nedd4 proteins and infected with virus encoding Myc-tagged GyrB-Flt-1 were separated using SDS-PAGE and detected by western blotting. Panel A shows that HA-Nedd4 and Flt-1 proteins were precipitated and that an increased amount of Flt-1 co-immunoprecipitated in cells treated with coumermycin. Whole cell lysate samples (Panel B) show the expression of Nedd4, Flt-1, the phosphorylation status of the receptor and the expression levels GFP, a marker for viral infection. These blots are representative of three independent experiments.

7.2.4 *Cbl and Cbl 70Z weakly associate with GyrB-Flt-1 and show striking localisation at the plasma membrane*

As Cbl has been implicated in the degradation of VEGF receptor-1 we sought to investigate whether wild type Cbl and Cbl 70Z associate with Myc-GyrB-Flt-1. Figure 7.7 shows that a small amount of GyrB-Flt-1 co-immunoprecipitates with wild type Cbl and Cbl 70Z however the amounts do not increase when GyrB-Flt is activated by coumermycin. In addition the wild type Cbl and Cbl 70Z proteins that are precipitated with GyrB-Flt-1 are not phosphorylated in response to GyrB-Flt-1 activation. HA immunoprecipitates of lysates from cells infected with mock virus do not show co-immunoprecipitation of any proteins that are detected by the Myc antibody.

In co-immunofluorescence experiments Cbl, detected using a HA antibody and a secondary antibody conjugated to a blue fluorescent dye, was strikingly localised to the periphery of cells treated and untreated with coumermycin. In addition the regions of the plasma membrane stained with the HA antibody were also highlighted by a Myc antibody and secondary antibody conjugated to texas red indicating the GyrB-Flt and Cbl co-localise.

PAE/VEGF receptor-2
IP (α -HA)



PAE/VEGF receptor-2
WCL

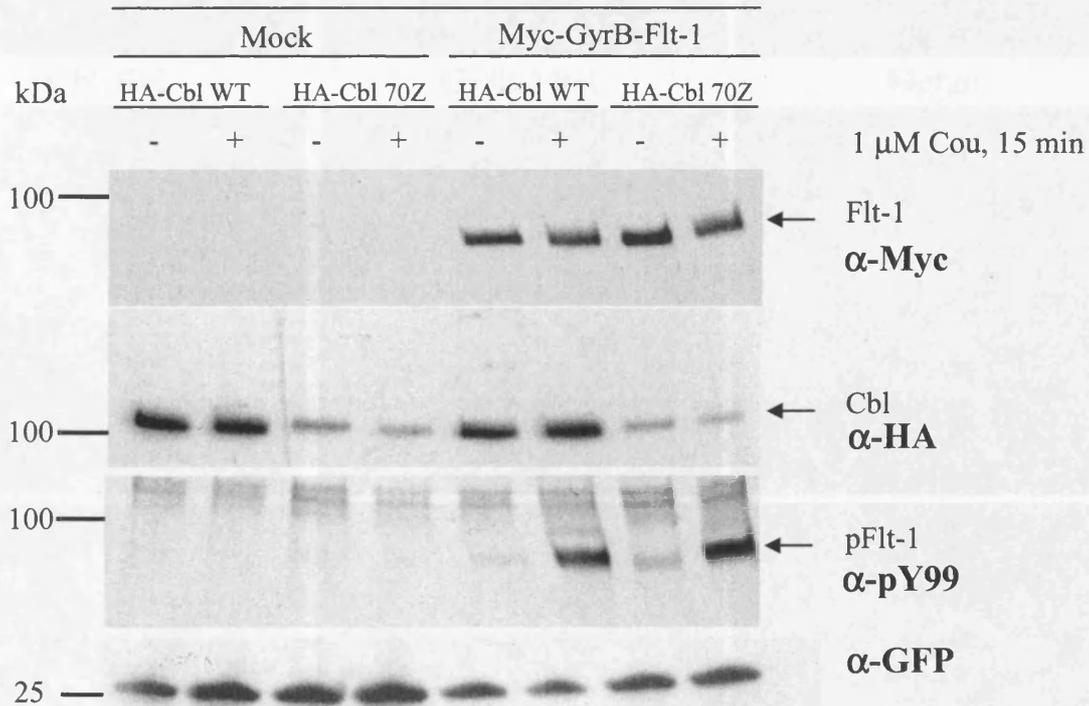


Figure 7.7 – HA-tagged Cbl proteins associate with Myc-GyrB-Flt-1 in PAE/VEGF receptor-2 cells. Proteins in immunoprecipitates of HA-Cbl from PAE cells stably expressing VEGF receptor-2, transiently expressing Cbl proteins and infected with virus encoding Myc-tagged GyrB-Flt-1 were separated using SDS-PAGE and detected by western blotting. Blots of the immunoprecipitated proteins shows that Cbl and Flt-1 proteins were precipitated. Whole cell lysate samples show the expression of Flt-1, the phosphorylation status of the receptor, the relative expression levels of wild-type Cbl and Cbl70Z and the levels of GFP, a marker for viral infection. These blots are representative of three independent experiments.

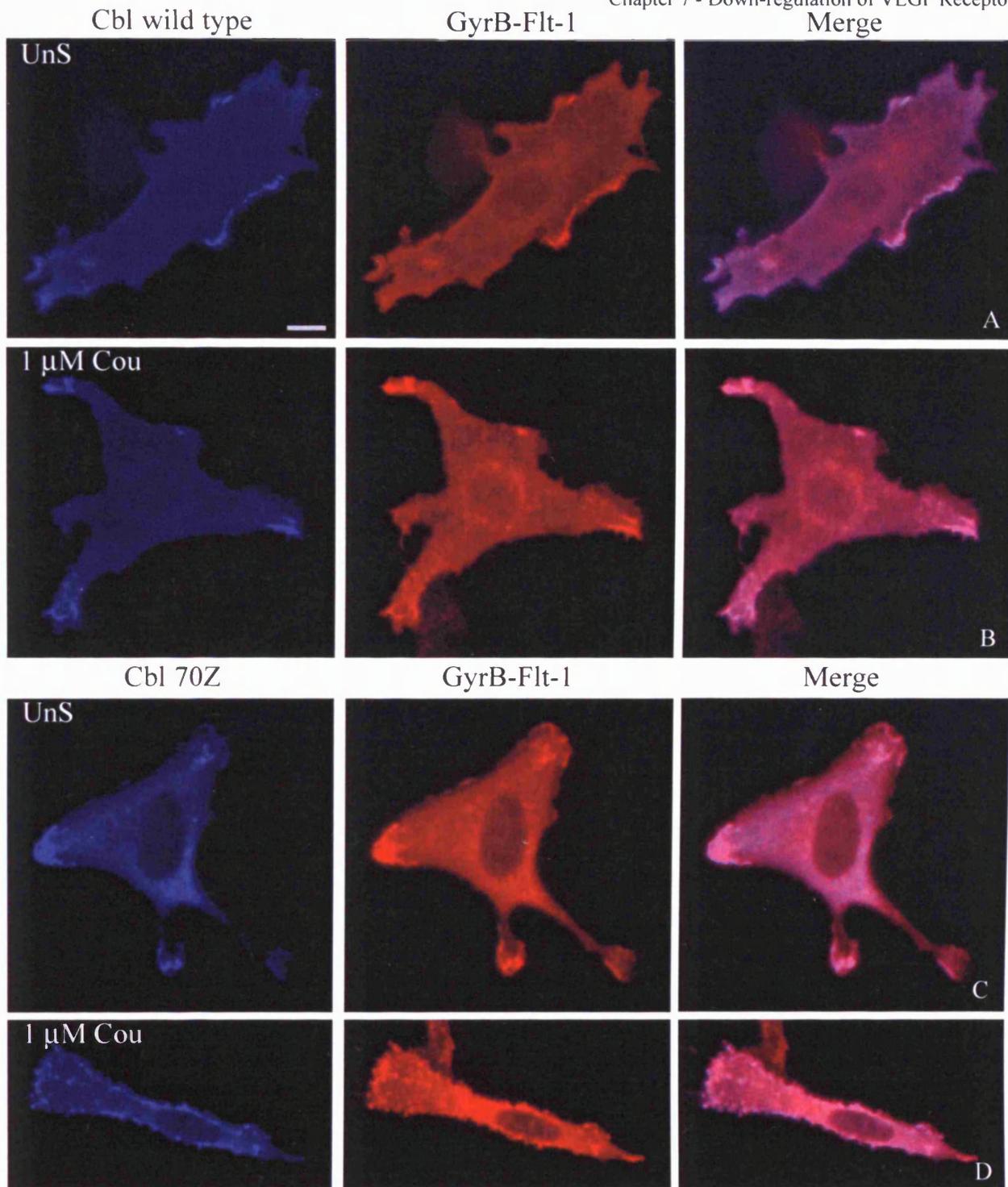


Figure 7.8 - HA-tagged Cbl localises with Myc-GyrB-Flt-1 at regions of the plasma membrane in PAE/VEGF Receptor-2 cells. PAE cells stably expressing VEGF receptor-2 were infected with virus encoding Myc-GyrB-Flt-1 and transiently transfected with HA-Cbl, wild type or the 70Z mutant. Images shown are of cells that were serum starved (Unstimulated) or treated with 1 μ M Coumermycin A1 for 15 minutes. Flt-1 is immunofluorescently labelled using an antibody against Myc and a secondary antibody conjugated to Texas Red. Cbl is immunofluorescently labelled using an antibody against HA and a secondary antibody conjugated to AMCA. These are representative cells from a single experiment. The scale bar represents 10 μ m.

7.2.5 *Over-expression of Nedd4, but not Cbl, increases the rate of GyrB-Flt degradation*

As both Nedd4 and Cbl associate and co-localise with GyrB-Flt-1 we investigate which, if either, of these ubiquitin ligases facilitated the degradation of GyrB-Flt-1. By comparing the level of GyrB-Flt-1, in cells expressing the receptor only and cells expressing the receptor with either wild type Cbl or wild type Nedd4, over a time course of activation of GyrB-Flt-1 with coumermycin, we observed that the over-expression of Nedd4 promoted the degradation of the receptor whereas over-expression of Cbl did not have the same effect (Figure 7.9). Western blots using antibodies to HA and GFP show an equal expression of Cbl and Nedd4 and the same infection efficiency of virus respectively over the time course of coumermycin treatment. The phosphotyrosine blots shows maximal activation of GyrB-Flt-1 and the vinculin blots show that approximately the same number of cells were analysed for each coumermycin time-point, coumermycin is not therefore toxic to these cells at this concentration. Protein levels of GyrB-Flt-1 were normalised to that of vinculin and over a 90-minute period 57% of GyrB-Flt-1 was degraded in cells expressing only the receptor but this increased to 82% when Nedd4 was also over-expressed in a small percentage of cells. In contrast only 45% of GyrB-Flt-1 was degraded when Cbl was over-expressed. The efficiency of transfection for HA-Nedd4 and HA-Cbl constructs in PAE/VEGF receptor-2 cells was gauged during immunofluorescence imaging of the tagged proteins and from western blots of whole cell lysates. In our opinion the transfection efficiency was low for each construct however the expression of HA-Cbl was more efficient than that of HA-Nedd4. The normalised graphs in Figure 7.9 however take into consideration this differing level of protein expression.

Figure 7.9 – Degradation of Myc-GyrB-Flt-1 is enhanced by Nedd4 but not by Cbl. Whole cell lysate proteins from serum starved (unstimulated) or coumermycin treated PAE cells, stably expressing VEGF receptor-2, transiently expressing HA-Nedd4 or HA-Cbl and infected with virus encoding Myc-tagged GyrB-Flt-1 were separated using SDS-PAGE and detected by western blotting. Blots show the levels of Flt-1, the phosphorylation status of the receptor, the level of vinculin (used to normalise all samples), the relative expression levels of HA-Cbl and HA-Nedd4 and the level of GFP, a marker for viral infection. The graphs show the levels of Flt-1, as normalised to vinculin, and quantified using image quant. These blots are from a single experiment.

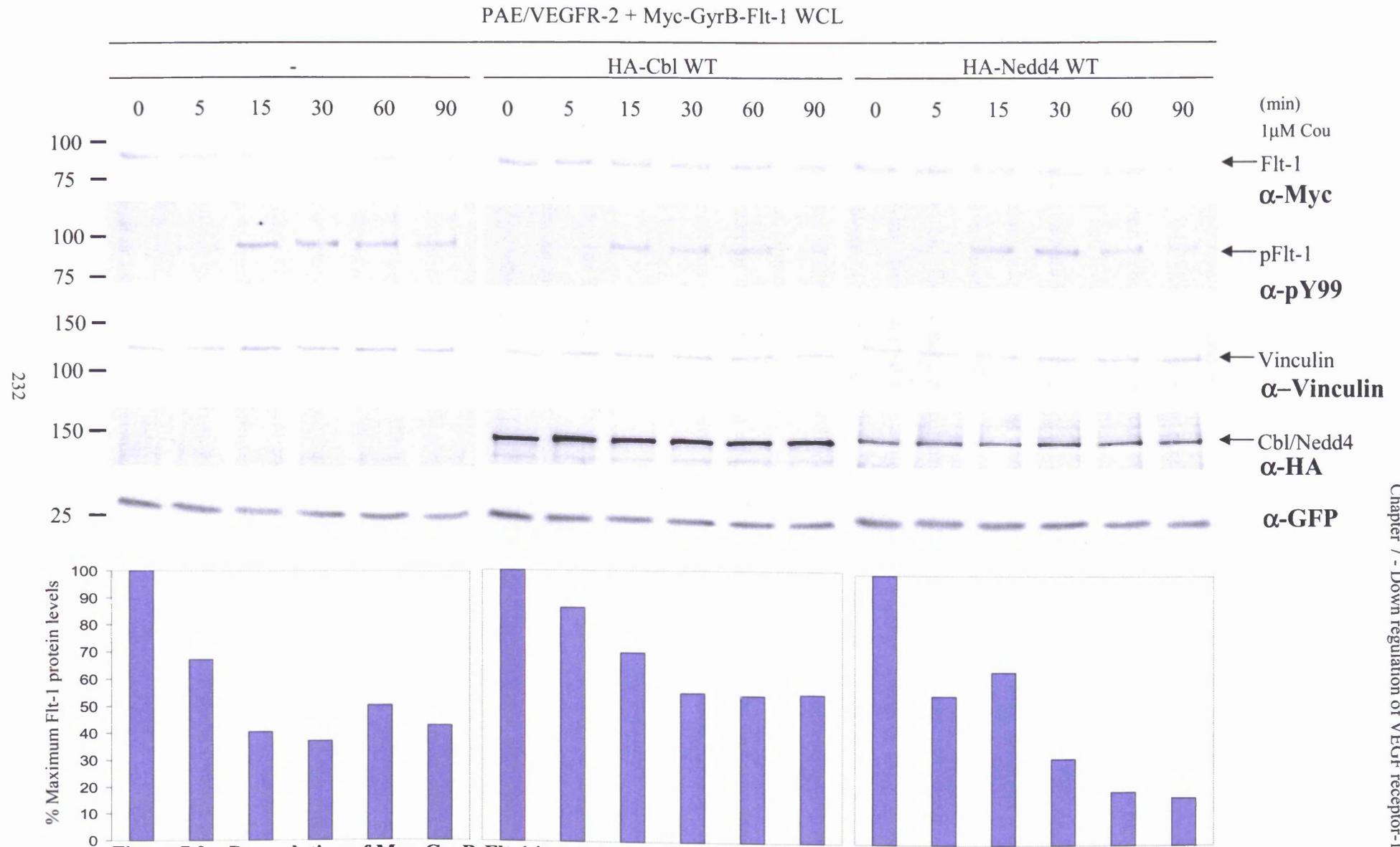


Figure 7.9 – Degradation of Myc-GyrB-Flt-1 is enhanced by Nedd4 but not by Cbl

7.3 - Discussion

A substantial amount of evidence has been generated to suggest that signalling downstream of the chimeras Myc-GyrB-Flt-1 and GyrB-KDR is representative of the endogenous VEGF receptors 1 and 2 respectively. GyrB-Flt-1 is efficiently phosphorylated in response to coumermycin A1 and this study has shown that this results in the direct association of proteins with the receptor. When comparing the association between GyrB-Flt-1 and PLC γ 1 with that of GyrB-Flt-1 and wild type Nedd4 we have noticed differences however. GyrB-Flt-1 shows the greatest amount of phosphorylation when activated by a 1 μ M dose of coumermycin for 15 minutes and although it is phosphorylated by a 5 μ M dose the levels are lower. PLC γ 1 appears to associate with GyrB-Flt-1 when cells are activated with 5 μ M coumermycin. Nedd4 on the other hand, associates during the peak of receptor phosphorylation, resulting from activation using a 1 μ M dose, and there is very little association when the levels of phospho-GyrB-Flt-1 are lower. These variations indicate that the status of the receptor is different when activated by different doses of coumermycin for the same length of time. Signalling events downstream of VEGF receptor-1 may occur at a greater rate when the receptor is activated with a high dose of coumermycin A1 and therefore after 15 minutes of activation at this dose some proteins, such as Nedd4, have dissociated whereas others, such as PLC γ 1, are associated.

As mentioned previously, the association of Nedd4 with VEGF receptor-1 may not involve phosphotyrosine residues as direct binding is mediated by one of its WW domains. In this study we have shown that unphosphorylated GyrB-Flt-1 co-immunoprecipitates with Nedd4 in resting cells however we have detected a greater amount of receptor associated with Nedd4 when it is activated by coumermycin. This indicates that association of Nedd4 is promoted by the presence of phosphotyrosine residue in the intracellular domain of the receptor. The association of the WW domain of Nedd4 with the PPPDY motif present in the C-terminal tail of GyrB-Flt-1 may explain the co-immunoprecipitation detected in resting cells however there are a few possibilities regarding the increase in association upon activation of the receptor. WW domains are able to bind to phosphoserine and phosphothreonine residues (Lu et al., 1999) as well as proline rich motifs. The activation of GyrB-Flt-1 results in the activation of Akt, ERK, p38, probably PKC downstream of PLC γ 1 (Knight et al., 2000) and possibly other serine/threonine kinases that have not been investigated. It is feasible therefore that activation of GyrB-Flt-1 results in phosphorylation of serine and/or threonine residues by a downstream

signalling protein as well as the autophosphorylated tyrosine residues thereby providing additional sites of interaction for the WW domains of Nedd4. PKC for example has been shown to mediate the down-regulation of VEGF receptor-2 by phosphorylating serine residues 1188 and 1191 in the C-terminal tail of the receptor (Singh et al., 2005). In addition the activation of Akt occurs downstream of PI3K, a lipid kinase. The C2 domain of Nedd4 may therefore bind to phospholipids generated in the plasma membrane by the activation of GyrB-Flt-1 and increase the local concentration of protein in the vicinity of the receptor. Furthermore Grb10 has been shown to associate constitutively with Nedd4s C2 domain (Morrione et al., 1999) and the SH2 domain of Grb10 may bind to phospho-Flt-1 providing an indirect interaction in activated cells. The interaction between Nedd4 and GyrB-Flt-1 is substantiated by the observation that a proportion of these proteins can co-localise at regions of the plasma membrane.

The Nedd4 C→S mutant was also able to associate with GyrB-Flt-1 however the association is not positively regulated by the activation of the receptor as seen for the wild type Nedd4. This implies that activation of GyrB-Flt-1 initiates Nedd4-mediated ubiquitination of the receptor, or an additional protein, that increases Nedd4 association. The ubiquitination and relocalisation of a protein that normally acts to inhibit Nedd4 binding to GyrB-Flt-1 is an attractive model to explain these results however further investigation is required to determine if this is the case and to identify potential candidates.

In immunoprecipitation experiments we have detected Nedd4 in phosphotyrosine immunoprecipitates, which suggests that Nedd4 may be tyrosine phosphorylated. This is not conclusive evidence however as Nedd4 may co-immunoprecipitate with another phosphorylated protein. The phosphorylation status of wild type and ligase inactive Nedd4 may decrease upon activation of GyrB-Flt-1. The validity of this result is questionable however as the efficiency of the phosphotyrosine precipitation from each cell lysate is difficult to determine. Analysis of immunoprecipitation of phospho-GyrB-Flt-1 can be used as an indication that phosphorylated proteins were successfully precipitated however a guide to the efficiency of the precipitation can only be achieved by the comparison of the immunoprecipitation western blot with the whole cell lysate western blot. These two blots shown in figure 7.6 are very similar indicating that the immunoprecipitations are a good representation of the phospho-proteins present in the cell lysates. Another factor to take into consideration however is the differences in expression level of Nedd4 and GyrB-Flt-1. All

cells express GyrB-Flt-1, as determined by the GFP marker that is used to detect infected cell, however only a small percentage express Nedd4. If for example a small proportion of phospho-proteins were not successfully precipitated from one of the cell lysates this may go unnoticed in the western blot of phospho-GyrB-Flt-1 but may make a considerable difference to the amount of phospho-Nedd4 detected. In conclusion these results suggest that Nedd4 may be phosphorylated under certain conditions but we are unable to unequivocally confirm that this phosphorylation is reduced upon activation of GyrB-Flt-1.

Phosphorylation of Nedd4 has been shown in resting mouse embryonic fibroblasts expressing exogenous Nedd4 and either Grb10 alone or Grb10 with the insulin receptor (IR) (Morrione et al., 1999). The stimulation of cells expressing IR, Grb10 and Nedd4 with insulin shows no change in the level of tyrosine phosphorylation of Nedd4 however insulin-like growth factor (IGF) stimulation of cells expressing Grb10 and Nedd4 shows an increase in tyrosine phosphorylation of Nedd4. Only a small fraction of Nedd4 is phosphorylated in resting cells or cells stimulated, with either stimulus, and only a small fraction of the phosphorylated Nedd4 associates with Grb10. In addition the amount of Grb10 that co-immunoprecipitates with Nedd4 does not change upon the stimulation of these cells. From these results Morrione et al propose that unphosphorylated Nedd4 binds preferentially to Grb10 and as insulin and IGF do not influence this association they suggest that it is phosphotyrosine independent. The Grb10 has been shown to serve as an adapter to form a bridge between Nedd4 and the IGF receptor to facilitate Nedd4-mediated receptor ubiquitination. It is also possible therefore that Grb10 could be used to recruit unphosphorylated Nedd4 to activated Flt-1 receptors in endothelial cells to facilitate receptor degradation. If Grb10 also prefers to associate with unphosphorylated Nedd4 then phosphorylation of Nedd4 may be used as an inhibitory mechanism to prevent its indirect association with Flt-1 and conversely dephosphorylation may promote this association and hence promote receptor degradation. If the decrease in Nedd4 phosphorylation upon stimulation of GyrB-Flt-1 can be verified then this provides a mechanism whereby dephosphorylation of Nedd4 promotes its association with a potential substrate. This situation would be contrary to that seen in EGF stimulated cells in which an increase in tyrosine phosphorylation of the Nedd4 family member Itch has been detected following EGF stimulation (Courbard et al., 2002).

The involvement of Nedd4 in GyrB-Flt-1 degradation is evident from figure 7.9, which shows that over expression of wild type Nedd4 increases the rate of degradation of the receptor. We

assume from this that Nedd4 ubiquitinates GyrB-Flt-1 and hence promotes its degradation however this study provides no evidence to suggest that the ubiquitin ligase activity of Nedd4 is essential to the receptor degradation. The apparent lack of inhibition of GyrB-Flt-1 degradation when Nedd4 C→S is co-expressed with the receptor, as shown in Figure 7.6, could be interpreted as an indication that the ligase activity of Nedd4 is not required for the degradation of GyrB-Flt. This interpretation may be false however and an explanation for this observation is apparent when considering the relative expression levels of the two proteins. Although both proteins are over-expressed the efficiency of adenoviral expression of GyrB-Flt-1 is much greater than that of the transfection method used to express Nedd4 C→S. Therefore the relatively small affect that Nedd4 C→S may have on the degradation of GyrB-Flt-1 is unlikely to be detected in this expression system.

The discovery that HECT E3 ligases and RING finger-containing ligases associate with each other has lead to the proposal that the two classes of proteins act cooperatively in ubiquitinating receptors and the accessory proteins required for their degradation (Magnifico et al., 2003). Although results thus far have implicated a role for Nedd4 in GyrB-Flt-1 degradation it is possible that Cbl activity may also contribute to this, especially as previously reports demonstrate that the Cbl/CD2AP complex is involved in VEGF receptor-1 ubiquitination and degradation (Kobayashi et al., 2004). Here we show that a small amount of Cbl protein is constitutively associated with GyrB-Flt-1 and the majority of protein is localised with the receptor in the same regions of the plasma membrane but we show no evidence of Cbl phosphorylation following activation of GyrB-Flt-1 or an increase in the rate of receptor degradation in cells expressing Cbl. It is possible that the PAE cells used in this study lack the CD2AP adapter protein and therefore phosphorylation and activation of Cbl is inhibited. The co-immunoprecipitation of the small amount of Cbl with GyrB-Flt-1 could therefore represent a basal level of association that is not sufficient to affect receptor degradation.

In summary we show that virally expressed GyrB-Flt-1 can be maximally phosphorylated by activation with coumermycin in PAE cells, and is degraded by a down-regulatory mechanism following sustained activation. Nedd4 is strongly implicated in the down-regulatory mechanism of GyrB-Flt-1 however the importance of its ligase activity has not yet been established. The relevance of the phosphorylation status of Nedd4 also requires further investigation and an alternative method is required to establish whether activation of GyrB-

Flt-1 mediates a change in the level of Nedd4 phosphorylation. In this cell system Cbl does not appear to participate in the degradation of GyrB-Flt-1 despite its association with the receptor. Co-expression of Cbl and CD2AP may however elucidate a cooperative role for Cbl and Nedd4 ubiquitin ligases in VEGF receptor-1 degradation.

Chapter 8

Using microscopy to study protein interactions in cells

Chapter 8 – Using microscopy to study protein interactions in cells

The use of GFP, a green fluorescent protein from the jellyfish, *Aequorea victoria*, has revolutionised the ability to study protein activities and interactions in single cells and correlate observations to changes in cell morphology, cell migration and their position within the cell cycle (reviewed in Lippincott-Schwartz et al., 2001; Wouters et al., 2001). The addition a fluorescent tag to a protein of interest is a unique tool that allows the study of protein chimeras in vivo, in real time and in living cells. The fluorescent tagging of proteins is advantageous because i) it provides a sensitive method of detection, ii) it is specific to the protein of interest and iii) introduction into cells is relatively non-invasive. When using chimeric proteins it is important however to establish that the protein function is not altered, especially in the case of enzymes such as receptor tyrosine kinases, and that the protein tag does not sterically hinder interactions with other proteins, especially in the case of adapter proteins. The use of biochemical techniques to compare chimeric and wild type proteins can provide confidence that these tagged proteins behave as they should.

By mutating key residues in GFP, proteins that emit light at different wavelengths can be created. The use of site directed mutagenesis to create blue-, yellow- and cyan-fluorescent proteins has therefore increased the number of fluorescent tags available. In addition further mutations have created enhanced GFP and GFP-derivatives, which are more resistant to photobleaching. The Dsred fluorescent protein from the sea anemone, *Discosoma striata*, and its greatly improved DsredII form, are also available. By engineering cells to express two or more proteins, tagged with different fluorophores, we can simultaneously visualise the cellular location of each of these proteins. In addition, the use of fluorescently tagged ligands can report on the localisation of receptors. Several complementary and/or alternative labelling techniques have been used to indirectly detect multiple proteins of interest. These often involve the use of antibodies conjugated to fluorescent dyes. For example, microinjection of fluorescently conjugated primary antibodies into living cells and immuno-labelling using fluorescently conjugated secondary antibodies to label proteins in fixed cells.

8.1 *Co-localisation studies*

Fluorescence microscopy has been used considerably in this study and by using a number of different approaches we have investigated early signalling events in VEGF stimulated cells. In the simplest of experiments the cellular localisations of GFP-tagged proteins or immuno-

labelled proteins were observed in serum starved and stimulated cells. More informative data was obtained however by observing dual fluorescent images of immuno-labelled proteins, such as VEGF receptors, with other GFP-tagged or immuno-labelled proteins that are implicated in VEGF receptor signalling or degradation. Some of this data provides evidence for the co-localisation, and hence the possible association, of adapters and ligases with VEGF receptors. It is often difficult however to make unambiguous conclusions from imaging a limited number of cells that may show varying degrees of protein co-localisation. The conclusions we have made in this study based on data derived from microscopy are generally supported by results from biochemical methods. Observations of co-localisation, for example, are supported by co-immunoprecipitation experiments. Conclusions from positive co-immunoprecipitation data do however have limitations. Although this indicates that two proteins can interact it does not necessarily mean that they do so *in vivo*. The procedure for these experiments involves cell lysis and the disruption of the organelles within them, so proteins that may be compartmentally separated in live cells are able to interact and associate in the total cell lysis. A complementary image of the two proteins localised to the same cellular structure(s) therefore reinforces the likelihood of their association in intact cells. An example shown in this study however highlights the importance of both techniques. Imaging studies of GFP-Sck, GFP-Sck Δ SH2 and GFP-Sck Δ PTB suggest that all three proteins can co-localise with VEGF receptor-2 at the plasma membrane. Co-immunoprecipitation experiments however show that only wild type Sck and Sck Δ PTB can associate directly with the receptor. Our interpretation of these results is that the SH2 domain mediates the direct interaction between Sck and VEGF receptor-2 and the binding of the PTB domain to phospholipids in the plasma membrane can localise Sck in the vicinity of VEGF receptor-2.

From the images we have shown, the co-localisation of proteins with VEGF receptors is apparent in membrane ruffles at the periphery of cells. These are regions of the cell at which the plasma membrane, and hence cell surface receptors, are particularly concentrated. The images presented here also show that VEGF receptor-2 is localised in punctuate structures across the whole of the cell surface. GFP-tagged adapter proteins are not obviously localised in the same structures however. This is likely to be due to the high level of GFP expression throughout the whole cell that may be masking proteins localised to punctate structures. To minimise the interference from intracellularly localised GFP tagged proteins deconvolution, a technique that uses the mathematical manipulation of digital images, can be applied to create clearer images of the membrane (McNally et al., 1999). In cells expressing high levels of

fluorescence a low number of out of focus components, from either above or below the plane of focus, build up to produce a flare of fluorescence that is detected in two-dimensional images (shown diagrammatically in Figure 8.1). This haze, created by the scattering of emitted light throughout the three-dimensional cell, is modelled during the deconvolution process. This involves taking images through the depth of a cell, at intervals of 0.1-0.2 μm , and applying a calculated point spread function that is based on the distribution of light intensity in the microscope system used. The out of focus light contamination in the digital image of each layer is then removed (volume deconvolution) or reassigned to the plane of focus from which it originated (3D restoration) using a mathematical algorithm. Fine details in individual two-dimensional images can be more clearly observed in deconvolved images of single focal planes and the combination of these images can produce a predicted three-dimensional model of the cell (Wallace et al., 2001).

Deconvolution microscopy was used in several experiments during this study in an attempt to minimise the fluorescence resulting from intracellularly localised GFP-tagged proteins and to visualise areas of the plasma membrane that were not contaminated by intracellular proteins. The endothelial cells in which we expressed these GFP-tagged proteins are large and, when three-dimensional models are created, appear to be very flat. The generation of single layers to study the plasma membrane therefore proved to be difficult and the deconvolved images obtained did not show any additional details to those observed in the raw images. The process of deconvolution requires a large amount of computer time and memory and this is especially true when we consider the large size of the endothelial cells used. Unfortunately the facilities we have available at present do not have enough memory for the 3D restoration of images obtained from cells of this size. This process however is preferential to volume deconvolution as it does not subtract any data from the image. After careful consideration we decided that the process of volume deconvolution is more likely to take away information from our images and therefore we have presented raw images for co-localisation studies. These have been digitally contrasted and enhanced to highlight key features and to manually remove background fluorescence only.

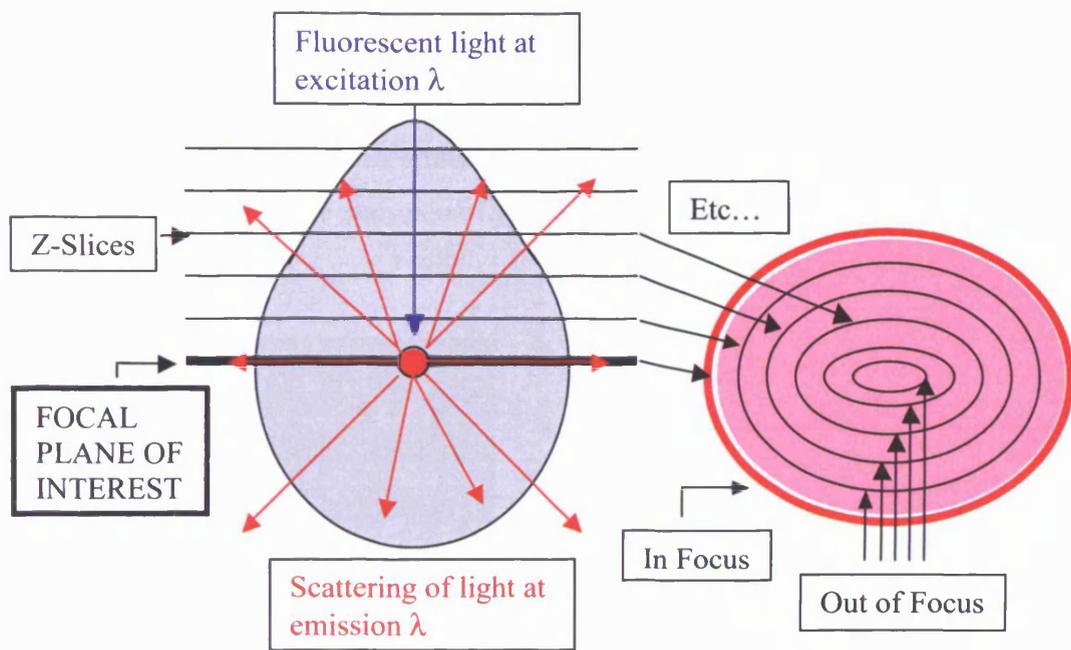


Figure 8.1 - Visualising fluorescent proteins causes light scattering within samples. Haze detected in images of fluorescent cells can be corrected using deconvolution however several images at z-slices throughout the sample must be taken for the out of focus light contamination to be calculated.

In studies in which the co-localisation of proteins is predicted a technique called fluorescence resonance energy transfer (FRET) microscopy can be used to confirm protein-protein interactions (van Roessel and Brand, 2002). FRET is defined as “a process by which radiation-less transfer of energy occurs from a fluorophore in the excited state to an adapter molecule in close proximity”. Efficient energy transfer is limited to fluorophores that are between 50 and 100 angstroms apart therefore the detection of a FRET signal is a good indicator of direct protein-protein interactions. The energy transfer does also depend on the relative orientation of the donor and acceptor so if two fluorophores are proximal but the orientation is unfavourable then FRET will not occur. The use of two fluorescently tagged proteins, each conjugated to a different derivative of GFP, provides a donating and accepting fluorophore as long as there is substantial overlap of the emission spectrum of the donor fluorophore with the absorption spectrum of the accepting fluorophore. The use of cyan fluorescent protein (CFP) as a donor fluorophore and yellow fluorescent protein (YFP) as an acceptor fluorophore has been studied previously (Sorkin et al., 2000) and will therefore be used as an example. In this system FRET is achieved by the excitation of CFP using light at a wavelength of 436 nm. This will result in the emission of light at 470 nm if YFP is not in

close proximity. If the distance between, and the orientation of, the two fluorophores is optimal then the energy from the excited CFP fluorophore will be transferred to YFP resulting in absorption of 470 nm light and emission of light at 535 nm by YFP. In summary excitation with light at 436 nm will result in either the emission of cyan fluorescent light if CFP and YFP are not co-localised or the emission of yellow fluorescent light if FRET occurs.

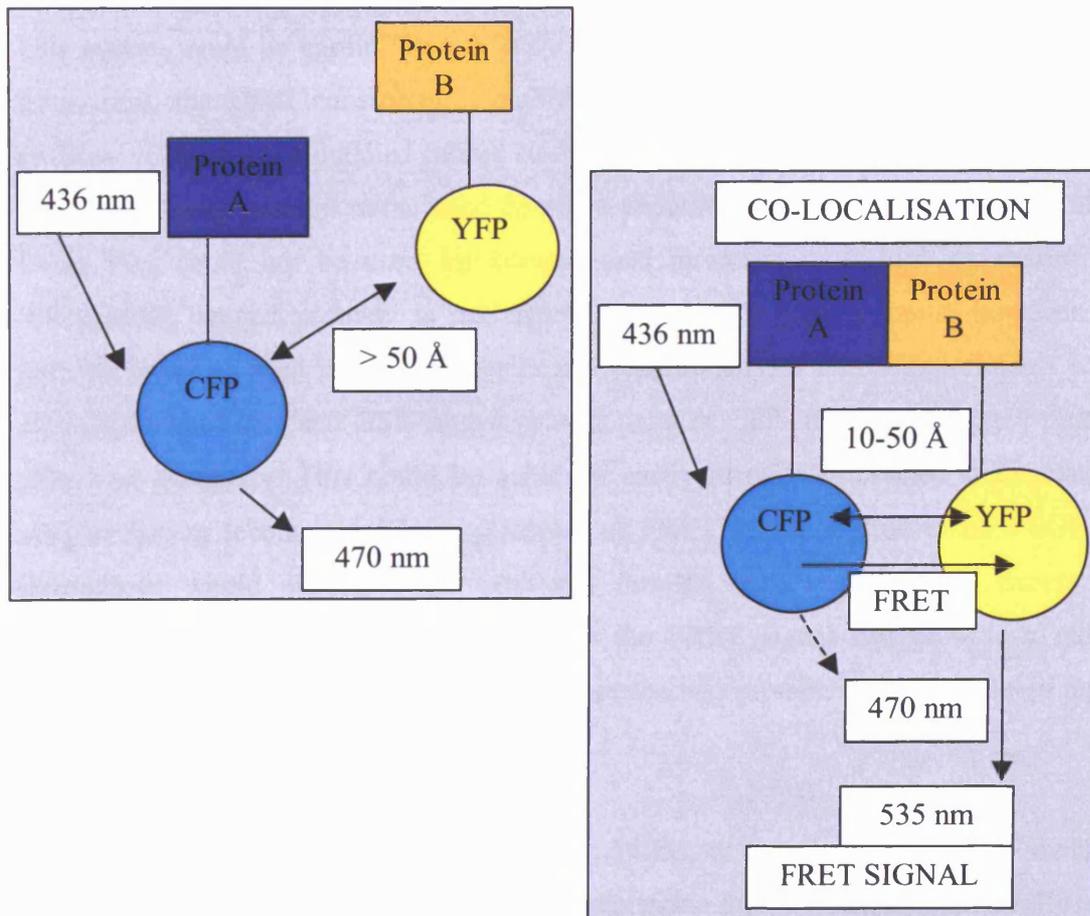


Figure 8.2 - A diagrammatic illustration of the transfer of energy that occurs during the FRET process. The overlap between the emission spectra of CFP and the excitation spectra of YFP allows the transfer of energy from CFP to YFP when the two fluorophores are in close proximity.

This system has been used to visualise the co-localisation of EGF-CFP and Grb2-YFP in living cells (Sorkin et al., 2000). EGF stimulation of PAE cells, lacking endogenous EGFR but expressing EGFR-CFP and transiently expressing Grb2-YFP, results in the recruitment of Grb2 to cellular compartments that contain EGFR-CFP. The co-localisation is evident as a large increase in the FRET signal amplitude, which in this case is observed at membrane

ruffles and in endosomes. In addition, the detection of FRET between Blue Fluorescent Protein (BFP) as a donor fluorophore and GFP as an acceptor fluorophore has been used to monitor the activity of Caspase 3, a protease that is important in the events leading to programmed cell death (Xu et al., 1998). In this study BFP and GFP are covalently linked together by a short peptide that contains a Caspase 3 recognition sequence. The loss of FRET is indicative of Caspase 3-mediated cleavage of the linker peptide.

This system could be applied to the study of interactions within VEGF receptor complexes. At present, the DNA constructs available in our lab encode for GFP- or DsredII-tagged proteins. Although not detailed in this study, GFP-tagged VEGF receptor constructs are also available. These proteins were used in early experiments however due to low expression levels they could not be used for conventional microscopy. A high expression level of fluorescently tagged proteins is not essential for FRET measurements however so these proteins could be used in future experiments. Additionally, if FRET experiments were to be attempted then GFP- and BFP-tagged protein pairs or CFP- and YFP- tagged protein pairs would be necessary. This could be achieved easily using site directed mutagenesis or by simple cloning techniques. The application of FRET in future studies of VEGF receptor interactions would eliminate the problems detailed here regarding an excess of GFP fluorescence. Additionally measurements of the FRET signal can provide a quantitative evaluation of the degree of co-localisation between two proteins in serum starved and VEGF stimulated cells.

Fluorescence Lifetime Imaging Microscopy, FLIM, is a technique that has derived from FRET and has also been applied in studies monitoring the interactions between fluorescently tagged molecules (Wouters and Bastiaens, 1999). In this technique measurements of the emission fluorescence lifetime of the donor (CFP in figure 8.2) are made. This parameter differs depending on whether or not FRET occurs i.e. in the absence of FRET a greater fluorescence lifetime is detected for the donor fluorophore. The value of this parameter therefore represents two different molecular situations and measurements can be used to quantify imaging data. To determine the population of molecules in an associated or disassociated state the fluorescence lifetime of the donor fluorophore has to be determined for the two situations. This has been applied to the study of GFP-tagged EGF receptor phosphorylation (Verveer et al., 2000). In this case FRET occurs, during EGF receptor activation, by the transfer of energy from the donor fluorophore, GFP, to a Cy3 fluorescent

tag (the acceptor fluorophore) bound to a phosphotyrosine antibody. As FLIM only measures the emission fluorescence of the donor fluorophore the Cy3-antibody can be added in excess and the binding of this antibody to other phospho-proteins is irrelevant. The fluorescence lifetime of GFP is first measured in the presence of Cy3-labelled antibodies, which represents the state in which FRET can occur. Then to measure the fluorescence lifetime of GFP in the absence of FRET, the Cy3 fluorophore is photobleached, which abolishes FRET and the fluorescence lifetime of GFP is measured again. From these values the efficiency of FRET can be calculated, but in addition, because each of the two states have a unique GFP fluorescence lifetime, they can be used in “global analysis” to determine the populations of phosphorylated and unphosphorylated EGF receptors. This approach can also provide data that is more informative than the qualitative immunofluorescence images obtained in this study as it allows the determination of relative concentrations of phosphorylated or associated proteins in a quantitative manner.

8.2 *Detection of intracellular molecules*

Imaging techniques, using fluorescent reporter constructs as sensors of cellular protein activity or physiological state, have been applied to a wide range of studies. A common example, which uses FRET, is that of a calcium-sensing fusion protein. This is similar to the Caspase 3 activity sensor mentioned above except in this case calcium brings together fluorescently labelled calmodulin and the M13 calmodulin binding-domain to induce FRET (Miyawaki et al., 1997). Probes have also been designed to detect ATP, GTP or camp (reviewed in Wouters et al., 2001).

FRET however is not essential for all fluorescently labelled reporter proteins. Intracellular lipid second messengers can be detected using fluorescently tagged lipid-binding protein domains. Examples of these include the C1 domain of PKC that identifies diacylglycerol (Oancea et al., 1998), the PA domain of Raf that binds to phosphatidic acid (Rizzo et al., 2000) and PH domains, which associate with selective phosphoinositides (Haugh et al., 2000; Oatey et al., 1999). In this study we have used the full-length general receptor for 3'phosphoinositides, Grp-1, to report on the locations at which PtdIns (3,4,5) P₃ is produced by PI3K. Other techniques, such as thin layer chromatography (TLC) or high performance liquid chromatography (HPLC), which also measure the production of PtdIns (3,4,5) P₃, only provide information about its total cellular synthesis and mass. We generated DNA constructs to encode DsredII fusion proteins of full length Grp-1 and of the PH domain alone.

Surprisingly the PH domain of Grp-1 localised to the nucleus in serum starved and VEGF stimulated endothelial cells so it could not be used as a marker of PtdIns (3,4,5) P₃ concentrations at the plasma membrane. The DsredII-tagged Grp-1 protein however shows peripheral membrane localisation, which is likely to be mediated by its PH domain. The use of DsredII-Grp-1 was a successful reporter for the production of PtdIns (3,4,5) P₃ by PI3K in response to VEGF.

The use of immunofluorescently tagged antibodies to detect the incorporation of 5-Bromo-2'-deoxy-Uridine, BrdU, into newly synthesised DNA can report on the physiological state of cells. Using this technique we were able to identify actively proliferating cells. By choosing a microscope detection system we were able to make comparisons between serum-starved and VEGF stimulated cells and, more significantly, between those expressing GFP-tagged wild type and mutant Sck proteins. We believe that this approach is more sensitive than that of other biochemical methods, such as incorporation of ³[H] thymidine, because we were able to consider only those cells expressing GFP-tagged proteins. This method was therefore advantageous in investigating a potential role for the phosphorylation of tyrosine residues in Sck after VEGF stimulation of endothelial cells.

8.3 *Real time re-localisation studies*

In studying VEGF receptor signalling we have investigated cellular events following receptor activation. In most experiments, that use both biochemical and microscopy techniques, we have applied a time course of ligand treatment to capture the status of populations of cells at various points after receptor stimulation. Rapid changes, to protein localisation and/or protein activity, at any point within the time course, are unlikely to be detected by methods such as western blotting and fixed cell imaging however. Real time microscopy, of live cells expressing fluorescently tagged proteins, does provide a method by which we can study both rapid and sustained events following receptor activation. This method, like the time courses used in other experiments, is of course discontinuous and we are unable to comment on the events that occur in between time points. With real time microscopy however we are able to capture images within time intervals as short as a second. By the generation of movies from these individual images protein complexes can be tracked in frames that are close to real time. An additional advantage of live cell imaging is that the physiological environment in which the proteins are visualised has not been disrupted. The lysis or fixation of cells that is required for other protein detection methods could introduce changes to cell structure and/or proteins

status. Furthermore, unlike methods that consider a population of cells at a given time point, results from real time imaging can compare changes in individual cells, before and after stimulation for example. This can provide valuable information, but if observations are inconsistent between different cells, or difficult to interpret, it can be time consuming to analyse multiple cells, especially if the expression frequency of fluorescently tagged proteins is low.

Several studies have used live cell imaging to visualise the relocalisation of proteins in response to cellular stimuli. Matsuda et al measured the relative intensities of plasma membrane, and intracellularly localised, red fluorescently tagged-PLC γ 1 and PLC γ 2, from images taken of individual A431 cells before and after EGF stimulation (Matsuda et al., 2001). Kinetic analysis of this data clearly shows that these proteins translocate to the membrane and create a distinct ring-like pattern around the whole periphery of the cell. Gillham et al show that during activation of an EGF receptor/ErbB3 receptor chimera GFP-tagged p85 proteins cluster and form patches at the cell surface (Gillham et al., 1999). In this case rhodamine red labelled EGF is used as the cell stimulus, and as a marker for EGF receptors, allowing the comparison of the localisation of GFP-p85 to that of activated receptors. A similar approach used by Carter et al shows that texas red labelled EGF and GFP-tagged EGF receptors co-localise, and endocytic vesicles containing these proteins rapidly migrate towards a perinuclear compartment (Carter and Sorokin, 1998). They also observe that vesicles containing GFP-EGF receptor swell and fuse together. The authors of this study claim that the real time optical analysis of vesicle trafficking in individual cells allows the visualisation of rapid dynamics that would not be preserved in chemically fixed and permeabilised cells.

In this study we provide an example of GFP-p85 clustering in response to activation of ErbB4/HER4, a member of the EGF receptor family, by its ligand heregulin. We were advised that activation of this system results in an obvious clustering of GFP-p85, and was therefore used as a positive control for the subsequent study of PI3K signalling in response to VEGF receptor activation. In our cell system GFP-p85 is mainly localised to focal adhesions and we were unable to show any obvious signs of its relocalisation in response to VEGF stimulation. We therefore investigated the product of PI3K activation and were able to show, using real time imaging that punctate clusters of DsredII-tagged Grp-1 appear to shuttle to and from the membrane. Although this suggests that PI3K is active in these cells the movie

showing Grp-1 dynamics does not convincingly show an overall change in Grp-1 localisation or the formation of distinct structures. This illustrates that real time imaging is not always constructive. If events, such as protein relocalisation or the formation of cellular structures, are obvious then this technique can provide dynamic information that is difficult to obtain by other methods. If however, only random movements are visualised, which cannot be correlated to other cellular events, then limited conclusions can be made.

8.4 *Summary of the microscopy used in this study*

Overall, the use of microscopy in this study has provided some valuable data that in conjunction with biochemical methods has led to the proposal of several conclusions. The observations made from the localisation of Grp-1 and the results obtained from labelling BrdU in newly synthesised DNA would have been difficult to generate by other methods. The co-localisation studies of Shc, Sck and VEGF receptor-2 are supportive of co-immunoprecipitation results and, although the localisation of Sck Δ SH2 and Sck Δ PTB did correlate entirely with the co-immunoprecipitation data, it provided evidence that the PTB domain of Sck is required for localisation to the plasma membrane. Future work using FRET and FLIM may reinforce conclusions regarding the association between VEGF receptors and adapter proteins or ligases. These techniques are superior to co-immunoprecipitation studies as they can confirm direct protein-protein associations in intact cells. Unfortunately in the real time imaging experiments, using Grp-1 as a reporter for phospholipid production, we could not make any clear conclusions with regards to the reason for this relocalisation. This approach is still potentially very useful, although imaging of multiple proteins may clarify the apparently random trafficking of a single tagged protein. In conclusion, fluorescence microscopy allows scientists to investigate protein interactions and activities *in vivo* and in real time, which is a valuable tool in studies of signal transduction mechanisms.

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