Signalling pathways downstream of vascular endothelial growth factor receptors

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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Department of Biochemistry
University of Leicester

September 2001

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Abstract

Emma L. Knight

Vascular endothelial growth factor (VEGF) is an essential angiogenic factor for formation of the embryonic vasculature, and also has important roles in pathological conditions such as diabetic retinopthy, rheumatoid arthritis and cancer. Although the signalling pathways induced by VEGF have been well-studied, the precise molecular mechanisms remain to be determined, particularly with respect to the relative contribution of the individual receptors.

Two main VEGF receptors are expressed in endothelial cells: VEGFR-1 and VEGFR-2. To identify novel effectors downstream of these receptors, their intracellular domains were used as bait to screen yeast two-hybrid cDNA libraries. The association of potential effector proteins with the VEGFRs were assessed in both yeast and mammalian cells, and the identity of the interacting residues was probed using site-directed mutagenesis and peptide competition experiments. These data revealed previously unreported interactions betweeen VEGFRs and WW domain-containing proteins – interactions that could have important implications for the mechanisms regulating VEGFR expression.

Analysis of phosphorylation-dependent interactions downstream of VEGFR-1 in mammalian cells, which is generally hindered by the lack of ligand-induced receptor phosphorylation, was enabled by the development of chimeric receptors. In these constructs, the intracellular domains of the VEGFRs were fused downstream of the dimerization domain of the GyrB subunit of bacterial DNA gyrase. The chimeric receptors were significantly activated in response to ligand, thereby enabling studies of their signalling with the putative effectors PKB/Akt, PLCγ and ERK1/2. Both chimeric receptors were able to activate these effectors. In addition, the observed dependence of some of these effects on a specific tyrosine residue within VEGFR-2 suggested that the chimeric receptors couple to downstream effectors in a manner analogous to that of the full-length receptors.

Acknowledgements

I would like to thank Sally Prigent for giving me the opportunity to do a research project in such an exciting field, and for irreplaceable help throughout my PhD. I also thank other members of the laboratory for their help and support, not only during my time in the lab but also throughout my writing-up period, as well as many members of the Biochemistry department and my committee members Nick Brindle and Bill Brammar. Thanks also to the BBSRC for supporting my research financially.

A huge thanks must undoubtedly go to my mum, who has not only witnessed my ups and downs throughout this PhD, but has actually experienced them with me – I'm sure she's breathing a huge sigh of relief now it's finally over! Mum – I promise I'll never put you through anything like this ever again! (I'll stick to canoeing with hippos!)

I am also eternally grateful to the rest of my family and friends for their continuous support and encouragement – I'm really looking forward to spending more time with you all. I'd also like to take this opportunity to apologize to my sister for not taking my fair share of the strife that has rocked our family in recent years.

Finally, throughout this past year I have been striving to establish myself in a new full-time job while slaving away at my thesis most evenings and weekends. I would therefore like to thanks my new colleagues for their tremendous support and friendship, and also apologize for my apparent social ineptitude! Huge apologies also to Tanya, who has had to endure living with the most unsociable flatmate ever for the past 10 months – I will no longer turn down the offer of alcohol or a party!

Abbreviations

3-AT, 3-amino-1,2,4-triazole
AD, activation domain
AKAP, A (protein kinase A)-kinase anchoring protein
Akt/PKB, protein kinase B
Amp, ampicillin
Ang, angiopoietin
APS, ammonium persulfate
BAEC, bovine aortic endothelial cell
bp, base pair
BR3P, Balbiani ring 3 protein
BrdU, bromodeoxyuridine
BSA, bovine serum albumin
CAM, chorioallantoic membrane
cDNA, complementary DNA
Cou, coumermycin A_1
CSF, colony stimulating factor
D-AKAP, dual-specifity A-kinase anchoring protein
DAG, diacylglycerol
dATP, deoxy adenosine 5´ triphosphate
DBD, DNA-binding domain
ddNTP, dideoxy nucleotide triphosphate
DMEM, Dulbecco's modified Eagle medium
DMF, N,N-dimethyl formamide
DMPC, dimethyl pyrocarbonate
DMSO, dimethyl sulfoxide
DNA, deoxyribonucleic acid
DNAase/DNase, deoxyribonuclease
DNMT1, DNA methyltyransferase 1
dNTP, deoxy nucleotide triphosphate
DOC, deoxycholate
DOM, drop-out mix
DTT, dithiothreitol
EC, endothelial cell
ECL, enhanced chemiluminescence
EDTA, diaminoethanetetra-acetic acid (disodium salt)
EFGR, epidermal growth factor receptor
EGTA, ethylene glycol-bis)b-aminoethyl ether)-N, N, N'. N',-tetraacetic acid
ENaC, the amiloride-sensitive distal renal epithelial sodium channel

eNOS, endothelial nitric oxide synthase
Eph, ephrin receptor
ERK, extracellular signal-regulated kinase
EST, expressed sequence tag
EtBr, ethidium bromide
FA, formaldehyde agarose
FAK, focal adhesion kinase
FCS, fetal calf serum
FGF, fibroblast growth factor
Flk, fetal liver kinase
Flt, Fms-like tyrosine kinase
G-CSF, granulocyte colony stimulating factor
GAP, GTPase-activating protein
GST, glutathione S-transferase
GyrB, the B subunit of bacterial DNA gyrase
h, hour
HECT, homologous to the E6-AP carboxyl terminus
HEK, human embryonic kidney
HMVEC, human microvascular endothelial cell
HRP, horse radish peroxidase
HSC, haematopoietic stem cell
HUVEC, human umbilical vein endothelial cell
IAP, inhibitor of apoptosis
IL, interleukin
Ins(3,4,5)*P*3, inositol (3,4,5) trisphosphate
IP, immunoprecipitation
IPTG, isopropyl-b-D-thiogalactopyranoside
JNK, c-Jun N-terminal kinase
kb, kilobase
kDa, kilodalton
KDR, kinase insert domain containing receptor
LAT, linker for activated T cells
LB, Luria Bertani
LMP2A, latent membrane protein 2A
MAPK, mitogen activated protein kinase
min, minute
MMP, matrix metalloproteinase
mRNA, messenger RNA
NaAc, sodium acetate

ND, not determined
NE, no effect
Neo, neomycin
NFAT, nuclear factor of activated T cell
NIP, neuropilin-1-interacting protein
NLS, nuclear localization signal
NO, nitric oxide
NOS, nitric oxide synthase
NTP, nucleoside triphosphate
OD, optical density
ONPG, O-nitrophenyl-β-D-galactopyranoside
p45/NF-E2, erthryoid-specific transcription factor
PA, plaminogen activator
PAE, porcine aortic endothelial
PBS, phosphate buffered saline
PBST, phosphate buffered saline with Tween
PCR, polymerase chain reaction
PD-ECGF, platelet-derived endothelial cell growth factor
PDGF, platelet-derived growth factor
PECAM-1, platelet endothelial cell adhesion molecule 1
PEG, polyethylene glycol
PFIV, platelet factor IV
pH, percentage hydrogen ions
PI3K, phosphatidylinositide 3-kinase
PKA, protein kinase A
PKC, protein kinase C
PLC, phospholipase C
PlGF, placental growth factor
PMSF, phenyl methyl sulfonyl fluoride
PTB, phosphotyrosine binding domain
PtdIns(3)*P*3, phosphoinositide (3) monophosphate
PtdIns(3,5)*P*3, phosphoinositide (3,5) bisphosphate
PtdIns(3,4)*P*3, phosphoinositide (3,4) bisphosphate
PtdIns(3,4,5)*P*3, phosphoinositide (3,4,5) trisphosphate
PY, phosphotyrosine
RIα, type 1 regulatory subunit of protein kinase A
RIIα, type II regulatory subunit of protein kinase A
RNA, ribonucleic acid
RNase, ribonuclease

rpm, revolutions per minute
RTK, receptor tyrosine kinase
SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE, standard error
SEC, sinusoidal endothelial cell
sFlt, soluble Flt-1
SH, Src homolgy
SHP, SH2-containing phosphatase
SLP-76, SH2 domain containing leukocyte protein of 76 kDa
SRE, serum response element
TBS, tris-buffered saline
TBST, tris-buffered saline with Tween
TCR, T cell receptor
TEMED, N,N,N′,N′-tetramethyl ethylenediamine
TGF, transforming growth factor
TNF, tumour necrosis factor
UAS, upstream activating sequence
UTR, untranslated region
UV, ultraviolet
V, volts
VE-cadherin, vascular endothelial cadherin
VEGF, vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor
VPF, vascular permeability factor
VRAP, VEGF receptor associated protein
VRP, VEGF-related protein
WBC, white blood cell
WBP, WW domain binding protein
wt, wild-type
X-gal, 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside
YAP65, yes-associated protein of 65 kDa

Contents

Abstract 2
Acknowledgements 3
Abbreviations 4
Contents page 8
List of Figures 15
List of Tables 16

Chapter 1: General Introduction 17
1.1 Development of the vascular system 18
1.2 Pathological implications of misregulated angiogenesis 20
1.3 Angiogenic factors 20
1.4 Vascular endothelial growth factor 22
1.4.1 VEGF ligands 23
1.4.1 (i) VEGF-A 24
1.4.1 (ii) VEGF-B 26
1.4.1 (iii) VEGF-C 26
1.4.1 (iv) VEGF-D 27
1.4.1 (v) VEGF-E 27
1.4.1 (vi) PlGF 28
1.4.1 (vii) Regulation of *VEGF* gene expression 28
1.4.2 VEGF receptors 29
1.4.2 (i) VEGFR-1 30
1.4.2 (ii) VEGFR-2 31
1.4.2 (iii) VEGFR-3 32
1.4.2 (iv) Neuropilin-2 32
1.4.2 (v) Receptor variants 32
1.4.3 Angiogenic roles of the Tie receptors 33
1.4.4 Angiogenic roles of ephrins 34
1.5 Therapeutic manipulation of angiogenesis 35
1.5.1 Anti-angiogenic therapy 35
1.5.2 Pro-angiogenic therapy 37
1.6 VEGF-induced signalling cascades 38
1.6.1 Receptor tyrosine kinase effectors 38
1.6.1 (i) Cell proliferation 41
1.6.1 (ii) Apoptosis 42
1.6.1 (iii) Cell migration and intracellular trafficking 42
1.6.2 Activation of VEGFRs 1 and 2 43
1.6.3 Endothelial cell responses mediated through VEGFRs 44

1.6.3 (i) Cell proliferation 44
1.6.3 (ii) Vascular permeability 46
1.6.3 (iii) Cell migration 47
1.6.3 (iv) Cell survival 49
1.6.4 Responses mediated by VEGFRs in non-endothelial cell types 50
1.6.5 Molecular signalling through VEGFRs 1 and 2 50
1.6.5 (i) PLCγ 51
1.6.5 (ii) PI3K 51
1.6.5 (iii) Other SH2-containing effectors 52
1.6.5 (iv) ERK1/2 53
1.6.5 (v) Summary and objectives 54

Chapter 2: General materials and methods 56
2.1. Suppliers of reagents 57
2.2 General materials 57
2.3 General methods 58
2.3.1 Common buffers 58
2.3.2 DNA manipulations 58
2.3.2 (i) DNA purification 58
2.3.2 (ii) Restriction digests of DNA 58
2.3.2 (iii) Phenol–chloroform extraction 58
2.3.2 (iv) Ethanol precipitation of DNA 59
2.3.2 (v) Alkaline phosphatase treatment 59
2.3.2 (vi) DNA ligations 59
2.3.2 (vii) Separation of DNA by agarose gel electrophoresis 59
2.3.2 (viii) Extraction of DNA from agarose gels 59
2.3.2 (ix) Standard DNA minipreps 60
2.3.2 (x) Midi-DNA preps: the polyethylene glycol (PEG) method . 60
2.3.2 (xi) Synthesis of primers 61
2.3.2 (xii) Amplification of DNA by the polymerase chain reaction. 61
2.3.2 (xiii) Double-stranded DNA sequencing 61
2.3.2 (xiv) Site-directed mutagenesis 62
2.3.3 Protein manipulations 62
2.3.3 (i) Sodium dodecyl sulfate polyacrylamide gel electrophoresis 62
2.3.3 (ii) Electro-transfer of proteins to nitrocellulose 63
2.3.3 (iii) Immunodetection of proteins bound to nitrocellulose membrane 63
2.3.3 (iv) Expression and purification of GST fusion proteins 64
2.3.4 Bacterial cell culture 65
2.3.4 (i) Luria-Bertani medium (LB) 65

2.3.4 (ii) Maintenance of *Escherichia coli* DH5α 65
2.3.4 (iii) Preparation of transformation-competent DH5α 65
2.3.4 (iv) Transformation of plasmid DNA into DH5α 65
2.3.5 Mammalian cell culture 66
2.3.5 (i) PAE cell medium 66
2.3.5 (ii) Maitenance of PAE cell lines 66
2.3.5 (iii) Freezing down cells for storage 66
2.3.5 (iv) Recovery of frozen cells 66
2.3.5 (v) Whole cell lysate preparations for SDS–PAGE 66
2.3.5 (vi) Protein precipitation with GST fusion proteins 67
2.3.6 Yeast two-hybrid assays 67
2.3.6 (i) YPD medium 67
2.3.6 (ii) Drop-out media 67
2.3.6 (iii) X-gal medium 68
2.3.6 (iv) Maintenance of *Saccharomyces cerevisiae* L40 68
2.3.6 (v) Preparation of transformation-competent L40 69
2.3.6 (vi) Transformation of plasmid DNA into competent L40 69
2.3.6 (vii) Preparation of yeast whole cell lysates for SDS–PAGE ... 69
2.3.6 (viii) Yeast two-hybrid growth assay 69
2.3.6 (ix) X-gal plate assay 69
2.3.6 (x) β-galactosidase filter assay 70
2.3.6 (xi) β-galactosidase solution assay 70
2.3.6 (xii) Protein assay 70

Chapter 3: Screening yeast two-hybrid cDNA libraries for novel effectors of VEGF receptors 71
3.1 Introduction 72
3.1.1 Effectors implicated in VEGF-induced signalling cascades 72
3.1.2 Sreening for protein–protein interactions: the yeast two-hybrid system 73
3.1.3 Enhancing the tyrosine phosphorylation of VEGFR-1 76
3.2 Materials and methods 78
3.2.1 Construction of a hMVEC cDNA library in pVP16 78
3.2.1 (i) DMPC-treating solutions 78
3.2.1 (ii) Extraction of total RNA from hMVEC lysates 78
3.2.1 (iii) RNA separation by formaldehyde-agarose gel electrophoresis 78
3.2.1 (iv) Purification of mRNA from total RNA 79
3.2.1 (v) Reverse transcription of mRNA into cDNA 79
3.2.1 (vi) Amplification of cDNAs 79
3.2.1 (vii) Ethidium bromide estimation of DNA concentration 79

3.2.1 (viii) Electroporation of the pVP16 library into ELECTROMAX DH12S™ competent cells 80
3.2.1 (ix) SOB medium 80
3.2.1 (x) Harvesting library DNA 80
3.2.2 Construction of bait plasmids for yeast two-hybrid assays 81
3.2.2 (i) Bait construction primers 81
3.2.2 (ii) Bait mutagenesis primers 81
3.2.3 Screening yeast two-hybrid cDNA libraries 81
3.2.3 (i) Library screen transformation 81
3.2.3 (ii) Isolation of plasmid DNA from positive transformants 82
3.2.3 (iii) Preparation of HB101 electrocompetent cells 82
3.2.3 (iv) Electroporation of plasmids into electrocompetent HB101 82
3.2.3 (v) M9 medium 82
3.2.3 (vi) Isolation of DNA from library plasmids in HB101 cells ... 83
3.2.3 (vii) Sequencing primers 83
3.3 Results 84
3.3.1 Construction of a cDNA library from hMVECs 84
3.3.1 (i) Extraction of total cellular RNA 84
3.3.1 (ii) Isolation of mRNA from total cellular RNA 84
3.3.1 (iii) Transcription of mRNA into double-stranded cDNA 84
3.3.1 (iv) Ligation of cDNAs into pVP16 and transformation into DH5α 85
3.3.2 Construction of bait proteins 87
3.3.3 Characterization of bait proteins 88
3.3.3 (i) Fusion protein expression and phosphorylation 88
3.3.3 (ii) Intrinsic transactivation properties of bait proteins 89
3.3.4 Library screening 91
3.3.4 (i) Introduction 91
3.3.4 (ii) Screening with RIα–VEGFR-1 91
3.3.4 (iii) Further characterization of identified clones 92
AKAPs 93
PLCγ 94
DNMT1 and Grb2 97
3.4. Discussion 98
3.4.1 Summary of library screening results 98
3.4.2 AKAPs 98
3.4.3 The VEGFR-1–PLCγ interaction 99
3.4.4 Significance of the VEGFR-1 interaction with Grb2 and DNMT1 101
3.4.5 Future screening potential 101

Chapter 4: Construction and characterization of cell lines expressing chimeric VEGFRs 102
4.1 Introduction 103
4.1.1 Limitations of current systems for studying VEGF signalling pathways 103
4.1.2 Using chimeras to study receptor-specific signalling cascades 104
4.2 Materials and methods 107
4.2.1 Construction primers 107
4.2.2 Kinase treatment of myc oligonucleotides 107
4.2.3 Stable transfections 107
4.2.4 Immunoprecipitation of proteins from PAE cells 108
4.2.5 BrdU incorporation assay 108
4.3 Results 110
4.3.1 Construction of chimeric receptor constructs 110
4.3.2 Generation of PAE cell lines stably expressing chimeric VEGFRs 112
4.3.3 Characterization of chimeric VEGFRs 112
4.3.3 (i) Ligand-induced autophosphorylation 112
4.3.3 (ii) Activation of ERK1/2 116
4.3.3 (iii) Stimulation of cell proliferation 117
4.3.3 (iv) Activation of PLCγ 120
4.3.3 (v) Activation of Akt/PKB 121
4.3.3 (vi) Responses mediated by a GyrB–VEGFR-2(Y1175F) mutant 121
4.4 Discussion 126
4.4.1 Use of chimeric VEGFRs in studying VEGF signalling cascades 126
4.4.1 (i) Ligand-induced autophosphorylation 126
4.4.1 (ii) Responses mediated by the chimeric VEGFRs 127
4.4.1 (iii) The mechanisms of chimeric VEGFR signalling 128
4.4.1 (iv) Future use of chimeric receptors 131

Chapter 5: Interactions between VEGFR-1 and SH2-domain-containing proteins 133
5.1 Introduction 134
5.1.1 The role of SH2 domains in intracellular signalling cascades 134
5.1.2 The role of 'novel' effectors in VEGF signalling cascades 135
5.1.2 (i) Grb2-like proteins and VEGFR signalling 135
5.1.2 (ii) Shc-like proteins and VEGFR signalling 137
5.2 Materials and methods 139
5.2.1 Maintenance of Jurkat T cells 139
5.2.2 Isolation of monocytes from a white blood cell preparation 139
5.2.3 Whole cell lysate preparations for SDS–PAGE 139

5.2.3 (i) Jurkats 139
5.2.3 (ii) Monocytes 139
5.3 Results 140
5.3.1 Interactions of SH2-containing proteins with VEGFRs in yeast 140
5.3.2 Precipiation of chimeric VEGFRs from PAE cells with GST–SH2 fusion proteins 142
5.3.3 Identification of VEGFR-1 residues responsible for binding SH2-domain-containing proteins 145
5.3.4 Coexpression of SH2-domain-containing proteins and VEGFR-1 in mammalian cells 147
5.4 Discussion 149
5.4.1 Interactions between VEGFR-1 and Grb2-like proteins 149
5.4.2 Interactions between VEGFR-1 and Shc-like proteins 151

Chapter 6: Interactions between VEGFR-1 and WW-domain-containing proteins 153
6.1 Introduction 154
6.1.1 The role of WW domains in intracellular signalling cascades 154
6.1.2 WW domains and VEGF signalling cascades 155
6.1.2 (i) Nedd4 155
6.1.2 (ii) YAP65 157
6.2 Materials and methods 159
6.2.1 Bait construction primers 159
6.2.2 Bait mutagenesis primers 159
6.2.3 Peptide synthesis 159
6.3 Results 160
6.3.1 Interaction of Nedd4 and YAP65 with VEGFRs in yeast 160
6.3.2 Precipitation of chimeric VEGFRs from PAE cells with Nedd4 and YAP65 GST fusion proteins 163
6.3.3 Identification of VEGFR-1 residues responsible for binding Nedd4 in yeast 165
6.3.4 Identification of VEGFR-1 residues responsible for binding Nedd4 and YAP65 GST fusion proteins 166
6.4 Discussion 169
6.4.1 Interaction of Nedd4 with VEGFR-1 169
6.4.2 Physiological relevance of the VEGFR-1–Nedd4 interaction 170
6.4.3 Relevance of YAP65 to VEGFR signalling 172

Chapter 7: Discussion 174

Appendices 180
Publications 181

Bibliography 182

List of figures

Chapter 1

1.1 The formation of blood vessels 19
1.2 The exons encoded by *VEGF* splice varaints 24
1.3 Structure of the VEGF receptors 30
1.4 Receptor specificity of VEGF ligands 33
1.5 Signalling pathways downstream of receptor tyrosine kinases 40

Chapter 3

3.1 Maps of pVP16 and pBTM116 yeast two-hybrid vectors 74
3.2 The yeast two-hybrid system 75
3.3 Isolation of total cellular RNA from hMVEC lysates 84
3.4 Amplification of cDNA library inserts 85
3.5 Restriction analysis of library inserts 87
3.6 Expression and phosphorylation of bait proteins 88
3.7 Transactivation properties of bait proteins 90
3.8 Putative translation product of the male testis cDNA clone isolated during a yeast two-hybrid library screen 93
3.9 PLCγ clones isolated from yeast two-hybrid screens 94
3.10 Expression and phosphorylation of VEGFR-1 mutant bait proteins 95
3.11 VEGFR-1 residues responsible for the interaction with PLCγ in yeast 96

Chapter 4

4.1 Strategy to dimerize VEGFRs using coumermycin-regulatable chimeras 106
4.2 pcDNA3/Neo vectors encoding the GyrB–VEGFR chimeras..... 111
4.3 Stable expression of the GyrB–VEGFR chimeras in PAE cells 112
4.4 Coumermycin-induced phosphorylation of chimeric VEGFRs 113
4.5 Coumermycin-induced phosphorylation requires receptor dimerization 114
4.6 Coumermycin-induced phosphorylation requires receptor kinase activity 115
4.7 Coumermycin-induced ERK1/2 activation 117
4.8 Coumermycin-induced DNA synthesis 119
4.9 PLCγ is phosphorylated downstream of GyrB–VEGFR activation 120
4.10 Akt is phosphorylated downstream of GyrB–VEGFR activation 121
4.11 Coumermycin-induced phosphorylation kinetics of a Y1175F GyrB–VEGFR2 mutant 123
4.12 ERK1/2 activation downstream of GyrB–VEGFR-2(Y1175) 124
4.13 Y1175 mutation in GyrB–VEGFR-2 ablates PLCγ but not Akt phosphorylation..... 125

Chapter 5

5.1 Domain structure of Grb2 and Gads 136
5.2 Domain structure of Shc-like proteins 137
5.3 Interaction of VP16–SH2 fusion proteins with VEGFRs in yeast 141
5.4 Precipitation of chimeric VEGFRs from PAE cells with GST–Shc SH2 fusion proteins 143
5.5 Precipitation of chimeric VEGFRs from PAE cells with GST–Grb2 SH2 fusion proteins 144
5.6 Identification of receptor residues responsible for interactions between Gads, Grb2, Sck and VEGFR-1 146
5.7 Coexpression of Gads and VEGFR-1 in monocytes 148

Chapter 6

6.1 Domain structure of Nedd4 proteins 155
6.2 Domain structure of YAP65 proteins 157
6.3 Interactions of VP16–WW fusion proteins with VEGFRs in yeast 161
6.4 sYAP and lYAP constructs are expressed in yeast 162
6.5 Precipitation of GyrB–VEGFR-1 from PAE cells with GST–WW fusion proteins 164
6.6 Expression of VEGFR-1 mutant bait proteins 165
6.7 Identification of receptor residues responsible for the interaction between Nedd4 and VEGFR-1 166
6.8 A PY-containing peptide competes with VEGFR-1 for binding Nedd4 168
6.9 VEGFRs contain a WW-like domain in their juxtamembrane domains 172

Chapter 7

7.1 Distinct signalling pathways mediated by GyrB–VEGFR-1 and GyrB–VEGFR2 176
7.2 Effectors linking GyrB–VEGFR-2 to ERK1/2 activation 177
7.3 Possible roles of Nedd4 and YAP65 with VEGFRs 178

List of tables

1.1 Characteristics of angiogenic factors 21
1.2 Amino acid identity of members of the PDGF family of cytokines 23
3.1 Optimization of vector: insert ligation before library construction 86
3.2 Clones identified from library screens 92

Chapter 1

General introduction

Chapter 1: General introduction

1.1: Development of the vascular system

Establishment of a highly intricate, well-organized vascular system is essential for successful growth and development. The majority of blood vessel growth occurs during embryogenesis, via two related processes known as vasculogenesis and angiogenesis (reviewed in Risau and Flamme, 1995; Risau, 1997). Vasculogenesis is defined as the formation of blood vessels by the *in situ* development of endothelial cells from angioblasts (committed endothelial cell precursors). This process accounts for the initial formation of blood vessels during embryogenesis and produces, for example, the extraembryonic vessels of the yolk sac and early vascular structures such as the heart and the dorsal aorta (Coffin and Poole, 1988). By contrast, angiogenesis refers to the sprouting of new capillaries from pre-existing vessels. Once the early vessels have been formed by vasculogenesis, angiogenesis takes over to become the predominant mechanism by which blood vessels form both during the later stages of embryonic development and throughout adulthood. Vessels produced by angiogenesis include the intersomitic arteries and those associated with organs (Coffin and Poole, 1988).

Vasculogenesis is initially induced, in response to signals from the endoderm, in the extraembryonic mesoderm of the yolk sac. This mesoderm differentiates into angioblasts and haematopoietic stem cells (HSCs; blood cell precursors), and these then assemble into blood islands – structures in which peripheral angioblasts surround central HSCs (Fig. 1.1). The angioblasts and HSCs then differentiate into endothelial and haematopoietic cells, respectively, and adjacent blood islands fuse to produce an endothelial-lined tube containing luminal haematopoietic cells. Growth and migration of the endothelial cells, along with continued blood island fusion, generates primary capillary plexi, which are then expanded and remodelled by angiogenesis. This process requires the initial dissolution of endothelial cell basement membranes and formation of a provisional matrix into which proliferating endothelial cells can extend filapodia and migrate. Cells behind the proliferating capillary tip adhere to each other and produce a new basement membrane. As the branching vasculature matures, pericytes and smooth muscle cells are recruited to the small and large vessels, respectively, to provide both structural support and a source of paracrine signals for the endothelial cells.

Blood vessel growth throughout embryogenesis is highly proliferative, reflecting the vascularization of developing tissues and organs. However, once organogenesis is complete, neovascularization becomes contrastingly rare (Engerman *et al.*, 1967). In adulthood, physiological angiogenesis is tightly regulated, being observed only periodically during the female reproductive cycle (Phillips *et al.*, 1990; Shweiki *et al.*, 1993) and wound healing (Brown *et al.*, 1992; Peters *et al.*, 1993).

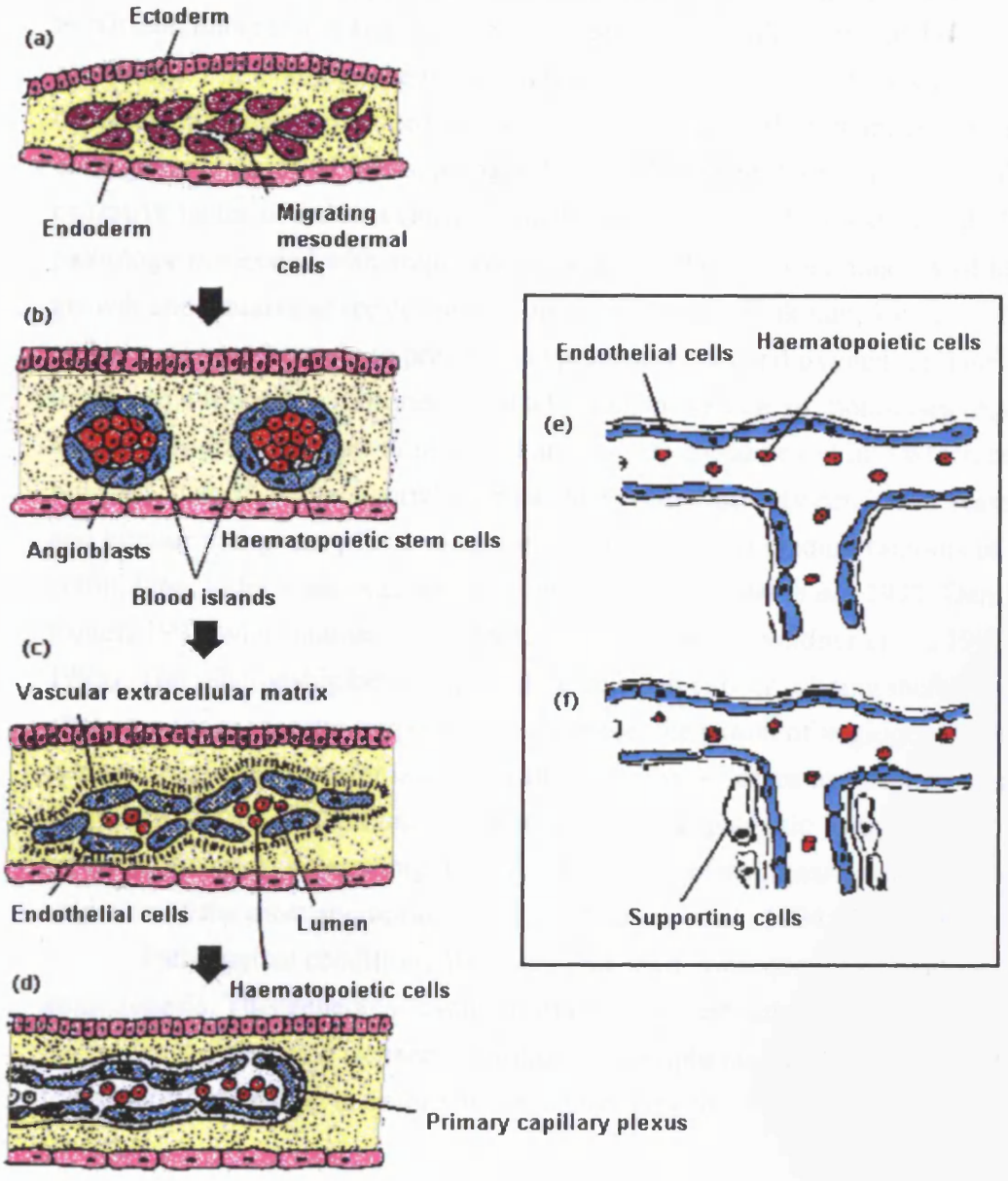


Fig. 1.1: The formation of blood vessels. During blood vessel formation, mesoderm differentiates into angioblasts and haematopoietic stem cells (a). These then assemble into blood islands (b) and differentiate into endothelial and haematopoietic cells, respectively (c). Adjacent blood islands fuse (c) which, together with endothelial cell growth and migration, produces primary capillary plexi (d). The vessels within these plexi are remodelled by angiogenesis (e), and supporting cells (smooth muscle cells and pericytes) are recruited (f)

1.2: Pathological implications of misregulated angiogenesis

Persistent, unregulated angiogenesis has been linked with several pathological conditions. For example, the invasion of the vitreous by new retinal capillaries can cause the blindness associated with diabetic retinopathy (Folkman, 1987), and angiogenic invasion of joints, along with the subsequent destruction of cartilage, is a causative factor of arthritis (Fava *et al.*, 1994). However, the most well-studied pathology associated with angiogenesis is cancer. For the vast majority of tumours, both growth and metastasis are dependent on angiogenesis (Folkman, 1990; 1992). Without an intimate blood supply to provide adequate nutrients and oxygen, tumour volume is limited to a few cubic millimeters. In addition, the release of metastases requires both a sufficiently sized primary tumour and an intimate blood vessel into which cells can be released. Indeed, a direct correlation has been observed between blood vessel density and tumour malignancy for a variety of solid tumours, including tumours of the breast, colon, lung, kidney and bladder (Albo *et al.*, 1994; Bosari *et al.*, 1992; Denijn and Ruiter, 1993; Macchiarini *et al.*, 1992; Toi *et al.*, 1993; Weidner *et al.*, 1991; 1992; 1993). The relationship between blood vessels and cancer is being increasingly exploited for diagnostic purposes. For example, the extent of angiogenesis in biopsy specimens of breast cancer is used as an indication of future metastatic risk (reviewed in Gasparini, 2001). In addition, the identities of the angiogenic factors found in the blood or urine of cancer patients might reflect the degree of malignancy and/or facilitate selection of the most appropriate therapy (Nguyen *et al.*, 1994; Watanabe *et al.*, 1992).

Pathological conditions also exist that are a consequence of inadequate angiogenesis. This generally results from oxygen deprivation and is seen, for example, in duodenal ulcers, and ischaemic cardio- and peripheral vascular diseases. In contrast to anti-tumour therapy therefore, these pathologies could benefit from proangiogenic therapy.

1.3: Angiogenic factors

The *in vivo* angiogenic potential of a given molecule is often assessed using either the chick chorioallantoic membrane (CAM) assay (Ausprunk *et al.*, 1974) or the rabbit corneal pocket assay (Gimbrone *et al.*, 1974). These assays quantify blood vessel growth towards an implanted disc that has been impregnated with the molecule whose angiogenic potential is under investigation. Such assays have identified many *in vivo* angiogenic factors, including low-molecular weight non-peptide factors (e.g. prostaglandins and nicotinamide), as well as polypeptide growth factors and cytokines (reviewed in Klagsbrun and D'Amore, 1991; Hamawy *et al.*, 1999; Heldin and Westermark, 1999; Liekens *et al.*, 2001). Examples of these angiogenic factors are given in Table 1.1.

Table 1.1: Characteristics of angiogenic factors

Angiogenic factor	*In vivo* angiogenesis	EC migration	EC proliferation	EC specific	Secreted
α and β FGF	+	+	+	–	–
VEGF-A	+	+	+	+	+
PDGF	+	+	+	–	+
PD-ECGF	+	+	NE	+	–
TGF-α	+	+	+	–	+
Angiogenin-1	+	+	NE	+	+
Angiotropin	+	+	NE	+	+
TGF-β	+	NE	–	–	+
TNF-α	+	NE	–	–	+
Angiopoietin-1	+	+	NE	+	+

Abbreviations: EC, endothelial cell; FGF, fibroblast growth factor; NE, no effect; ND, not determined; PD-ECGF, platelet-derived endothelial-cell growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

Many factors that have been shown to stimulate angiogenesis in *in vivo* assays directly stimulate endothelial cell proliferation and/or migration. Factors that fail to stimulate either of these responses directly [e.g. transforming growth factor (TGF)-β] might either stimulate them indirectly, through activation of another angiogenic factor, or directly stimulate an alternative endothelial-cell response required for angiogenesis. In the case of TGF-β, both of these strategies are used. This cytokine attracts monocytes, which then release angiogenic factors that act directly on the endothelial cells (Wahl *et al.*, 1987). In addition, TGF-β induces the differentiation of mesenchyme into smooth muscle cells and pericytes (Rohovsky *et al.*, 1996), and stimulates matrix deposition (Desmouliere *et al.*, 1993), both of which are crucial to the angiogenic response. With respect to the low-molecular weight factors, whether they are intrinsically angiogenic, or whether they enhance the angiogenic response by acting as cofactors and nutrients, remains to be determined.

The existence of numerous angiogenic factors, ranging from growth factors and their receptors to cell adhesion molecules and transcription factors, could suggest a certain degree of functional redundancy. However, targeted deletions of the individual genes encoding many of these factors [e.g. PDGF-B (Lindahl *et al*, 1997), TGF-β1 (Dickson *et al.*, 1995) and angiopoietin-1 (Suri *et al.*, 1996)] are embryonic lethal as a consequence of vascular defects, thereby implying that each factor has a crucial role in blood vessel formation that cannot be supported by the remaining factors.

The formation of an appropriate vascular network is regulated by the interplay between positive and negative factors. Currently characterized negative factors include

thrombospondin, TGF-β, tumour necrosis factor (TNF)-α, γ-interferon, protamine, platelet factor IV (PFIV) and angiostatic steroids such as cortisone (reviewed in Klagsbrun and D'Amore, 1991; Liekens *et al.*, 2001). The proposed mechanisms by which these factors inhibit angiogenesis include preventing the interaction between proangiogenic factors and their receptors (the mechanism adopted by PFIV), inhibiting endothelial cell migration and proliferation (TGF-β), and inducing capillary regression by degrading endothelial cell basement membranes (angiostatic steroids). The activities of one class of angiogenesis inhibitor require cleavage of the factors from inactive precursors. These inhibitors include angiostatin (O'Reilly *et al.*, 1994) and endostatin (O'Reilly *et al.*, 1997), which are internal fragments of plasminogen and collagen XVIII, respectively, as well as internal fragments of prolactin (Clapp *et al.*, 1993), PFIV (Maione *et al.*, 1990), fibronectin (Homandberg *et al.*, 1985), epidermal growth factor (EGF) (Nelson *et al.*, 1995), thrombospondin (Tolsma *et al.*, 1993), laminin (Grant *et al.*, 1989) and collagen XV (Ramachandran *et al.*, 1999). Studies specifically ablating the expression of plasminogen or collagen XVIII (Bugge *et al.*, 1995; O'Reilly *et al.* 1997) using targeted gene-deletion strategies have shown that these inhibitors are not essential for correct development of the embryonic vasculature. However, this type of inhibitor seems to have been preferentially chosen by tumours for the intrinsic regulation of tumour angiogenesis (O'Reilly *et al.*, 1994; 1997). Indeed, the growth of metastases that often accompanies surgical removal of a primary tumour (for examples see Gorelik, 1983; Himmele *et al.*, 1986) is believed to be associated with the concurrent loss of such inhibitory factors, previously produced by the primary tumour (reviewed in Folkman, 1998). It is also possible that these endogenous inhibitors function in the control of physiological angiogenesis during wound healing and menstruation.

1.4: Vascular endothelial growth factor

VEGF is the only known angiogenic factor that is secreted, endothelial-cell specific and able to induce both endothelial-cell migration and proliferation directly. Hence, VEGF could be the most important factor for the induction of angiogenic activities in endothelial cells. In addition, whereas the lethality associated with ablating the expression of most angiogenic factors, including PDGF-B (Lindahl *et al*, 1997), TGF-β1 (Dickson *et al.*, 1995), angiopoietin-1 (Suri *et al.*, 1996), vascular endothelial (VE)-cadherin (Carmeliet *et al.*, 1999a), tissue factor (Carmeliet *et al.*, 1996a) and the transcription factors COUP-TFII (Pereira *et al.*, 1999) and MEF2C (Bi *et al.*, 1999), is caused by defects in remodelling of the primary capillary plexus and recruitment of smooth muscle cells and pericytes, ablating the expression of VEGF receptor (VEGFR)-2 actually prevents blood vessel formation (Shalaby *et al.*, 1995). This

suggests that the crucial role of VEGF signalling in the formation of blood vessels precedes that of other angiogenic factors.

Besides the VEGFRs, the only other receptors for angiogenic factors whose expression is virtually exclusive to endothelial cells are Tie 2 (also known as Tek) and Tie 1 (Partanen *et al.* 1992, Dumont *et al.* 1992). Although targeted deletions of the genes encoding these receptors were embryonic lethal as a consequence of vascular defects, primary capillary plexi were formed (Sato *et al.*, 1995; Dumont *et al.*, 1994; Puri *et al.*, 1995), thereby suggesting that the crucial role of Tie and Tek signalling in angiogenesis succeeds that of VEGFR-2.

1.4.1: VEGF ligands

So far, five genes for VEGF (A–E) have been characterized which, along with placental growth factor (PlGF), form a subgroup within the platelet-derived growth factor (PDGF) family of cytokines (Heldin *et al.*, 1993). Members of this family are characterized by a cystine knot motif – a structure containing eight conserved cysteine residues, six of which participate in intramolecular disulfide bonds to maintain the subunit structure and two of which form intermolecular disulfide bonds that hold two subunits together as a dimer (McDonald and Hendrickson, 1993). The relative sequence identities of various growth factors within this family are shown in Table 1.2 (Oloffson *et al.*, 1996; Joukov *et al.*, 1996; Achen *et al.*, 1998; Ogawa *et al.*, 1998; Wise *et al.*, 1999; DiSalvo *et al.*, 1995; Tischer *et al.*, 1991). The complexity of the VEGF family is further increased by the existence of multiple splice variants.

Table 1.2: Relative amino acid identity between various members of the PDGF family of cytokines

	Amino acid identity (%)						
Growth factor	VEGF-A (%)	VEGF-B (%)	VEGF-C (%)	VEGF-D (%)	VEGF-E (%)	PlGF (%)	PDGF (%)
VEGF-A	-	43	30	31	25–43	46	18–20
VEGF-B	-	-	27	28	34	30	20
VEGF-C	-	-	-	48	24	25	22–24
VEGF-D	-	-	-	-	27	32	ND
VEGF-E	-	-	-	-	-	20–34	15
PlGF	-	-	-	-	-	-	18–20

Abbreviations: ND, not determined; PDGF, platelet-derived growth factor; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

1.4.1 (i) VEGF-A

VEGF-A_{165} (generally referred to as VEGF throughout the literature and the rest of this thesis) was the first VEGF family member to be characterized, originally as a vascular permeability factor (VPF) in tumour ascites fluids (Senger *et al.*, 1983), and later as an endothelial cell-specific growth factor secreted from folliculo stellate cells (Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989). Five mRNA transcripts, encoding polypeptides of 121, 145, 165, 189 and 206 amino acids in length, are produced by alternative splicing of the *VEGF* gene (reviewed in Neufeld *et al.*, 1999) (Fig. 1.2).

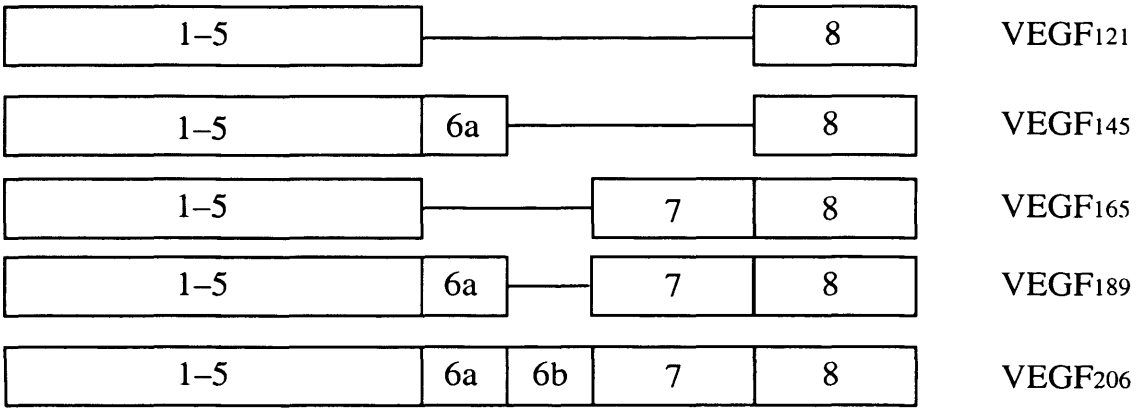


Fig. 1.2: The exons encoded by *VEGF* splice variants. Of the eight exons encoded by the *VEGF* gene, exons 6 and 7 are subject to alternative splicing, yielding five isoforms of 121, 145, 165, 189 and 206 amino acids in length.

Of the eight exons, those subject to alternative splicing are exons six and seven, both of which are highly basic in charge: 50% and 20%, respectively (Tischer *et al.*, 1991). Such basicity is characteristic of heparin-binding domains. In support of such a role, all VEGF isoforms except $VEGF_{121}$, which lacks both exons six and seven, can bind heparin (reviewed in Neufeld *et al.*, 1999).

The ability of growth factors to bind heparin has been associated with their solubility upon secretion: instead of diffusing away from the cell, a heparin-binding growth factor can be retained at the cell surface through interaction with heparan sulfate-containing proteoglycans in the extracellular matrix (Rusnati and Presta, 1996). Such retention represents a mechanism by which to control growth factor availability and activity, with the stored growth factors being activated by proteolytic release from the extracellular matrix when required. Alternatively, heparin-binding growth factors might be active while anchored, stimulating receptors expressed on adjacent cells. In the case of VEGF, all five isoforms are secreted, although the longer the isoform, the more tightly the protein remains attached to the extracellular matrix upon secretion (reviewed

in Neufeld *et al.*, 1999). However, even the anchored forms of $VEGF_{189}$ and $VEGF_{206}$ have been reported to stimulate endothelial cell proliferation (Park *et al.*, 1993).

The binding of growth factors to heparin has also been proposed to increase the affinity of, and even be essential for, the interaction between a growth factor and its receptor (Yayon *et al.*, 1990). However, although initial experiments with VEGF reported an enhanced binding of the growth factor to VEGFR-2 in the presence of heparin (Gitay-Goren *et al.*, 1992; Tessler *et al.*, 1994; Keyt *et al.*, 1996), this has since been attributed to heparin-mediated restoration of receptor-binding ability, which had been lost by VEGF oxidation during the iodination process (Gitay-Goren *et al.*, 1996). Such heparin-mediated protection might be important *in vivo* during wound healing, hypoxia-induced angiogenesis and inflammation; that is, in situations that produce oxidants and free radicals.

All five *VEGF* splice variants induce endothelial cell proliferation (Houck *et al.*, 1991; 1992; Poltorak *et al.*, 1997; Park *et al.*, 1993), although some studies have reported that $VEGF_{121}$ has a slightly weaker mitogenic potency than $VEGF_{165}$ (Keyt *et al.*, 1996). In addition, although some studies have suggested that the bioactivity of $VEGF_{189}$ and $VEGF_{206}$ requires their proteolytic release from the extracellular matrix (Plouet *et al.*, 1997; Houck *et al.*, 1992), others have shown that the membrane-bound versions of these factors are also active (Park *et al.*, 1993).

Expression of VEGF coincides, both temporally and spatially, with periods of angiogenic activity. During embryogenesis, expression is high in regions associated with angiogenesis; for example, the developing brain (Breier *et al.*, 1992, 1995; Flamme *et al.*, 1995). However, once organ vascularization has ceased (i.e. in adulthood), this expression decreases dramatically, being upregulated only transiently during wound healing (Brown *et al.*, 1992; Peters *et al.*, 1993) and the female reproductive cycle (Phillips *et al.*, 1990; Shweiki *et al.*, 1993).

$VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are often simultaneously expressed, with $VEGF_{121}$ and $VEGF_{165}$ being the most abundant forms (Bacic *et al.*, 1995; Neufeld *et al.*, 1996). Some tissue preference has been observed for the expression of these isoforms; for example, $VEGF_{189}$ is predominantly expressed in heart and lung, $VEGF_{165}$ in brain and kidney, and $VEGF_{121}$ in kidney and lung (Bacic *et al.*, 1995). By contrast, the expression of $VEGF_{206}$ (Houck *et al.*, 1991) and $VEGF_{145}$ (Poltorak *et al.*, 1997; Charnock-Jones *et al.*, 1993; Cheung *et al.*, 1995) appears to be restricted to embryonic and reproductive tissues, respectively.

Targeted deletion of the *VEGF* gene showed that even heterozygous knockouts were embryonic lethal, dying *in utero* between E8.5 and E11 (Ferrara *et al.*, 1996; Carmeliet *et al.*, 1996b). This implies that the regulation of embryonic blood vessel development by VEGF is tightly dose-dependent. The observed lethality was a consequence of severe vascular defects, resulting from the formation of immature and

disorganized blood vessels. The subsequent generation of mice null for only the 164 and 188 amino acid isoforms, demonstrated that $VEGF_{120}$ was unable to compensate for the loss of these factors: the mice died as a consequence of ischaemic cardiomyopathy during embryogenesis (Carmeliet *et al.*, 1999b).

1.4.1 (ii) VEGF-B

In 1996, a partial cDNA clone with homology to *VEGF* was isolated from a yeast two-hybrid library screen, and the corresponding protein, an endothelial cell-specific mitogen, was named VEGF-B (Olofsson *et al.*, 1996a). The gene encoding VEGF-B was subsequently shown to generate two transcripts by alternative splicing, encoding polypeptides of 167 and 186 amino acids (Olofsson *et al.*, 1996b). Because this splicing event enforces a frameshift on nucleotides C-terminal to exon 5, the two VEGF-B isoforms have distinct C-termini. The C-terminus of $VEGF\text{-}B_{167}$ is highly basic, can interact with heparin and remains anchored to the extracellular matrix upon secretion (Olofsson *et al.*, 1996a). By contrast, the C-terminus of $VEGF\text{-}B_{186}$ is only weakly basic, instead being rich in proline, serine and threonine residues (Olofsson *et al.*, 1996b). Unlike $VEGF\text{-}B_{167}$, $VEGF\text{-}B_{186}$ is secreted as a soluble factor. Both isoforms are expressed in a wide variety of tissues, often alongside VEGF (Olofsson *et al.*, 1996a; 1996b). In addition, their expression is particularly abundant in myocytes (Olofsson *et al.*, 1996a). The coexpression of VEGF and VEGF-B in many tissues has led to the suggestion that VEGF–VEGF-B heterodimers, which have been observed *in vitro* (Olofsson *et al.*, 1996a; 1996b), might also be relevant *in vivo*. Preventing VEGF-B expression by specifically deleting the corresponding gene did not preclude vascular development, although the embryos did have smaller hearts than their wild-type counterparts (Aase *et al.*, 2001; Bellomo *et al.*, 2000).

1.4.1 (iii) VEGF-C

Identification of an EST (expressed sequence tag) clone with homology to VEGF led to the cloning of full-length VEGF-related protein (VRP) from a human glioma cDNA library (Lee *et al.*, 1996). Simultaneously, VEGF-C, which was later shown to be identical to VRP, was isolated as the factor in conditioned media of PC-3 prostatic adenocarcinoma cells that was responsible for VEGFR-3 activation (Joukov *et al.*, 1996). VEGF-C does not possess the highly basic domains found in VEGF and VEGF-B family members, but instead has a unique C-terminal cysteine-rich region (Joukov *et al.*, 1996). This domain contains four copies of a motif similar to a cysteine-rich motif found in the Balbiani ring 3 protein (BR3P), a major protein of the larval saliva of the midge *Chironomus tentans*. These motifs have been suggested to compensate for the absence of heparin-binding domains, mediating interactions between VEGF-C and either the extracellular matrix or the cell membrane upon secretion;

indeed, VEGFR-3 was shown to bind to the membranes of cells expressing VEGF-C (Lee *et al.*, 1996).

Also unlike the previously identified VEGF family members, VEGF-C is synthesized as a precursor with N- and C-terminal extensions (Joukov *et al.*, 1997). Although these terminal propeptides can be completely removed following the secretion of dimeric precursor proteins (Joukov *et al.*, 1996; 1997), wild-type VEGF-C molecules are usually partially processed (Joukov *et al.*, 1996; 1997).

VEGF-C has been shown to induce vascular permeability, and the migration and proliferation of endothelial cells (Lee *et al.*, 1996; Joukov *et al.*, 1996; 1997; Witzenbichler *et al.*, 1998; Cao *et al.*, 1998). In addition, this growth factor enhanced both vasculogenesis and angiogenesis in *in vitro* and *in vivo* assays (Hamada *et al.*, 2000; Pepper *et al.*, 1998; Witzenbichler *et al.*, 1998). However, VEGF-C preferentially promotes the growth of lymphatic, rather than vascular, endothelia (Jeltsch *et al.*, 1997; Oh *et al.*, 1997). Consistent with this observation, during embryogenesis the expression of VEGF-C is particularly predominant in regions associated with either an abundance, or the sprouting, of lymphatic vessels (Kukk *et al.*, 1996). Expression is maintained throughout adulthood, predominantly in the lung, heart and kidney (Lee *et al.*, 1996; Kukk *et al.*, 1996; Joukov *et al.*, 1996).

1.4.1 (iv) VEGF-D

Human VEGF-D was identified from database searches on the basis of its homology with other VEGF proteins (Achen *et al.*, 1998). VEGF-D resembles VEGF-C in that it contains N- and C-terminal extensions that are subject to proteolytic cleavage (Achen *et al.*, 1998). In addition, the C-terminal domain, similar to that of VEGF-C, contains BR3P-like cysteine-rich motifs.

VEGF-D potently induces the proliferation of endothelial cells (Achen *et al.*, 1998). Despite the ability to bind both VEGFRs 2 and 3, VEGF-D was observed to stimulate the proliferation of lymphatic endothelia in preference to that of vascular endothelia (Wilting *et al.*, 1996). The expression of this factor is relatively low during early embryogenesis, and a marked upregulation, specifically in embryonic lung, is observed as embrogenesis proceeds (Farnebo *et al.*, 1999). VEGF-D is expressed in several adult tissues, but is particularly abundant in heart, lung, skeletal muscle, colon and small intestine (Achen *et al.*, 1998; Yamada *et al.*, 1997).

1.4.1 (v) VEGF-E

The fifth gene encoding a VEGF-like protein, VEGF-E, was discovered in Orf viruses on the basis of its sequence similarity with $VEGF_{121}$ (Lyttle *et al.*, 1994; Cottone *et al.*, 1998; Rziha *et al.*, 1998). This viral gene is thought to have derived from either the *VEGF* gene, or an as yet unidentified VEGF family member (Wise *et al.*, 1999).

VEGF-E polypeptides do not possess basic heparin-binding domains and cannot bind heparin (Ogawa *et al.*, 1998). They also lack the N- and C-terminal extensions found in VEGF-C and -D (Wise *et al.*, 1999). Instead, these proteins have a C terminus rich in threonine and proline residues (Meyer *et al.*, 1999).

VEGF-E proteins induce microvascular permeability, and endothelial cell proliferation and migration, with a similar potency to $VEGF_{165}$ (Ogawa *et al.*, 1998; Meyer *et al.*, 1999).

1.4.1 (vi) PlGF

PlGF was isolated from a placental cDNA library on the basis of its homology with VEGF (Maglione *et al.*, 1991). Three splice variants of PlGF exist, PlGF-1, -2 and -3, encoding polypeptides with 131, 152 and 201 amino acid residues, respectively (Cao *et al.*, 1996a; 1997). Of these, only PlGF-2 contains a basic exon, and only this isoform can bind heparin.

PlGF has been reported to stimulate endothelial cell proliferation and migration, angiogenesis and blood vessel permeability (Sawano *et al.*, 1996; Ziche *et al.*, 1997a; Midgal *et al.*, 1998). However, other groups found either only a weak induction of proliferation (Maglione *et al.*, 1991; Hauser and Weich, 1993), or even that PlGF could only act to potentiate the responses induced by low levels of VEGF (Park *et al.*, 1994). PlGF was also shown to induce the migration of monocytes (Clauss *et al.*, 1996). Interestingly, the rat GS-9L glioma cell line has been shown to secrete VEGF–PlGF heterodimers, as well as both types of homodimer, and the endothelial mitogenicity induced by these heterodimers was comparable to that induced by VEGF homodimers (DiSalvo *et al.*, 1995). In addition, PlGF–VEGF heterodimers have been shown to have *in vivo* angiogenic activity (Cao *et al.*, 1996a; 1996b).

The expression of PlGF is restricted to placental tissue, trophoblastic tumours and cultured human endothelial cells (Maglione *et al.*, 1991; 1993; Hauser and Weich, 1993). Interestingly, targeted deletion of the gene encoding PlGF did not affect embryonic angiogenesis, although a compensatory increase in VEGF levels was observed in these $PLGF^{-/-}$ embryos (Carmeliet *et al.*, 2001). However, the angiogenesis associated with ischaemia, inflammation, wound healing and cancer was impaired, suggesting an important role for this factor in pathological, rather than physiological, angiogenesis. In support of a role for PlGF in enhancing VEGF-induced responses, VEGF-induced migration, growth and survival were all impaired in endothelial cells null for PlGF.

1.4.1 (vii) Regulation of VEGF *gene expression*

Many factors have been reported to induce expression of the *VEGF* gene, including cytokines such as TGF-β and PDGF, hormones, and oncogenes such as the activated

forms of Src and Ras (Mukhopadhyay *et al.*, 1995; Rak *et al.*, 1995; Detmar *et al.*, 1994). In addition, hypoxia has been consistently shown to upregulate *VEGF* both *in vitro* and *in vivo*. This upregulation occurs at two distinct levels: hypoxia increases the transcription of *VEGF* via binding of hypoxia-inducible factor-1 (HIF-1) to a hypoxia response element (HRE) in the *VEGF* promoter, and also the stability of *VEGF* mRNAs via an element in the 3′ untranslated region (Shweiki *et al.*, 1992; Minchenko *et al.*, 1994; Ikeda *et al.*, 1995; Levy *et al.*, 1995, 1996; Liu *et al.*, 1995; Damert *et al.*, 1997; Shima *et al.*, 1995).

Interestingly, it appears that expression of the various VEGF proteins can be induced selectively. For example, whereas hypoxia is a major stimulator of *VEGF* transcription, it has no effect on the mRNA levels of PlGF (Gleadle *et al.*, 1995), VEGF-B or VEGF-C (Enholm *et al.*, 1997). Similarly, VEGFs A and C are upregulated, to differing degrees, by various growth factors, whereas VEGF-B is insensitive to such factors (Enholm *et al.*, 1997; Ristimäki *et al.*, 1998).

1.4.2: VEGF receptors

To date, four receptors for VEGF have been characterized. Three of these, VEGFR-1 (Flt-1, Fms-like tyrosine kinase; Shibuya *et al.*, 1990), VEGFR-2 (KDR/Flk-1, kinase insert domain containing receptor/fetal-liver kinase-1; Matthews *et al.*, 1991) and VEGFR-3 (Flt-4; Aprelikova *et al.*, 1992; Pajusola *et al.*, 1992), belong to the Class III receptor tyrosine kinase (RTK) family (the characteristics of which are reviewed in Yarden and Ullrich, 1988). This family also includes the receptors for PDGF-α and -β, colony stimulating factor-1 (CSF-1) and stem cell factor. The extracellular domains of Class III RTKs typically contain five C2-type immunoglobulin-like domains; however, those of the VEGF receptors contain seven (Fig. 1.3). Conserved cysteine residues within these domains form intramolecular disulfide bonds that ensure the correct folding of the ligand-binding domain (Williams, 1989). A second characteristic shared by members of this receptor family is the presence a long peptide insertion in the intracellular kinase domain. This sequence, at least in the case of PDGFR, is believed to mediate selective binding of the receptors to downstream signalling molecules (Cantley *et al.*, 1991).

Omitting the highly variable insert, the tyrosine kinase domains of the three VEGFRs are ~80% identical to each other and ~60% identical to the corresponding domains of other Class III RTKs (Shibuya *et al.*, 1990; Pajusola *et al.*, 1992). The extracellular domains of the three VEGFRs are ~35% identical (Pajusola *et al.*, 1992; Matthews *et al.*, 1991), although that of VEGFR-3 is distinct in that it is cleaved into two polypeptide chains, which are held together by disulfide bonds (Pajusola *et al.*, 1994).

The fourth VEGF receptor, neuropilin-1, was previously characterized as a receptor for the semaphorin/collapsin family of proteins (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997), with a proposed role in axon growth and guidance during embryonic development (Kawakami *et al.* 1995; Takagi *et al.* 1995). Neuropilin-1 is structurally unrelated to the Class III RTKs (Fig. 1.3).

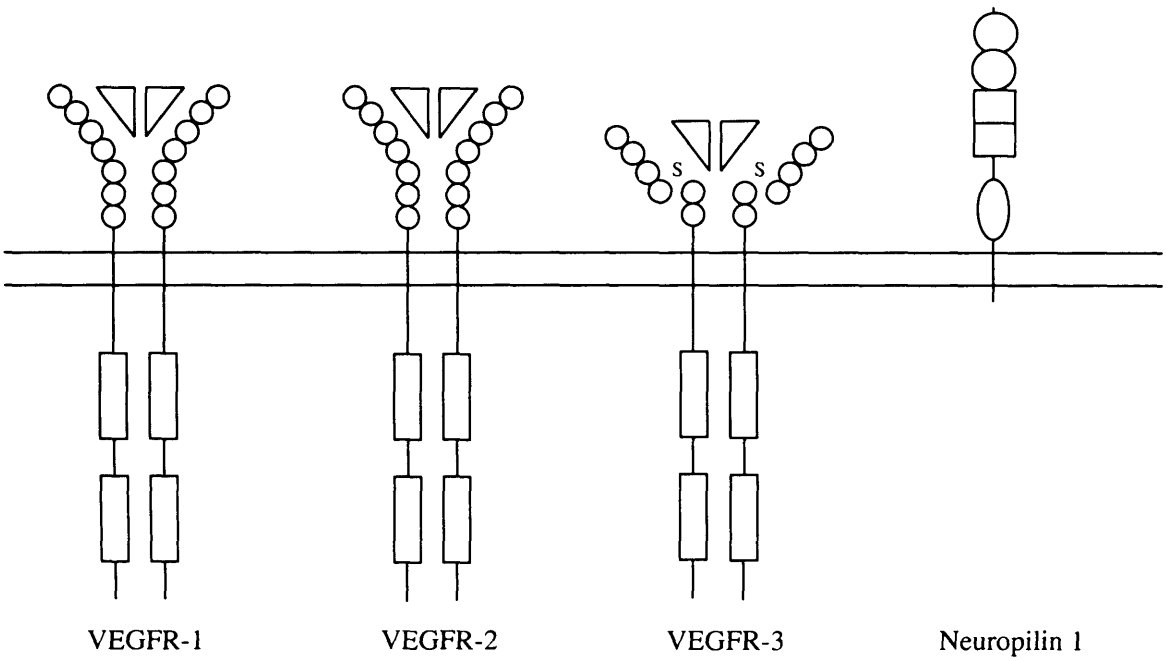


Fig. 1.3: Structure of the VEGF receptors. VEGFRs 1, 2 and 3 belong to the Class III family of receptor tyrosine kinases. They have seven immunoglobulin-like domains in their extracellular domains, a single-pass transmembrane domain and a cytoplasmic domain containing a split tyrosine kinase domain. The VEGF receptor neuropilin-1 is structurally unrelated to the other VEGFRs.

1.4.2 (i) VEGFR-1

The expression of VEGFR-1 is first detected in the angioblasts and endothelial cells of the yolk sac blood islands (Peters *et al.*, 1993). Throughout embryogenesis and during adulthood, expression remains almost exclusively restricted to endothelial cells (Peters *et al.*, 1993; Shibuya *et al.*, 1990). VEGFR-1 is highly expressed during organogenesis, concurrent with formation of the embryonic vasculature (Peters *et al.*, 1993). However, expression is maximal in the quiescent adult vasculature, thereby suggesting that VEGFR-1 is important in the maintenance, as well as in the formation, of blood vessels.

Gene deletion studies have shown that *VEGFR1*$^{-/-}$ homozygotes are embryonic lethal, dying *in utero* between E8.5 and E11 as a consequence of severe vascular defects (Fong *et al.*, 1995). Angioblasts are still able to differentiate into endothelial cells and

blood vessels do form, although these are immature, disorganized and abnormally large. This disorganization is evident even in the blood islands: angioblasts and haematopoietic cells are intermixed rather than segregated. The inability of VEGFR-1 knockouts to produce an organized network of mature blood vessels suggests a crucial role for this receptor, subsequent to endothelial cell differentiation, in the organization and maturation of embryonic vessels. Interestingly, one report has shown that mice expressing a VEGFR-1 mutant encoding just the extracellular and transmembrane domains of this receptor escape embryonic lethality, suggesting that only the extracellular domain of VEGFR-1 has a crucial role in embryonic vascular development (Hiratsuka *et al.*, 1998).

1.4.2 (ii) VEGFR-2

The expression of VEGFR-2 is initially detected, before any morphological evidence of endothelial cell precursors, in mesodermal cells that ultimately differentiate into angioblasts (Yamaguchi *et al.*, 1993). As development proceeds, expression of this receptor, similar to that of VEGFR-1, becomes restricted to the angioblasts of yolk sac blood islands and to the endothelium of all major embryonic and extraembryonic blood vessels as they form (Millauer *et al.*, 1993; Yamaguchi *et al.*, 1993; Quinn *et al.*, 1993; Oelrichs *et al.*, 1993; Matthews *et al.*, 1991). However, in contrast to the expression of VEGFR-1, that of VEGFR-2 is maximal during organogenesis, being drastically reduced in quiescent endothelium (Millauer *et al.*, 1993).

Mice in which expression of VEGFR-2 has been ablated are embryonic lethal, dying *in utero* between E8.5 and E11 as a consequence of severe vascular defects (Shalaby *et al.*, 1995). Unlike the angioblasts in *VEGFR1*$^{-/-}$ homozygotes, those in VEGFR-2 knockouts are unable to differentiate into endothelial cells, thereby precluding blood vessel formation. The crucial roles of VEGFR-2, therefore, might be in the differentiation and subsequent proliferation of endothelial cells. It is of note that *VEGFR2*$^{-/-}$ homozygotes also contained severely reduced numbers of HSCs and no intravascular blood cells. This receptor might, therefore, be crucial for the development of both endothelial and haematopoietic lineages. Alternatively, the lack of haematopoietic cells might be secondary to the absence of a suitable endothelial-lined microenvironment.

Interestingly, endothelial cells are produced and blood vessels do form in the absence of VEGF [see section 1.4.1 (i)]. Their absence in *VEGFR2*$^{-/-}$ mice therefore suggests that the crucial role of VEGFR-2 in embryonic development precedes that of VEGF, and also that a factor other than VEGF is able to activate VEGFR-2 during this developmental period. This hypothesis is supported by the observation that VEGFR-2 expression precedes that of VEGF (Yamaguchi *et al.*, 1993; Breier *et al.*, 1995; Flamme *et al.*, 1995). Several VEGF-related proteins exist that can activate VEGFR-2 (Fig. 1.4).

In particular, it is of note that VEGF-C, which can activate both VEGFR-2 and VEGFR-3, is expressed at E7, one and half embryonic days earlier than VEGFR-3 (Kukk *et al.*, 1996; Kaipainen *et al*, 1995).

1.4.2 (iii) VEGFR-3

VEGFR-3 is also expressed in endothelial cells during embryogenesis (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). However, as development proceeds, in contrast to VEGFRs 1 and 2, which specifically label vascular endothelia, VEGFR-3 becomes exclusively restricted to lymphatic endothelia, and expression is then maintained in mature lymphatic vasculature (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). Therefore, this receptor might be important in lymphangiogenesis rather than in angiogenesis, and in the subsequent maintenance of lymphatic vessels throughout adulthood. Interestingly, mice homozygous for targeted deletion of the *VEGFR3* gene die *in utero* as a consequence of vascular defects before the initiation of lymphangiogenesis (Dumont *et al.*, 1998), indicating that this receptor must also have an essential role in development of the embryonic vasculature. This role appears to be in the remodelling and maturation of large vessels. Recently, the importance of VEGFR-3 for formation of the embryonic vasculature has been attributed to a regulatory role of the receptor (Hamada *et al.*, 2000). These studies showed that VEGFR-2 signalling was influenced by VEGFR-3 expression levels as these regulate the concentration of VEGF-C available to activate VEGFR-2.

1.4.2 (iv) Neuropilin-1

Neuropilin-1 is not only expressed in neurons (Kawakami *et al.*, 1995), but also in both vascular endothelial cells and a variety of tumour-derived cells (Soker *et al.*, 1996; 1998). This receptor is structurally unrelated to the Class III RTKs (Fig. 1.3) and, as yet, only one binding partner has been identified for its relatively short cytoplasmic domain, namely neuropilin-1-interacting protein (NIP; Cai and Reed, 1999). This could support current opinion about neuropilin-1; that is, rather than having intrinsic signalling capabilities, this receptor modulates the activity of other receptors (Nakamura *et al.*, 1998a; Feiner *et al.*, 1997). Indeed, neuropilin-1 has been shown to augment the VEGF-induced response mediated by VEGFR-2, seemingly by increasing the affinity of VEGF for this receptor (Soker *et al.*, 1998). Importantly, mice in which neuropilin-1 expression has been specifically ablated die *in utero* as a consequence of vascular defects (Kitsukawa *et al.*, 1997).

1.4.3 (v) Receptor variants

In addition to these four receptors (their ligand specificities are depicted in Fig. 1.4), a natural soluble version of VEGFR-1 (sFlt-1) has been identified in several cell types

(Kendall and Thomas, 1993). This receptor, which encodes only the extracellular domain of VEGFR-1, binds VEGF with an affinity comparable to that of the full-length receptor. Consequently, sFlt-1 might function to modulate VEGF-induced responses by sequestering ligand or by recruiting full-length VEGFR monomers into inactive heterodimeric complexes (Kendall *et al.*, 1996). A similar role could be envisaged for a C-terminally truncated version of VEGFR-2, a transcript for which has been detected in rat retina (Wen *et al.*, 1998).

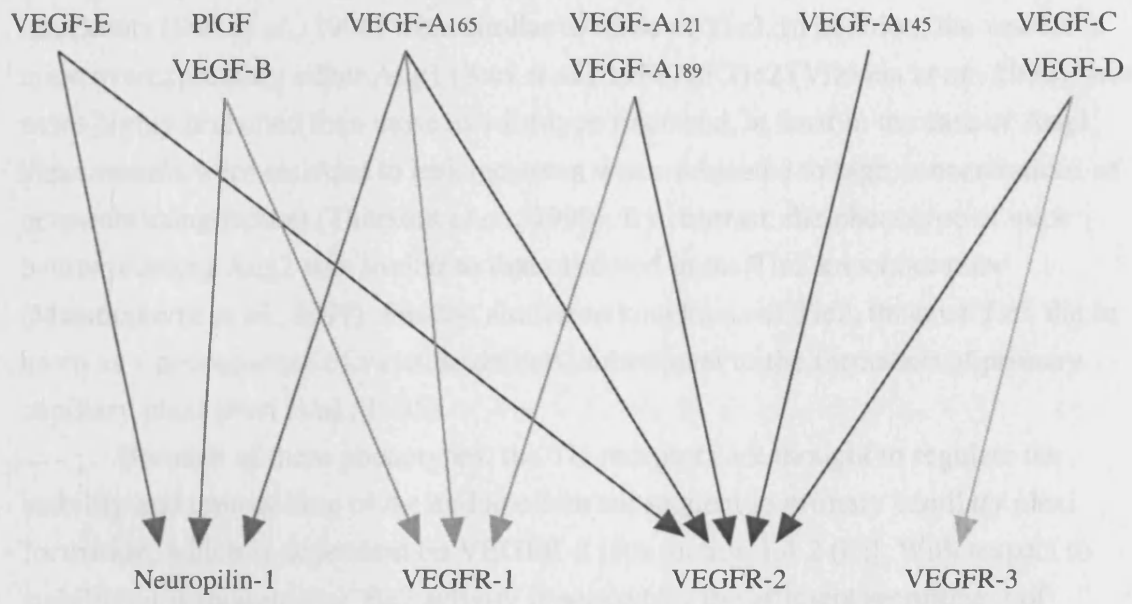


Fig. 1.4: Receptor-specificity of VEGF ligands. VEGFR-1 is activated by PlGF, VEGF-B, $VEGF_{165}$, $VEGF_{121}$ and $VEGF_{189}$; VEGFR-2 by VEGF-E, $VEGF_{165}$, $VEGF_{121}$, $VEGF_{189}$, $VEGF_{145}$, VEGF-C and VEGF-D; and VEGFR-3 by VEGF-C and VEGF-D. Neuropilin-1 is able to bind VEGF-E, PlGF, VEGF-B and $VEGF_{165}$. Not shown in this figure is that the abilities of VEGF-C and VEGF-D to bind VEGFRs 2 and 3 depend on the processed state of the ligand. In addition, the ability of VEGF-E proteins to bind neuropilin-1 seems to be strain-specific; a VEGF-E protein that is 43% identical to VEGF binds neuropilin-1, whereas an isoform with only 25% identity does not. (References: Park *et al.*, 1994; Lee *et al.*, 1996; Kukk *et al.*, 1996; Joukov *et al.*, 1996; 1997; Achen *et al.*, 1998; Ogawa *et al.*, 1998; Migdal *et al.*, 1998; Wise *et al.*, 1999; Makinen *et al.*, 1999.)

1.4.3: Angiogenic roles of the Tie receptors

As mentioned in section 1.4, apart from the VEGFRs, Tie1 and Tie2 are the only receptors for angiogenic factors that exhibit virtually exclusive expression in endothelial cells. Several ligands, the angiopoietins (Ang), have been identified for Tie2; Tie1, as yet, remains an orphan receptor (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997;

Valenzuela *et al.*, 1999). The ligands so far identified have opposing activites: Ang1 and Ang4 activate Tie2, whereas Ang2 and Ang3 inhibit (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999).

Targeted deletion of the gene encoding Tie2 was embryonic lethal as a consequence of insufficient expansion and maintenance of the primary capillary plexus (Sato *et al.*, 1995; Dumont *et al.*, 1994). Vessels were simplified (i.e. had only a few branches) and endothelial cells were poorly associated with underlying pericytes and smooth muscle cells. Probably as a consequence of this insufficient support, the embryos displayed widespread vascular haemorrhage. The phenotypes of Ang1 knockouts (Suri *et al.*, 1996) were simliar to those of Tie2. In addition, the vessels in mice overexpressing either Ang1 (Suri *et al.*, 1998) or Tie2 (Vikkula *et al.*, 1996) were more highly branched than those in wild-type mice and, at least in the case of Ang1, these vessels were resistant to leakage, even when subjected to high concentrations of permeabilizing factors (Thurston *et al.*, 1999). By contrast, the phenotype of mice overexpressing Ang2 was similar to that observed in the Tie2 knockout mice (Maisonpierre *et al.*, 1997). Finally, similar to knockouts of Tie2, those of Tie1 die *in utero* as a consequence of vascular defects, subsequent to the formation of primary capillary plexi (Puri *et al.*, 1995).

Because of these phenotypes, the Tie receptors are thought to regulate the stability and remodelling of the endothelium subsequent to primary capillary plexi formation, which is dependent on VEGFR-2 [see section 1.4.2 (ii)]. With respect to stability, it is thought that Tie2 activity is needed for the efficient recruitment of supporting cells to newly formed blood vessels. For angiogenic remodelling, the association between the supporting cells and the endothelium needs to be reversibly regulated, presumably by the coordinated actions of the various angiopoietins. In support of this theory, the expression of Ang2 (an antagonistic ligand) is highest during early angiogenesis and so could potentially prevent the recruitment of supporting cells while new vessels are sprouting (Maisonpierre *et al.*, 1997). This expression then declines as that of Ang1 (an agonist) increases, allowing Ang1 to initiate processes that stabilize and mature the vessel (Maisonpierre *et al.*, 1997).

1.4.4: Angiogenic roles of ephrins

A role in angiogenesis subsequent to formation of primary capillary plexi has also being attributed to a class of molecules relatively new to the angiogenic field: the ephrins. This system houses a large number of factors, with 8 ephrins (ligands) and 14 Eph receptors being known in mammals (reviewed in Adams and Klein, 2000). Ephrins differ from VEGFs and angiopoietins in that they are membrane bound and, therefore, their signalling is restricted to adjacent cells. The most well-characterized role for ephrins at present is their regulation of nerve fibre pathfinding. In this process,

interactions between ephrins and their receptors are repulsive: migrating nerve cells expressing one component of the pair turn away from surrounding cells expressing the other (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Brennan *et al.*, 1997; Krull *et al.*,1997; Wang and Anderson, 1997). Mice with specific deletions of the genes encoding ephrinB2 (Adams *et al.*, 1999; Wang *et al.*, 1998), EphB4 (Gerety *et al.*, 1999) and EphB2/B3 (i.e. the double knockout; Adams *et al.*, 1999) have been generated. Knockouts of ephrinB2 and EphB4, and a percentage of the EphB2/B3 knockouts, were embryonic lethal as a consequence of defects in vascular remodelling. In particular, vessels were poorly organized and were not remodelled into a network of large and small vessels. In addition, endothelial cells were poorly associated with underlying pericytes and smooth muscle cells. It therefore appears that the ephrins have angiogenic roles in vessel stabilization and remodelling, subsequent to formation of primary capillary plexi. In analogy to their relationship in nerve patterning, ephrins and their Eph receptors might exhibit repulsive interactions during angiogenesis. Evidence in support of this hypothesis comes from studies with intersomitic vessels (which express EphB4) and adjacent somites (which express ephrinB2). These vessels do not usually infiltrate the somites; however, in ephrinB2 knockouts, such invasion is observed, suggesting the removal of a repulsive factor (Helbling *et al.*, 2000).

1.5: Therapeutic manipulation of angiogenesis

1.5.1: Anti-angiogenic therapy

As previously mentioned (section 1.2), inappropriate activation of angiogenesis is associated with diabetic retinopathy, rheumatoid arthritis and cancer. Many strategies have been adopted with the goal of developing anti-angiogenic therapies, including the use of endogenous inhibitors, soluble growth factor receptors, ribozymes and small molecule inhibitors, as well as gene therapies, antibody therapies and antisense approaches (reviewed in Hamby and Showalter, 1999; Cao, 2001). The VEGF signalling cascades in particular could be attractive targets for the treatment of pathological angiogenesis. For example, the formation of blood vessels that contributes to diabetic retinopathy has been attributed to an upregulation of VEGF in the eye (Adamis *et al.*, 1994). In addition, the angiogenic factors most often secreted by tumours (to attract a blood supply into the tumour mass) are FGF and/or VEGF (Nguyen *et al.*, 1994; Ferrara and Henzel, 1989; Senger *et al.*, 1990; Gospodarowicz *et al.*, 1989; Cao *et al.*, 1996a).

Tumours secreting VEGF include neuroblastomas (Levy *et al.*, 1989), haemangiomas (Takahashi *et al.*, 1994) and glioblastomas (Millauer *et al.*, 1994). In addition, upregulation of VEGF has been associated with the progression of human astrocytomas into the more malignant glioblastomas (Plate *et al.*, 1992). These relationships between VEGF and tumours, along with the original isolation of VEGF

from tumour ascites (Senger *et al.*, 1983), strongly implicates a role for this factor in tumour vascularization. In addition, VEGF expression is induced by hypoxia [discussed in section 1.4.1 (vii)], a characteristic of the internal environment of tumours.

The complete dependence of the malignancy of most tumours on angiogenesis makes this process an attractive therapeutic target. Indeed, anti-angiogenic therapy has several potential advantages over more conventional chemotherapy. For example, in the latter, the continuous bombardment of rapidly proliferating, genetically unstable tumour cells with cytotoxic drugs frequently causes the selection of mutated cancer cells with acquired drug resistance. By contrast, the tumour-associated endothelial cells targeted by anti-angiogenic therapy proliferate much more slowly and are consequently less likely to acquire drug resistance.

Several agents that interfere with angiogenic signal transduction have successfully inhibited tumour growth in experimental models. Signalling pathways targeted by these agents include those initiated by VEGF, angiopoietin-1 and bFGF, although only those relating to VEGF are discussed here.

In one experiment, three human tumourigenic cell lines, A673 rhabdomyosarcoma, G55 glioblastoma multiforme and SK-LMS-1 leiomyosarcoma, were injected into nude mice (Kim *et al.*, 1993). Administration of a monoclonal anti-VEGF antibody, either simultaneously with the tumour cells or one week later, inhibited tumour cell growth by up to 96%. This inhibition was secondary to a reduction in the number of blood vessels. That the therapy was effective whether applied with or after establishment of the tumour cells, implies that this strategy could be of use to disrupt both the initiation and maintenance of tumour establishment. In a second study, cells harbouring a recombinant retrovirus encoding a signalling incompetent version of VEGFR-2 (i.e. C-terminally truncated lacking the cytoplasmic domain) were co-implanted into nude mice with C6 rat glioblastoma cells (Millauer *et al.*, 1994). Expression of this receptor dramatically inhibited tumour growth, presumably by recruiting full-length receptors into signalling-incompetent heterodimers and/or by sequestering ligand. As in the first approach, the inhibition of tumour growth was secondary to a decreased density of blood vessels and was observed whether the virus-containing cells were implanted with, or after, the tumour cells. A third approach was to use sFlt-1 (Goldman *et al.*, 1998). HT-1080 fibrosarcoma tumour cells, transfected with the sFlt-1 cDNA, were either implanted or injected into nude mice. Expression of sFlt-1 inhibited both the growth of tumour cells and the implantation of lung metastases. Furthermore, mice receiving intracranial injections of the sFlt-1-expressing glioblastoma cells survived twice as long as those injected with untransfected glioblastoma cells. Finally, studies using SU5416, a small molecule that selectively inhibits endothelial cell proliferation and migration induced by VEGF, demonstrated the

ability of this agent to inhibit the growth of many tumour cell lines in mice and also to induce the regression of established tumours (Angelov *et al.*, 1999; Fong *et al.*, 1999).

Other potential candidates for anti-angiogenic tumour therapy are the endogenous inhibitors that are produced as precursors (see section 1.3); indeed, the use of these factors by tumours themselves supports their suitability for this type of angiogenic suppression. In mouse models, both endostatin (O'Reilly *et al.*, 1997) and angiostatin (O'Reilly *et al.*, 1994; 1996) successfully inhibited the growth of primary tumours and the emergence of metastases. Furthermore, cycled endostatin therapy, in which there were alternate periods of treatment and tumour recovery, led to permanent inhibition of tumour growth, even when therapy was discontinued (Boehm *et al.*, 1997). In addition, treatment with a combination of angiostatin and endostatin was shown to completely eradicate tumours in mice (Boehm *et al.*, 1997). Throughout these studies, there has been no evidence of acquired drug resistence nor toxicity with either agent. Moreover, in contrast to another antiangiogenic agent (AGM-1470/TNP-470, a derivative of fumagillin isolated from the fungus *Aspergillus fumigatus fresnius*), neither endostatin nor angiostatin have been reported to have any deleterious effects on endometrial maturation, corpus luteum formation, wound healing or embryo growth in pregnant mice (Klauber *et al.*, 1997; Brem *et al.*, 1997; O'Reilly *et al.*, 1994; 1997).

Many anti-angiogenic agents are currently in clinical trials (for reviews see Cao, 2001; Sun and McMahon, 2000; Taraboletti and Margosio, 2001; Klohs and Hamby, 1999; Hamby and Showalter, 1999; Thompson *et al.*, 1999). In terms of VEGF signalling, these include a monoclonal anti-VEGF antibody, a monoclonal VEGFR-2 antibody and SU5416. Results so far indicate that these agents have significant biological activity and are well tolerated. The majority of agents used in current anti-angiogenic approaches are large molecules such as proteins. These molecules, often chosen because of their relevance to molecular signalling pathways, are generally highly specific and, therefore, probably less toxic than other agents. However, smaller molecules such as SU5416 have advantages in terms of bioavailability, pharmacokinetics, biostability, physical stability and manufacturing costs.

At least in the near future, the use of anti-angiogenic therapy will probably be limited to maintenance therapy following the removal of a primary tumour, or to combination therapy along with a conventional cytotoxic agent. Indeed, the combined use of anti-angiogenic and cytotoxic therapies has already been shown to eradicate tumours in animals that were insensitive to either agent alone (Teicher *et al.*, 1994).

1.5.2: Pro-angiogenic therapy

Pathological conditions resulting from oxygen deprivation; for example, peripheral and myocardial ischaemia, would benefit from the enhanced stimulation of angiogenesis, so-called therapeutic angiogenesis.

In animal models of peripheral and myocardial ishaemia, administration of VEGF or FGF has been shown to successfully promote the development of collateral vessels (for examples see Ferrara and Davis-Smyth, 1997; Ware and Simons, 1997; Mack *et al.*, 1998; Giordano, *et al.*, 1996; Baffour *et al.*, 1992; Takeshita *et al.*, 1994). This success was irrespective of whether recombinant growth factors or their genes were administered. Sufficient data were obtained from these preclinical studies to enable the extension of therapeutic angiogenesis to human patients with myocardial or peripheral vascular insufficiency. So far, with respect to VEGF, administration of either recombinant protein or the gene has shown some success in clinical trials, increasing the number of collateral vessels and enhancing perfusion of the ischaemic tissues (see examples in Henry *et al.*, 1998; Baumgartner *et al.*, 1998; Rosengart *et al.*, 1999). However, firm conclusions are lacking because of the small number of patients in these early trials. Although patients with a history of cancer have so far been excluded from these trials, there have been no reports of stimulation of undetected malignancies. In addition, even though diabetic patients have been included, no retinopathy has been observed. In general, treatment with VEGF has been well tolerated, although some oedema has been reported (Isner *et al.*, 1995).

In addition to treating peripheral and myocardial ischaemia, agents that stimulate blood vessel formation could also be used to treat gastric ulcers and to help heal chronic wounds. Indeed, patients with gastric ulcers are now being effectively treated with bFGF (Hull, 1995), and topical application of this growth factor has also been shown to accelerate the healing of chronic wounds (Robson *et al.*, 1992).

1.6: VEGF-induced signalling cascades

VEGF was originally isolated independently both as a vascular permeability factor (Senger *et al.*, 1983) and as an endothelial cell-specific mitogen (Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989). Subsequent studies have shown that VEGF can also induce endothelial cell survival, remodelling of the extracellular matrix, and the migration of endothelial cells and macrophages (see sections 1.6.3 and 1.6.4). However, the molecular mechanisms governing these responses remain to be clearly defined.

1.6.1: Receptor tyrosine kinase effectors

Signal transduction cascades mediated through the majority of RTKs are initiated upon ligand-induced dimerization of two receptor monomers (reviewed in Heldin, 1995). Dimerization activates the tyrosine kinase activity that resides within the intracellular domains of RTKs. Residues within the activation loop of the kinase are phosphorylated, and this further increases the kinase activity, leading to phosphorylation of residues outside the kinase domain. These sites then act as docking sites for selective downstream signalling molecules that contain Src homology (SH) 2 and/or

phosphotyrosine binding (PTB) domains. These domains recognize phosphotyrosine residues that are presented in a particular amino acid context: SH2 domains are selective on the basis of residues downstream of the phosphorylated tyrosine (Yxxx), whereas PTB domains recognize the preceding residues (xxxY) (reviewed in Kavanaugh and Williams, 1994). Effectors can also couple to RTKs independent of phosphorylation; for example, via the binding of SH3 domains to polyproline motifs (Feng *et al.*, 1994; Yu *et al.*, 1994). Once bound to the receptor, effector molecules might then themselves be substrates for receptor-mediated phosphorylation. These initial effectors can have intrinsic enzymatic activities and/or can function as adaptor molecules, coupling activated RTKs to effectors further downstream. The assembly of such multimeric protein complexes ultimately results in changes in gene expression and cellular responses (Heldin, 1995). A brief overview of some of the proteins frequently involved in RTK signal transduction pathways is given below and outlined in Fig 1.5.

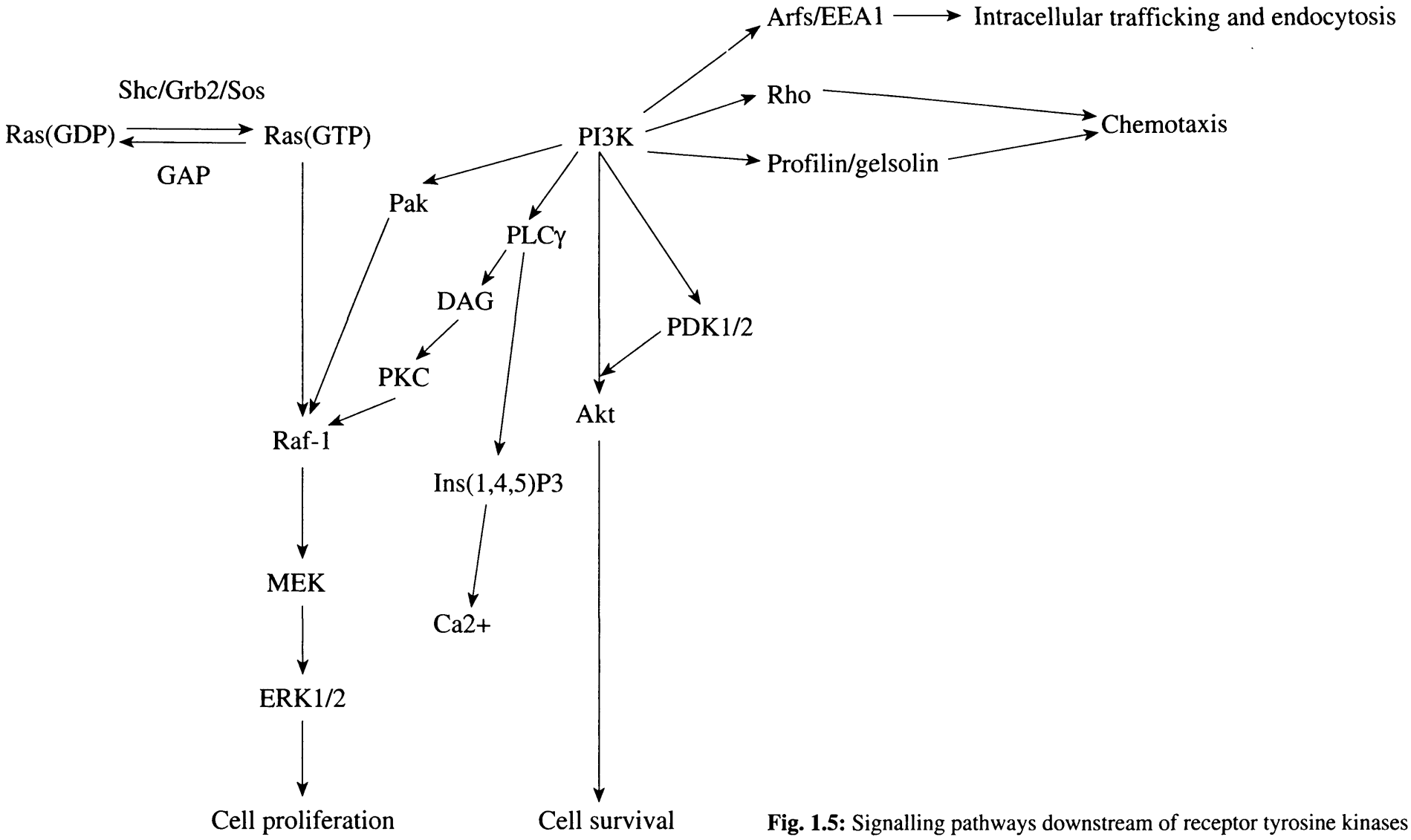


Fig. 1.5: Signalling pathways downstream of receptor tyrosine kinases

1.6.1 (i) Cell proliferation

RTK activation is often coupled to the induction of cell proliferation, usually by activation of a phosphorylation cascade termed the Ras/ERK pathway (reviewed in Widmann *et al.*, 1999; Su and Karin, 1996; Garrington and Johnson, 1999). In this prototypical pathway (Fig. 1.5), upon ligand-induced RTK activation, the adaptor protein Grb2 is recruited to the receptor, either directly or via the adaptor protein Shc. By docking with the transmembrane receptor, Grb2 recruits the guanine-nucleotide exchange factor Sos to the plasma membrane, which then activates the GTP-binding protein Ras. (This pathway is regulated by the GTPase-activating protein RasGAP). Activated Ras initiates a three kinase regulatory cascade by recruiting Raf, a serine/threonine kinase, to the plasma membrane. Raf activates MEK [a dual-specificity (i.e. tyrosine and threonine) kinase] and MEK then activates the serine/threonine mitogen-activated protein kinases (MAPKs) – ERKs 1 and 2. If sufficiently activated, the ERKs then translocate to the nucleus where they induce the transcription of immediate early (IE) genes required for cell proliferation (e.g. c-myc and c-fos).

Although only the Ras–Raf–MEK–ERK cascade is described above, there are multiple family members for each of these components. In particular, other MAPKs include the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs (reviewed in Widmann *et al.*, 1999). Whereas the ERK pathway is initiated by growth factors, those leading to activation of JNK and p38 are induced by cellular stresses such as irradiation, heat shock and DNA damage. Unlike ERK activation, which often couples to cellular proliferation, activation of JNK and p38 MAPKs is linked to induction of apoptosis.

The Ras/ERK pathway is not the sole route by which RTKs can effect cell proliferation. For example, phosphatidylinositide 3′-kinase (PI3K) has been reported to be required for activation of ERKs downstream of growth factor stimulation (for examples, see Yart *et al.*, 2001; Cross *et al.*, 1994; Marra *et al.*, 1995; Roche *et al.*, 1994). PI3K mediates this response through activation of either phospholipase Cγ (PLCγ, see below) or Pak, both of which then activate Raf-1. PI3K (for reviews see Funaki *et al.*, 2000; Rameh and Cantley, 1999) is a dimeric enzyme comprising a catalytic (p110) and a regulatory (p85) subunit. The localization of PI3K to the plasma membrane is mediated by an interaction between the SH2-containing p85 subunit and activated RTKs. Once at the membrane, PI3K phosphorylates the inositol ring of phosphoinositides. The products of this reaction, phosphatidylinositol (PtdIns)-3-monophosphate [PtdIns(3)*P*], PtdIns(3,5)bisphosphate [PtdIns(3,5)P_2], PtdIns(3,4)bisphosphate [PtdIns(3,4)P_2] and PtdIns(3,4,5)trisphosphate [PtdIns(3,4,5)P_3], act to recruit other signalling molecules to the plasma membrane.

The importance of PI3K activity in cell proliferation is not clear, although, at least in response to PDGF, preventing signalling through PI3K inhibits DNA synthesis (Kazlauskas *et al.*, 1992; Fantl *et al.*, 1992; Ronnstrand *et al.*, 1992; Valius *et al.*, 1993).

In addition, oncogenic forms of PI3K have been found (Chang *et al.*, 1997; Jimenez *et al.*, 1998) and PTEN, a phosphatase for the products of PI3K, is a tumour suppressor (Maehama and Dixon, 1998).

There is also controversy over whether PLCγ has a role in proliferation, with experiments either supporting (Valius and Kazlauskas, 1993; DeMali *et al.*, 1997; Roche *et al.*, 1996) or refuting (Mohammadi *et al.*, 1992; Peters *et al.*, 1992) such a requirement. This enzyme (reviewed in Williams, 1999) is a PtdIns(4,5)bisphosphate [PtdIns(4,5)P_2]-specific phosphodiesterase, whose activity produces diacylglycerol (DAG) and Ins(3,4,5)trisphosphate [Ins(3,4,5)P_3]. DAG then activates the serine/threonine kinase protein kinase C (PKC), and Ins(3,4,5)P_3 stimulates Ca^{2+} release from the endoplasmic reticulum, thereby increasing intracellular Ca^{2+} levels. PKC has been proposed to phosphorylate, and thereby activate, Raf (Glise *et al.*, 1995).

1.6.1 (ii) Apoptosis

Besides cell proliferation, ERK has been shown to be required for the prevention of apoptosis induced upon growth factor removal (Xia *et al.*, 1995). However, by far the most well-studied effector when it comes to survival signalling is PI3K. PI3K activity is essential for survival downstream of apoptotic signals induced by cellular stresses, growth factor withdrawal and cellular detachment from the extracellular matrix (for examples see Minshall *et al.*, 1996; Shimoke *et al.*, 1997; Spear *et al.*, 1997; ; Kulik *et al.*, 1997; Frisch and Francis, 1994; Khwaja *et al.*, 1997; Yao and Cooper, 1995; 1996). The mechanisms underlying this protection have been extensively studied, and require the PI3K-dependent activation of the serine/threonine kinase Akt/PKB (reviewed in Coffer *et al.*, 1998). Akt is the major target of the PI3K product PtdIns(3,4)P_2, and this interaction recruits Akt to the plasma membrane. Akt is then phosphorylated by PDKs [PtdIns(3,4,5)P_3-dependent kinases] 1 and 2, both of which are thought to be membrane targeted by interacting with the PI3K product PtdIns(3,4,5)P_3.

Several survival mechanisms seem to be employed by Akt. These include inducing the expression of anti-apoptotic proteins by activating the transcription factor NFκB (Mayo *et al.*, 1997; Alon *et al.*, 1995; Spyridopoulos *et al.*, 1997), inhibiting the expression of pro-apoptotic proteins by inactivating the AFX forkhead transcription factor (Brunet *et al.*, 1999), and permitting the survival activities of anti-apoptotic Bcl-2 proteins by sequestering the pro-apoptotic protein Bad in complexes with the 14-3-3 adaptor proteins (del Peso *et al.*, 1997; Datta *et al.*, 1997; Zha *et al.*, 1996).

1.6.1 (iii) Cell migration and intracellular trafficking

PI3K has also been shown to be essential for chemotaxis and membrane ruffling induced by PDGF (Wennstrom *et al.*, 1994; Kundra *et al.*, 1994). It has been suggested that these effects might be mediated by interaction of PI3K with small GTPases of the

Rho family (i.e. RhoA, Cdc42 and Rac1), which regulate the actin cytoskeleton (Carpenter *et al.*, 1997; Tall *et al.*, 2000; Tolias *et al.*, 2000; Hartwig *et al.*, 1995). In addition, PI3K has been shown to bind the actin-binding proteins profilin and gelsolin (Lassing and Lindberg, 1985).

Cell migration is intricately coupled to cell adhesion. Growth factor-induced cell motility is thought to be regulated by Src – a non-receptor tyrosine kinase. This kinase phosphorylates focal adhesion proteins such as focal adhesion kinase (FAK), p190RhoGAP and cortactin, leading to a concomitant loss of cell adhesion (reviewed in Parsons and Parsons, 1997).

A role for PI3K has also been implicated in intracellular trafficking and endocytosis. Indeed, inhibition of PI3K signalling has been shown to block vesicle trafficking of proteins both between intracellular compartments (Brown *et al.*, 1995; Davidson, 1995), and to and from the plasma membrane (Cheatham *et al.*, 1994; Haruta *et al.*, 1995; Joly *et al.*, 1995). The involvement of PI3K in vesicular trafficking could involve crosstalk with ADP-ribosylation factors (Arfs) – small GTP-binding proteins that function in vesicular trafficking and cytoskeletal regulation (reviewed in Jackson *et al.*, 2000). In particular, PtdIns(3,4,5)P_3 has been shown to recruit cytohesin and centaurin proteins, both of which activate Arf, to the plasma membrane.

1.6.2: Activation of VEGFRs 1 and 2

The vascular endothelial cell responses initiated by VEGF are presumably mediated through VEGFRs 1 and 2 as VEGFR-3 localizes to lymphatic endothelia and neuropilin-1 has no known intrinsic signalling capabilities (see section 1.4.2). However, whereas VEGF-induced phosphorylation of VEGFR-2 has been consistently demonstrated in mammalian cells (for examples see Takahashi and Shibuya, 1997; Quinn *et al.*, 1993; Waltenberger *et al.*, 1994), the intrinsic kinase activity of VEGFR-1 appears to be very weak, and ligand-induced phosphorylation of this receptor in intact cells is barely, if at all, detectable (Park *et al.*, 1994; Seetharam *et al.*, 1995; Waltenberger *et al.*, 1994). Even *in vitro*, ligand-induced autophosphorylation of VEGFR-1 is much lower than that of VEGFR-2 (Waltenberger *et al.*, 1994), so the difference does not appear to be a consequence of the presence of VEGFR-1-specific phosphatases in cells. In addition, when expressed in yeast and insect cells, the intracellular domain of VEGFR-1, similar to that of other RTKs, is constitutively phosphorylated (Cunningham *et al.*, 1995; Sawano *et al.*, 1997; Ito *et al.*, 1998). Therefore, the observed weak ligand-induced phosphorylation might be a consequence of suboptimal activation rather than of an intrinsic inability of the VEGFR-1 tyrosine kinase domain to become activated. It is possible that a productive interaction of ligand with this receptor requires cell-surface proteoglycans and/or co-receptors that are absent from both the cells used to assess VEGFR-1 phosphorylation and *in vitro* conditions.

VEGFR-1 has both a tenfold higher ligand affinity and a tenfold lower kinase activity than VEGFR-2 (Ferrara and Davis-Smith, 1997; Shibuya, 1995; Mustonen and Alitalo, 1995; Sawano *et al.*, 1996; 1997). This has led to the general opinion that VEGFR-1 functions as a ligand sink for VEGF, thereby regulating the activation of VEGFR-2. This proposal was supported by the observation that mice expressing a VEGFR-1 mutant encoding just the extracellular and transmembrane domains of the receptor escape the embryonic lethality imposed by the absence of the full-length receptor (Hiratsuka *et al.*, 1998).

The intracellular domains of the VEGFRs have been expressed in insect cells to identify autophosphorylation sites. [Residues identified in such systems for other RTKs have been shown to correlate well with those phosphorylated upon ligand-induced activation of the full-length receptors (Hsu *et al.*, 1990; Mohammadi *et al.*, 1996; Xia *et al.*, 1996).] For VEGFR-1, these studies have implicated that residues Y1213, Y1242 and Y1169 are the major autophosphorylation sites, with Y1327 and Y1333 being minor sites (Ito *et al.*, 1998; Sawano *et al.*, 1997). Expression of the VEGFR-2 cytosolic domain in bacteria led to the identification of four autophosphorylation sites: Y951, Y996, Y1054 and Y1059 (Dougher-Vermazen *et al.*, 1994).

1.6.3: Endothelial cell responses mediated through VEGFRs

Angiogenesis is a very complex process (reviewed in Risau and Flamme, 1995; Risau, 1997). The initial dissolution of the basement membrane and the subsequent remodelling of the extracellular matrix both require protease activity. In addition, endothelial cells at the leading edge of the invading sprout proliferate and migrate, whereas those behind the proliferating capillary tip adhere to each other and produce a new basement membrane. Supporting cells are then recruited to the endothelial-lined tubes. The exact molecular mechanisms by which these effects are mediated are the focus of much present-day research.

1.6.3 (i) Cell proliferation

The ability of VEGF to induce DNA proliferation specifically in endothelial cells was one of the characteristics that led to the identification of this factor (Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989). It has since been shown consistently that VEGF is mitogenic for primary endothelial cells, although the magnitude of this response varies depending on the cell type and the assay used. For example, in human umbilical vein endothelial cells (HUVECs), VEGF was shown to stimulate DNA proliferation in 65% of the cells [as measured by bromodeoxyuridine (BrdU) incorporation], compared with only 10% in the absence of VEGF (Thakker *et al.*, 1999). In addition, when DNA synthesis was measured by ^{3}H-thymidine incorporation (Wu *et al.*, 2000a), or extrapolated from the serum response element (SRE)-driven transcription of IE genes

(Thakker *et al.*, 1999), a sevenfold increase upon stimulation with VEGF was observed. However, in bovine aortic endothelial cells (BAECs), VEGF increased cell number by just twofold compared with controls (Bernatchez *et al.*, 1999).

Experiments in which cells were either pretreated with PD98059 (a specific MEK inhibitor) (Yu and Sato, 1999; Pedram *et al.*, 1998; Wu *et al.*, 2000a), or transfected with a dominant negative ERK2 construct (Pedram *et al.*, 1998), showed that ERK2 activity was important for the VEGF-induced proliferation of endothelial cells. However, using consitutively active and dominant negative mutants of JNK1, one group showed that ERK was only required to activate JNK1, and that it was the activity of JNK1 that was essential for VEGF-induced cell proliferation (Pedram *et al.*, 1998). In support of this, if JNK-interacting protein 1 (Jip-1) was overexpressed, JNK was retained in the cytoplasm and VEGF-induced proliferation was inhibited (Pedram *et al.*, 1998).

In HUVECs, inhibiting signalling through PI3K, either by pretreatment with wortmannin (a PI3K inhibitor) or by transfection of a dominant negative p85 construct, inhibited VEGF-induced proliferation by 50%, thereby suggesting an important role for PI3K in this response (Thakker *et al.*, 1999). Studies using the PI3K inhibitor LY294002 also demonstrated that PI3K activity was essential for VEGF-induced proliferation (Yu and Sato, 1999).

The PLCγ–PKC pathway has also been implicated in VEGF-induced mitogenesis (Xia *et al.*, 1996; Wellner *et al.*, 1999; Wu *et al.*, 2000a, Takahashi *et al.*, 1999). Indeed, pretreatment of cells with a specific PKC inhibitor suppressed VEGF-induced incorporation of ^{3}H-thymidine into DNA by 70% (Wu *et al.*, 2000a). Interestingly, in this study, the inhibition of PKC blocked ERK activation more severely than it did proliferation, suggesting that a second, ERK-independent, mechanism is also involved in the VEGF-induction of DNA synthesis.

VEGF-induced cell proliferation was also prevented by inhibition of nitric oxide synthase (NOS) activity, thereby showing a dependence on nitric oxide (NO) (Ziche *et al.*, 1997b; 1993; 1994; Morbidelli *et al.*, 1996; Parenti *et al.*, 1998). Induction of NO production in response to VEGF is thought to be mediated by PI3K in the short-term, and by Ca^{2+}-mediated activation of endothelial NOS (eNOS) in the long-term (Papapetropoulos *et al.*, 1997; Hood *et al.*, 1998).

Several studies have attributed the VEGF-induced proliferation of endothelial cells to VEGFR-2. VEGFs C, D and E – ligands that activate VEGFR-2 but not VEGFR-1 – can all stimulate endothelial cell proliferation (Lee *et al.*, 1996; Joukov *et al.*, 1996; 1997; Achen *et al.*, 1998; Ogawa *et al.*, 1998; Meyer *et al.*, 1999). In addition, the VEGF-induced proliferation of HUVECs was completely abrogated by expression of an antisense VEGFR-2 transcript (Bernatchez *et al.*, 1999), by overexpression of a phosphatase specific for VEGFR-2 (Huang *et al.*, 1999) and

by treatment with a VEGFR-2-specific inhibitor (Wu *et al.*, 2000a). Furthermore, stimulation of endothelial cells with VEGF mutants whose VEGFR-2-binding abililty had been reduced, were 20-fold less efficient in stimulating proliferation than wild-type VEGF ligands (Keyt *et al.*, 1996). Finally, VEGFR-2 mediates a VEGF-induced mitogenic signal when transfected into both PAE (Kroll and Waltenberger, 1997; Landgren *et al.*, 1998) and NIH3T3 (Takahashi and Shibuya, 1997) cells.

By contrast, transfecting antisense oligonucleotides to VEGFR-1 into cells had no effect on VEGF-induced proliferation (Bernatchez *et al.*, 1999). In addition, the mitogenicity of VEGF mutants with decreased VEGFR-1 binding ability was not impaired compared with wild-type VEGF (Keyt *et al.*, 1996). Furthermore, the VEGF-induced proliferative capacity of sinusoidal endothelial cells (SECs) expressing a version of VEGFR-1 lacking the intracellular domain, was not impaired compared with that of wild-type SECs (Hiratsuka *et al.*, 1998).

At present, there is controversy over whether the VEGFR-1-specific ligand PlGF can stimulate endothelial cell proliferation. The general consensus appears to be that if there is a PlGF-induced response, then this is only weak (Maglione *et al.*, 1991; Hauser and Weich, 1993; Wu *et al.*, 2000a; Bernatchez *et al.*, 1999). It has also been suggested that PlGF could act to enhance the mitogenicity induced by suboptimal concentrations of VEGF (Park *et al.*, 1994). Rather confusingly, VEGF-B, another VEGFR-1-specific ligand, has been reported to stimulate endothelial cell proliferation (Olofsson *et al.*, 1996a). However, no induction of proliferation has been observed in response to VEGF in either PAE–VEGFR-1 (Landgren *et al.*, 1998; Waltenberger *et al.*, 1994) or NIH3T3–VEGFR-1 (Seetharam *et al.*, 1995) cells.

1.6.3 (ii) Vascular permeability

VEGF was originally isolated as a vascular permeability factor (Senger *et al.*, 1983). The induction of vascular permeability leads to extravasation of plasma proteins such as fibrinogen from endothelial cells into extracellular spaces, resulting in the deposition of a provisional matrix that favours and supports the sprouting of new blood vessels.

The mechanisms by which VEGF regulates vascular permeability are not clear and several possibilities have been proposed. For example, VEGF has been shown to: (1) increase the activities of transmembrane transporters such as the glucose transporter, GLUT-1 (Sone and Kumagai, 1998); (2) stimulate the fusion of vesicular–vacuolar organelles, thereby generating continous channels between the vessel lumen and tissue spaces (Kohn *et al.*, 1992; Feng *et al.*, 1996a; 1997); and (3) regulate the integrity of endothelial cell–cell junctions (Kevil *et al.*, 1998a; Antonetti *et al.*, 1999). To date, most permeability studies have concentrated on the effects of VEGF on intercellular junctions.

VEGF has been shown to induce the disorganization and phosphorylation of junctional proteins, both of which have been associated with vascular permeability (Kevil *et al.*, 1998a; 1998b; Lampugnani *et al.*, 1992; Antonetti *et al.*, 1999; Abedi and Zachary, 1997; Esser *et al.*, 1998). Specifically, VEGF increases the phosphorylation of platelet–endothelial cell adhesion molecule 1 (PECAM-1), VE-cadherin, β-catenin, plakoglobin, occludin and zonula occluden 1 (ZO-1), and causes the redistribution of occludin and VE-cadherin at cell–cell junctions.

VEGF-induced permeability was shown to require ERK, but not PKC nor PI3K, activity in HUVECs (Kevil *et al.*, 1998a). However, an involvement of PKC was suggested in other studies (Haselton *et al.*, 1992; Aeillo *et al.*, 1997). VEGF-induced permeability has also been reported to involve NO (Murohara *et al.*, 1998; Kubes and Granger, 1992; Laszlo *et al.*, 1995; Nguyen *et al.*, 1995; Ziche *et al.*, 1997b). With respect to NO, the VEGF-induced permeability of cultured microvascular endothelial cells was shown to require caveolae-mediated transcytotic transport, which is now thought to target NO to the nucleus so it can influence gene transcription (Feng *et al.*, 1999a).

Both VEGF C (Joukov *et al.*, 1997) and E (Ogawa *et al.*, 1998; Meyer *et al.*, 1999) can induce vascular permeability, suggesting that the response can be mediated through VEGFR-2. In support of this, a VEGF-C mutant unable to activate VEGFR-2 failed to induce vascular permeability (Joukov *et al.*, 1998). However, a mutant of VEGF that could no longer activate VEGFR-2 retained the ability to induce vascular permeability (Stacker *et al.*, 1999). PlGF was incapable of inducing vascular permeability, although it did potentiate the response generated by low levels of VEGF (Murohara *et al.*, 1997; Park *et al.*, 1994; Sawano *et al.*, 1996; Ziche *et al.*, 1997a). The dispensibility of VEGFR-1 signalling for this response was also suggested by the observation that VEGF-induced permeabilty was not compromised in SECs expressing a VEGFR-1 mutant lacking the cytoplasmic domain (Hiratsuka *et al.* 1998).

1.6.3 (iii) Cell migration

During angiogenesis, endothelial cells need to migrate into the extracellular matrix to form new vessels. VEGF has been consistently shown to induce the migration of endothelial cells (for examples see Waltenberger *et al.*, 1994; Rousseau *et al.*, 2000; Bernatchez *et al.*, 1999). Cell migration requires the disruption and formation of multiple intercellular and cell–extracellular matrix contacts. Decreased cell–cell adhesion and increased cell migration has been associated with increased tyrosine phosphorylation of junction components (Kinch *et al.*, 1995; Lampugnani *et al.*, 1997). It is therefore of note that VEGF increases phosphotyrosine labelling at focal adhesions (specialized structures that play a crucial role in mediating cell adhesion and motility) and cell–cell contact areas (Esser *et al.*, 1998; Rousseau *et al.*, 2000; Abedi and

Zachary, 1997). In particular, VEGF induces the phosphorylation of FAK and paxillin, both of which are required for the formation of focal adhesions (Abedi and Zachary, 1997; Rousseau *et al.*, 2000). VEGF also induces the phosphorylation of two of the main endothelial cell–cell adhesion proteins, PECAM-1 and the cadherin–catenin complex, the latter of which has been shown to be important for regulation of endothelial cell migration (Breviario *et al.*, 1995; Vittet *et al.*, 1996; Esser *et al.*, 1998).

In addition to inducing phosphorylation, VEGF redistributes cadherin complexes at cell–cell contact areas into a pattern that has previously been associated with destabilization of endothelial junctions (Lampugnani *et al.*, 1995; Rabiet *et al.*, 1996; Esser *et al.*, 1998; Kevil *et al.*, 1998a). VEGF also increases the staining of vinculin and paxillin at focal adhesions (Rousseau *et al.*, 2000) and decreases that of occludin at tight junctions (Kevil *et al.*, 1998a). Furthermore, VEGF has been shown to induce the expression of $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha v\beta 3$ integrins in endothelial cells (Senger *et al* 1996; 1997), the first two of which have been shown to function in cell migration (Gardner *et al.*, 1996; Gotwals *et al.*, 1996; Skinner *et al.*, 1994; Keely *et al.*, 1995).

Using either a VEGFR-2-specific inhibitor (Wu *et al.*, 2000a) or neutralizing antibody (Rousseau *et al.*, 2000), it was shown that VEGF-induced FAK phosphorylation in HUVECs, as well as HUVEC migration itself, was dependent on this receptor. This is supported by the ability of VEGF to induce these responses in PAE–VEGFR-2 cells (Rousseau *et al.*, 2000; Landgren *et al.*, 1998). By contrast, neither PlGF nor VEGF could induce these responses in PAE–VEGFR-1 cells and PlGF was also inactive in HUVECs (Rousseau *et al.*, 2000; Landgren *et al.*, 1998; Wu *et al.*, 2000a). The requirement of VEGFR-2 for endothelial cell migration has also been implicated by the observations that HUVEC migration is inhibited by HCPTPA, a VEGFR-2-specific phosphatase (Huang *et al.*, 1999), and that treatment of cells with antisense VEGFR-2 oligonucleotides inhibited BAEC migration (Bernatchez *et al.*, 1999). By contrast, PlGF did not induce BAEC migration and VEGF-induced migration was not blocked by antisense VEGFR-1 oligonucleotides (Bernatchez *et al.*, 1999). Similarly, the redistribution of VE-cadherin observed upon VEGF stimulation was not seen following stimulation with PlGF (Esser *et al.*, 1998).

Interestingly, the removal of occludin from tight junctions required ERK activity (Kevil *et al.*, 1998a). However, ERK was not required for VEGF-induced migration (Rousseau *et al.*, 1997; Dudley *et al.*, 1995), nor for FAK phosphorylation (Abedi and Zachary, 1997). Instead, HUVEC migration was dependent on the VEGF-induced activation of p38 (Rousseau *et al.*, 1997). In addition, NO (Noiri *et al.*, 1997; Murohara *et al.*, 1999) and PI3K activity (Dimmeler *et al.*, 2000) were reported to be essential for VEGF-induced migration. These two effectors could be linked as Akt, a PI3K effector, has been shown to activate eNOS (Dimmeler *et al.*, 2000).

In addition to the regulation of cellular contacts, cell migration involves degradation of basement membranes and remodelling of the extracellular matrix (ECM), both of which require protease activity. Plasminogen activators (PAs) are key mediators of proteolytic activity, converting plasminogen to plasmin which can then, either directly or indirectly [e.g. via matrix metalloproteinases (MMPs)] degrade most of the ECM proteins (reviewed in Chapman, 1997). VEGF has been shown to induce PA activity in primary endothelial cells (Pepper *et al.*, 1991; Mandriota *et al.*, 1995; Bernatchez *et al.*, 1999) and also upregulates several MMPs in vascular smooth muscle cells (Wang and Keiser, 1998).

1.6.3 (iv) Cell survival

VEGF is thought to be the major survival factor for endothelial cells present in immature, newly formed blood vessels (Benjamin *et al.*, 1999; Alon *et al.*, 1995; Benjamin and Keshet, 1997). In addition, VEGF is essential for the viability of SECs (Yamane *et al.*, 1994) and acts as a survival factor for serum-deprived HUVECs (Fujio and Walsh, 1999; Gerber *et al.*, 1998a). VEGF-induced survival of HUVECs is dependent on the PI3K-mediated activation of Akt (Fujio and Walsh, 1999; Gerber *et al.*, 1998a). Indeed, a constitutively active Akt mutant was sufficient for cell survival in the absence of VEGF (Fujio and Walsh, 1999; Gerber *et al.*, 1998a). In addition, by studying the ability of VEGF to rescue both vascular endothelial cell monolayers and suspension cultures, it was demonstrated that the activation of Akt by VEGF needed cell–ECM interactions; if Akt was already active, this interaction was not necessary for cells to survive apoptosis (Fujio and Walsh, 1999; Gerber *et al.*, 1998a). The additional observations that VEGF could not rescue a monolayer of immortalized vascular endothelial cells derived from mice null for VE-cadherin, nor wild-type cells pretreated with neutralizing anti-VE-cadherin antibodies, suggested that VE-cadherin was involved in the cell–ECM interaction required for VEGF-induction of Akt (Carmeliet *et al.*, 1999a).

VEGF also induces the expression of a second anti-apoptotic protein: Bcl-2 (Gerber *et al.*, 1998b). Similar to Akt, overexpression of this protein is sufficient for the survival of serum-deprived HUVECs in the absence of VEGF (Gerber *et al.*, 1998b). In addition, VEGF-induction of Bcl-2 requires VE-cadherin (Carmeliet *et al.*, 1999a). Finally, the expression of XIAP and survivin [members of the inhibitors of apoptosis (IAP) family of proteins] is also upregulated by VEGF (Tran *et al.*, 1999).

The VEGF-induction of Akt in HUVECs has been shown to be mediated by VEGFR-2 by using a VEGF mutant selective for VEGFR-2 (Gerber *et al.*, 1998a) and VEGFR-2-selective inhibitors (Wu *et al.*, 2000a). However, VEGF C and D (ligands that activate VEGFR-2 but not VEGFR-1) failed to support survival and were unable to activate Akt (Carmeliet *et al.*, 1999a). Because VEGF-induced Akt activation is

dependent on VE-cadherin, it might also be of relevance that VE-cadherin and VEGFR-2 have been found associated in a complex, which also contains β-catenin and PI3K (Carmeliet *et al.*, 1999a). No phosphorylation of Akt was observed in HUVECs stimulated with PlGF (Wu *et al.*, 2000a; Carmeliet *et al.*, 1999a; Gerber *et al.*, 1998a) or with a VEGFR-1-selective VEGF mutant (Gerber *et al.*, 1998a).

1.6.4: Responses mediated by VEGFRs in non-endothelial cell types

Although primarily expressed in endothelial cells, VEGFRs 1 and 2 are not completely restricted to this cell type. For example, macrophages, their monocytic precursors and smooth muscle cells, express VEGFR-1 but not VEGFR-2 (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Wang and Keiser, 1998). In addition, reports that VEGF induces the differentiation of osteoblasts (Midy and Plouet, 1994) and the production of insulin by beta cells (Oberg *et al.*, 1994) suggest that VEGFRs must also be expressed in these cell types.

Interestingly, in contrast to current opinion about the roles of VEGFRs in endothelial cells, VEGF-induced responses in macrophages have been shown to be mediated by VEGFR-1 rather than VEGFR-2. Both VEGF and PlGF stimulated the migration of monocytes (Clauss *et al.*, 1996; Barleon *et al.*, 1996), and these responses were completely ablated in monocytes expressing a VEGFR-1 mutant lacking the intracellular domain (Hiratsuka *et al.*, 1998). In addition, pretreating the cells with a neutralizing anti-VEGFR-2 antibody had no effect on VEGF-induced monocyte migration (Clauss *et al.*, 1996). Both VEGF and PlGF were also shown to induce the migration of smooth muscle cells, and the upregulation and secretion of the MMPs needed for this process (Wang and Keiser, 1998).

1.6.5: Molecular signalling through VEGFRs 1 and 2

As described above, VEGF promotes the proliferation, migration and survival of endothelial cells, as well as vascular permeability. However, despite much progress in recent years, the molecular interactions giving rise to these responses remain incompletely defined. An overview of some of the molecules frequently found to be associated with, and/or phosphorylated by, active RTKs was given in section 1.6.1. The putative involvement of these effectors in transducing VEGF-induced signals has been studied both in yeast and mammalian cells. However, in the latter, observations have been cell-type specific. This could reflect differences in the efficiency of VEGF activation of cell surface receptors or differences in the intracellular factors within the VEGF signalling pathways.

1.6.5 (i) PLCγ

A role for PLCγ in VEGF signalling pathways is supported by the VEGF-induced phosphorylation of this effector in primary endothelial cells (Abedi and Zachary, 1997; Seetharam *et al.*, 1995; Guo *et al.*, 1995; Wu *et al.*, 2000a). The intracellular domains of both VEGFR-1 and VEGFR-2 have been shown to bind to the SH2 domains of PLCγ in a phosphorylation-dependent manner in yeast two-hybrid assays (Cunningham *et al.*, 1997). In addition, the intracellular domain of VEGFR-1 precipitated PLCγ from NIH3T3 cell lysates (Sawano *et al.*, 1997) and a GST fusion protein of the PLCγ SH2 domain precipitated the VEGFR-1 intracellular domain from insect cell lysates (Cunningham *et al.*, 1997).

In HUVECs, VEGF, but not PlGF, induced the phosphorylation of PLCγ (Wu *et al.*, 2000a) and, in VEGFR-transfected PAE cells, VEGF promoted the formation of a PLCγ–VEGFR-2 complex but not a PLCγ–VEGFR-1 complex (Landgren *et al.*, 1998). However, in transfected NIH3T3 fibroblasts, VEGF induced significant PLCγ phosphorylation downstream of both receptors, and both PLCγ–receptor complexes were precipitated (Takahashi and Shibuya, 1997; Sawano *et al.*, 1997). The ability of VEGFR-2 to bind PLCγ indirectly, via a VEGF-induced interaction with the adaptor protein VRAP (VEGFR-associated protein), has also been suggested (Wu *et al.*, 2000b).

In an attempt to define the sites in VEGFR-1 responsible for binding PLCγ, peptides encompassing tyrosine residues derived from the receptor were bound to an Affi-Gel matrix and incubated with ^{35}S-metabolically labelled endothelial cell lysates (Ito *et al.*, 1998). Phosphopeptides encompassing Y1213 and Y1333 independently precipitated PLCγ from the lysates. However, VEGFR-1 intracellular domains precipitated PLCγ equally well whether wild-type, or mutant for either Y1213 or Y1333 (Ito *et al.*, 1998; Sawano *et al.*, 1997). By contrast, mutating the Y1169 residue significantly inhibited the binding of this domain to PLCγ (Sawano *et al.*, 1997). Furthermore, in yeast, mutating the Y1169 and Y794 residues inhibited the VEGFR-1–PLCγ interaction by 67% and 57%, respectively, and the Y794/1169F double mutant decreased binding by 97% (Cunningham *et al.*, 1997).

Cunningham *et al.* also tested the residues corresponding to Y794 and Y1169 (i.e. Y801 and Y1175) in VEGFR-2 for their ability to bind PLCγ (Cunningham *et al.*, 1997). As with VEGFR-1, mutation of these residues severely disrupted the VEGFR-2–PLCγ interaction in yeast. Another study in yeast showed that Y951 was essential for, and that Y1175 was involved in, the interaction between VEGFR-2 and PLCγ (Wu *et al.*, 2000b).

1.6.5 (ii) PI3K

Phosphorylation of p85 has been observed in response to VEGF stimulation of BAECs (Guo *et al.*, 1995) and VEGFR-2-overexpressing HUVECs (Thakker *et al.*, 1999).

Although an 80% increase in PI3K activity was also detected in the latter, neither p85 phosphorylation, nor an increase in PI3K activity, was detected in response to VEGF stimulation of native HUVECs (Abedi and Zachary, 1997).

The intracellular domain of VEGFR-1 has been repeatedly shown to bind p85 in yeast two-hybrid assays, and Y1213 is essential for this interaction (Igarashi *et al.*, 1998a, Cunningham *et al.*, 1995). By contrast, no such interactions have been detected between VEGFR-2 and p85, although several clones with high homology to p85 were isolated during a yeast two-hybrid screen using VEGFR-2 as bait (Igarashi *et al.*, 1998b).

No increase in PI3K activity or p85 phosphorylation has been observed upon VEGF stimulation of VEGFR-transfected cells (Waltenberger *et al.*, 1994; Takahashi and Shibuya, 1997; Seetharam *et al.*, 1995). However, a VEGF-induced interaction between p85 and VEGFR-2 was reported in the VEGFR-2-overexpressing HUVECs (Thakker *et al.*, 1999). Alternatively, it has been suggested that VEGFR-2 might interact with PI3K via VRAP (Wu *et al.*, 2000b).

PI3K transduces signals for cell survival downstream of VEGF by coupling to Akt (Fujio and Walsh, 1999; Gerber *et al.*, 1998a). Through the use of receptor-selective ligands (Gerber *et al.*, 1998a; Wu *et al.*, 2000a; Carmeliet *et al.*, 1999a) and inhibitors (Wu *et al.*, 2000a), induction of this PI3K–Akt pathway has been attributed to VEGFR-2.

1.6.5 (iii) Other SH2-containing effectors

A weak phosphorylation of Shc was detected *in vitro* in response to VEGF stimulation of VEGFR-2 (Kroll and Waltenberger, 1997). However, VEGF-induced Shc phosphorylation was barely detectable in intact cells, both in 3T3–VEGFR fibroblasts and in SECs (Seetharam *et al.*, 1995). It has been suggested that VEGF signalling might involve the Shc-like protein, Sck (also known as ShcB), rather than Shc itself. Indeed, VEGFR-2 was precipitated from PAE–VEGFR-2 cells with GST fusion proteins encoding the SH2 domain of Sck but not that of Shc (Warner *et al.*, 2000). In addition, VEGF induced the association of Sck and VEGFR-2 expressed in HEK293 cells (Warner *et al.*, 2000). In yeast, both VEGFR-2 and VEGFR-1 interacted with Sck, and the interaction between Sck and VEGFR-2 was shown to require the Y1175 residue (Igarashi *et al.*, 1998b; Warner *et al.*, 2000). The importance of the Y1175 residue was supported by studies in PAE–VEGFR-2 cells showing that the VEGFR-2–Sck interaction was specifically inhibited by a Y1175 phosphopeptide (Warner *et al.*, 2000).

VEGF-induced Nck phosphorylation has been detected in BAECs (Guo *et al.*, 1995). [Nck, as well as Crk, can substitute for Grb2 in recruiting Sos to signalling complexes at the plasma membrane (Matsuda *et al.*, 1994; Hu *et al.*, 1995).] In addition, ligand-activated VEGFR-2 has been shown to associate with both Grb2 and Nck *in*

vitro (Kroll and Waltenberger, 1997). Grb2 was also shown to bind the intracellular domain of VEGFR-1 and a VEGFR-1-derived Y1213 peptide, although this residue was not essential for the Grb2–VEGFR-1 interaction (Ito *et al.*, 1998). Interestingly, the Y1213 peptide also pulled down a Grb2-like protein, p27, from endothelial cell lysates (Ito *et al.*, 1998). In addition, Nck and Crk were shown to bind the VEGFR-1-derived Y1333 peptide (Ito *et al.*, 1998).

A VEGF-induced interaction between GAP and VEGFR-2, and a weak phosphorylation of GAP, have been observed *in vitro* (Waltenberger *et al.*, 1994; Kroll and Waltenberger, 1997). Significant VEGF-induced GAP phosphorylation has also been observed both in 3T3–VEGFR transfected cells and in primary endothelial cells (Seetharam *et al.*, 1995; Guo *et al.*, 1995).

Both VEGFR-1 and VEGFR-2 have shown weak, ligand-dependent association with the Src family members Fyn and Yes, although an increase in their phosphorylation was only observed downstream VEGFR-1-expressing cells (Waltenberger *et al.*, 1994).

Roles for the SH2-containing protein tyrosine phosphatases (SHPs) have been implicated downstream of both VEGFR-1 and VEGFR-2. SHP-2 was captured from endothelial cell lysates by the VEGFR-1-derived Y1213 peptide attached to an Affi-Gel matrix (Ito *et al.*, 1998). This residue was subsequently shown to be essential for the interaction between the VEGFR-1 intracellular domain and the phosphatase (Ito *et al.*, 1998). In addition, both SHP-1 and SHP-2 have been shown to bind VEGF-stimulated VEGFR-2 *in vitro* (Waltenberger *et al.*, 1994).

A third tyrosine phosphatase, HCPTPA, was identified during a yeast two-hybrid screen using VEGFR-2 as bait (Huang *et al.*, 1999). (No interaction has been reported between HCPTPA and VEGFR-1). Both the phosphorylated VEGFR-2 intracellular domain, and the full-length receptor, are substrates for HCPTPA (Huang *et al.*, 1999). In support of a role for this phosphatase in VEGF signalling, coexpression of HCPTPA and VEGFR-2 was demonstrated in two endothelial cell lines (Huang *et al.*, 1999). Moreover, infection of endothelial cells with an adenoviral construct overexpressing HCPTPA significantly inhibited VEGF-induced ERK activation, DNA synthesis and cell migration, and addition of the virus drastically impaired vascular sprout development in the rat aortic ring angiogenesis model (Huang *et al.*, 1999).

1.6.5 (iv) ERK1/2

Activation of ERKs 1 and 2 in response to VEGF has been demonstrated consistently in primary endothelial cells (Yu and Sato, 1999, Seetharam *et al.*, 1995, Takahashi and Shibuya, 1997, Wu *et al.*, 2000a, Abedi and Zachary, 1997, Pedram *et al.*, 1998). By using selective inhibitors, this induction has been shown to depend on MEK and PLCγ (Wu *et al.*, 2000a, Abedi and Zachary, 1997, Yu and Sato, 1999). The induction of ERK

activity in VEGFR-transfected NIH3T3 cells was shown to be mediated primarily by PKC (Takahashi and Shibuya, 1997). In addition, by using specific PKC inhibitors and a dominant negative Ras construct, this same group have shown that Raf activation requires PKC activity. By contrast, neither Raf activation nor ERK activation required Ras (Takahashi *et al.* 1999). Some studies found that PI3K was not required for VEGF-induced ERK activation (Wu *et al.*, 2000a, Yu and Sato, 1999). However, in studies using wortmannin (a selective PI3K inhibitor) and a dominant negative p85 construct, it was shown that 70% of the ERK activation induced by VEGF was dependent on PI3K activity (Thakker *et al.*, 1999). It has also been reported that VEGF-induced activation of ERK requires NO: both NOS and guanylate cyclase inhibitors blocked the activation of Erk1/2 induced by VEGF (Parenti *et al.*, 1998; Kroll and Waltenberger, 1997).

By using a VEGFR-2 neutralizing antibody, it has been shown that VEGF-induced ERK activation in HUVECs is mediated through this receptor (Rousseau *et al.*, 2000). The inhibition of VEGF-mediated ERK activation by overexpression of HCPTPA also suggests an involvement of VEGFR-2 in this response (Huang *et al.*, 1999), as does the ability of VEGF-E to activate ERK in primary endothelial cells (Ogawa *et al.*, 1998). By contrast, PlGF was not able to induce ERK activation in endothelial cells (Rousseau *et al.*, 2000; Wu *et al.*, 2000a). In accordance with these data, in transfected PAE cells, a significant VEGF-induced ERK activation was observed downstream of VEGFR-2-expressing, but not VEGFR-1-expressing, cells (Kroll and Waltenberger, 1997, Rousseau *et al.*, 2000, Landgren *et al.*, 1998). However, a twofold induction of ERK activity was reported in PAE–VEGFR-1 cells in response to PlGF (Landgren *et al.*, 1998), and VEGF-induced ERK activation was observed downstream of both receptors *in vitro* (Seetharam *et al.*, 1995; Takahashi and Shibuya, 1997).

1.6.5 (v) Summary and objectives

An abundance of data has been reported on VEGF-induced signalling cascades in recent years. However, several observations have suggested that these pathways might diverge from those of prototypical RTKs, leading to the possibility that VEGFR-specific effectors might exist in endothelial cells. The physiological pathways of VEGF signalling are far from being definitively determined, not least because of the discrepancies observed between different cell types. In addition, it is possible that the contribution of VEGFR-1 to VEGF-induced signalling pathways has been underestimated as a consequence of the inability of current methods to efficiently activate this receptor.

Searching for potential novel effectors of VEGFRs was one of the main aims of the work described in this thesis. The approach used was library screening using yeast two-hybrid technology. Any interactions resulting from these screens were to be

characterized. The second main aim was to develop a system in which ligand-induced VEGFR-1 phosphorylation could be efficiently and reproducibly acheived, so that signalling mediated by VEGFRs 1 and 2 could be fairly compared. A system exploiting chimeric receptors was developed for this purpose. It was also hoped that this system would enable the study of signalling mediated by the individual VEGFRs, in a native endothelial cell background, without interference from endogenous receptors.

Chapter 2

General materials and methods

Chapter 2: General materials and methods

2.1: Suppliers of reagents

Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK
Becton Dickinson, Sparks, MD, USA
Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK
Clontech Laboratories, Palo Alto, CA, USA
DYNAL, Wirral, UK
Fischer Scientific, Loughborough, UK
Fisons, Loughborough, UK
FMC Bioproducts, Rockland, ME, USA
Gibco BRL Life Technologies, Paisley, UK
Jackson Immunoresearch Laboratories, West Grove, PA, USA
Melford Laboratories, Suffolk, UK
National Diagnostics, Hull, UK
NEN™ Life Science Products, Boston, MA, USA
New England BioLabs, Hitchin, Hertfordshire, UK
Oxoid, Basingstoke, Hampshire, UK
Promega, Southampton, UK
Qiagen, Crawley, West Sussex, UK
R & D Systems, Abingdon, Oxford, UK
Roche Diagnostics, Lewes, East Sussex, UK
Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sigma, Poole, Dorset, UK
Stratagene, West Cedar Creek, TX, USA
Techne, Duxford, Cambridge, UK
Whatman International, Maidstone, UK

2.2: General materials

All chemical reagents were of analytical grade and were obtained, unless stated otherwise, from either Sigma, Fischer Scientific or Fisons. Reagents for bacterial cell culture were obtained from Oxoid. Reagents for yeast cell culture were obtained from Oxoid and Becton Dickinson. Cell culture reagents were obtained from Gibco BRL.

Porcine aortic endothelial (PAE) cells were kindly provided by Lena Claesson-Welsh (Ludwig Institute For Cancer Research, Uppsala, Sweden). VEGFR-1/pcDNA1 and VEGFR-2/pcDNA3 (cloned by Bruce Terman) were also provided by Lena Claesson-Welsh.

Antibodies recognizing VEGFR-2 (sc-6251), VEGFR-1 (sc-316), phosphotyrosine (sc-7020) and Lex A (sc-1725) were purchased from Santa Cruz

Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibodies were also purchased from Santa Cruz Biotechnology. The anti-GST antibody had been raised in rabbits against a GST fusion protein.

2.3: General methods

2.3.1 Common buffers

TE (pH 7.4): 10 mM Tris/HCl (pH 7.4), 1 mM EDTA (pH 8.0)
TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 8.0)
TBS: 50 mM Tris/HCl (pH 7.4), 150 mM NaCl
PBS: 1.5 mM $NaH_2PO_4.H_2O$, 8.0 mM Na_2HPO_4, 150 mM NaCl (pH 7.4)
2 x Laemmeli sample buffer: 100 mM Tris.Cl (pH 6.8), 4% SDS, 0.01% bromophenol blue, 20% glycerol; freshly supplemented with 100 mM dithiothreitol (DTT)
6 x agarose gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol
Solution I: 50 mM glucose, 25 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0)
Solution II: 0.2 M NaOH, 1% SDS
Solution III: 3M KOAc, 11.5% glacial acetic acid

2.3.2: DNA Manipulations

2.3.2 (i) DNA purification

Double-stranded DNA (100 bp – 10 kb) was routinely purified from primers, nucleotides, polymerases and salts using the PCR Purification Kit (Qiagen). This kit exploits the selective binding properties of a silica-gel membrane: DNA absorbs to the membrane whereas contaminants pass through the column.

2.3.2 (ii) Restriction digests of DNA

Restriction endonucleases and their corresponding buffers were purchased from Roche Diagnostics. 0.5–5.0 μg DNA was digested in a maximum volume of 30 μl using 10 units of enzyme per reaction. Digests were performed for 1–2 hours at 37°C. In the case of double digests for which the buffers were not compatible, digests were performed sequentially, with the products from the first digest being purified before use in the second digest.

2.3.2 (iii) Phenol–chloroform extraction to remove protein from DNA samples

1 vol of phenol : chloroform : isoamyl alcohol (25:24:1) (Gibco) was added to an equal volume of the DNA solution and the contents mixed by vortexing. Centrifugation at 13 000 rpm, 4°C for 2 min, then separated the DNA (top, aqueous layer) from protein (bottom layer).

2.3.2 (iv) Ethanol precipitation of DNA

0.1 vol NaAc (3 M with respect to Na and 5 M with respect to acetate) and 2 vol cold 100% ethanol were added to the DNA sample. The tubes were vortexed and left to stand at room temperature for 2 min. DNA was then pelleted by centrifugation at 13 000 rpm, 4°C for 5 min. The pellet was washed with 1 ml cold 70% ethanol, air dried and resuspended in 50 µl TE containing 1 µl DNAase-free pancreatic RNase A (10mg/ml; Sigma). The tubes were vortexed and left at room temperature for 30 min before storing at –20°C.

For precipitation of oligonucleotides, after addition of NaAc and ethanol, samples were incubated on dry ice for 20 min. Oligonucleotides were then pelleted by centrifugation at 13 000 rpm, 4°C for 20 min, washed in cold 70% ethanol, air dried and resuspended in 100 µl TE. The absorbance of the precipitated sample was measured at OD_{260}, and the oligonucleotide concentration was calculated assuming a solution with OD_{260} = 1 has a concentration of 20 µg/ml.

2.3.2 (v) Alkaline phosphatase treatment to remove 5´ phosphate groups

1 unit of calf intestinal alkaline phosphatase (Roche Diagnostics) was added to 0.5–5.0 µg digested, purified DNA, mixed with the appropriate amount of buffer and incubated at 45°C for 1 h.

2.3.2 (vi) DNA ligations

Ligation reactions contained an approximately equimolar ratio of insert to vector (~100 ng insert DNA and 100 ng vector DNA), or a slight excess of insert over vector. Approximately 5 units of T4 DNA ligase (New England Biolabs) were used per reaction, in a total volume of no more than 20 µl. The ligations were performed at 16°C overnight.

2.3.2 (vii) Separation of DNA by agarose gel electrophoresis

6 x gel loading buffer was added to DNA samples before electrophoresis. The samples were electrophoresed in 1–2% agarose gels (in 0.5 x TBE), containing 0.2 µg/ml ethidium bromide for visualization of DNA bands by low-intensity UV radiation. Electrophoresis was performed at 100 V in 0.5 x TBE.

2.3.2 (viii) Extraction of DNA from agarose gels

DNA fragments (100 bp – 10 kb) were routinely extracted from agarose gels using a Gel Extraction kit (Qiagen). This kit exploits the selective binding properties of a silica-gel membrane: DNA absorbs to the membrane whereas contaminants pass through the column.

2.3.2 (ix) Standard DNA minipreps

DNA was routinely extracted from bacterial minicultures using a protocol based on the alkaline lysis method described by Birnboim and Doly (Birnoim and Doly, 1979). 2 ml LB–Amp (100 μg/ml) was inoculated with a single colony of DH5α and the culture grown at 37°C, with agitation at 225 rpm, overnight. Cells were pelleted at 13 000 rpm, 4°C for 30 sec, and resuspended in 100 μl ice-cold solution I by vortexing. 200 μl fresh solution II was then added, the solutions mixed by inversion, and the samples placed on ice. 150 μl ice-cold solution III was added and the solutions mixed by gently vortexing upside down for 10 sec. The tubes were then incubated on ice for a further 5 min, before centrifugation at 13 000 rpm, 4°C for 5 min. The supernatant was then subjected to phenol–chloroform extraction (to remove protein from the DNA samples), and the DNA was then collected by ethanol precipitation.

2.3.2 (x) Midi-DNA preps: the polyethylene glycol (PEG) method

5 ml LB–Amp was inoculated with a single colony of DH5α and the culture grown at 37°C, with agitation at 225 rpm, overnight. The following day, 1 ml of this miniculture was used to seed 500 ml LB–Amp, and the culture was grown at 37°C, with agitation at 225 rpm, overnight. Cells were pelleted at 8000 rpm for 15 min, and resuspended in 4 ml cold solution I. 20 mg lysozyme was added, and the solution incubated at room temperature for 10 min. 10 ml fresh solution II was then added, and the solution transferred to ice for 10 min. 7.5 ml solution III was then added, and the solution incubated on ice for a further 10 min. The mixture was centrifuged at 12 000 rpm for 10 min, after which the supernatant was divided into 2 x 10 ml aliquots. 6 ml isopropanol was added to each aliquot. The tubes were then incubated at room temperature for 5 min, and centrifuged at 11 500 rpm for 10 min. Pellets were then washed with 4 ml 70% ethanol. Each pellet was resuspended in 1 ml TE and treated with 5 μl RNase A (10 mg/ml) for 30 min at 37°C. The solution was then divided into 500 μl aliquots and subjected to phenol–chloroform extraction. The aqueous layers were further divided into 250 μl aliquots, from which the DNA was collected by ethanol precipitation. DNA was resuspended in 1 ml TE. 0.4 vol 30% PEG 8000/1.6 M NaCl was added to the DNA, and the solution was incubated at 4°C overnight. The next day, the DNA was pelleted at 8000 rpm for 20 min, washed with 1 ml 70% ethanol and then resuspended in 100 μl TE. The absorbance of the DNA sample was measured at OD_{260}, and the DNA concentration was calculated assuming a solution with an $OD_{260} = 1$ has a concentration of 50 μg/ml.

2.3.2 (xi) Synthesis of primers

Oligonucleotide primers were synthesized, at the 40 nM scale, on an Applied Biosystems 394 synthesizer (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK). Oligonucleotides were purified by ethanol precipitation.

2.3.2 (xii) Amplification of DNA by the polymerase chain reaction (PCR)

To amplify cDNA sequences by PCR, a double-stranded template is denatured by heating to 94°C. The temperature is then lowered (to 58°C) to facilitate annealing of the primers (designed to delineate the ends of the desired fragment) to the denatured template. Increasing the temperature to 72°C then encourages polymerase activity, which synthesizes a strand complementary to the template from the position dictated by the annealed primer. Annealing of the second primer to this newly synthesized strand initiates the synthesis of a new complementary strand. These 'new' strands are then used as templates for subsequent reactions.

PCR reactions were conducted in 0.5 ml, thin-walled, polypropylene microcentrifuge tubes (Applied Biosystems). Each reaction contained 50–500 ng template, 1 μM primers, 200 μM of each dNTP (Ultrapure dNTP Set; Pharmacia Biotech.) and 10 x reaction buffer (Stratagene), in a total volume of 100 μl. The PCR was performed in a GENIUS Thermal Cycler (Techne). Unless otherwise stated, the cycling parameters were as follows: template was denatured for 6 min at 94°C before the addition of 2 units of Taq DNA Polymerase (Stratagene). 19 cycles of denaturation (94°C, 30 sec), annealing (58°C, 50 sec) and elongation (72°C, 2 min) were followed by an extra 7 min of elongation (72°C) to allow the synthesis of all initiated strands to be completed.

2.3.2 (xiii) Double-stranded DNA sequencing

DNA was sequenced using the dideoxy chain-termination method (Sanger *et al.*, 1977), as detailed in the T7 Sequenase Quick-Denature plasmid sequencing kit (Amersham). According to this method, annealing of a specific oligonucleotide primer to a single-stranded template initiates DNA synthesis, catalysed by a DNA polymerase. Nucleotide precursors are recruited from four reaction mixtures. These mixtures contain all four deoxynucleoside-5′-triphosphates (dNTPs) along with one (dideoxy) ddNTP analogue which, owing to the absence of a 3'-OH group, is unable to support DNA elongation. The ratios of dNTPs and ddNTP in each mixture are optimized to generate a population of transcripts in which termination at each successive nucleotide is represented. Inclusion of a radioactive nucleotide into the mixes enables visualization of the transcripts, and thereby elucidation of the DNA sequence, by autoradiography.

Sequencing reactions were conducted as described in the kit, which supplied all reagents except template, [^{35}S]dATP label and primer. Double-stranded templates were

denatured with alkali. Once neutralized, the primer was then allowed to anneal and initiate DNA synthesis. This was catalysed by T7 sequenase version 2 polymerase, a modified version of DNA polymerase. Once the reactions had been terminated, they were heated to 75°C for 2 min, immediately before loading onto a 0.4 mm thick, 5% polyacrylamide–urea gel.

Gels were made by mixing Long ranger™ Gel Solution (acrylamide : bisacrylamide = 19:1) (FMC Bioproducts) and 7.0 M urea, in 1 x TBE, to make a 5% acrylamide solution. The solution was de-gased by filtration, 0.05% ammonium persulfate (APS) and 0.05% TEMED were added, and the gel was poured. Once set, the gel was pre-run in 1 x TBE at 80 W until the temperature had reached 50°C. The preheated samples were then loaded and electrophoresis was conducted at 50°C for the desired length of time. The gel was dried on Whatman 3MM paper for 1.5 h at 80°C and then exposed to X-ray film.

2.3.2 (xiv) Site-directed mutagenesis

Mutagenesis of double-stranded DNA templates was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). For each specific mutagenesis reaction, complementary primers (described in the respective chapters) encoding the desired mutation were synthesized. The double-stranded template was denatured, and the primers were used simultaneously to initiate DNA synthesis catalysed by Taq polymerase. This produces double-stranded, mutated plasmids with staggered nicks. These products are then digested with DpnI, which digests only the parental, methylated DNA, leaving the mutated strands intact. DNA is then transformed into Epicurian Coli® XL1-Blue Supercompetent Cells [Stratagene; genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})], which repair the nicks, generating closed circular, double-stranded, mutant plasmids.

All reactions were conducted as described in the kit, using the reagents provided. NZY+ broth, required for transformation of the mutated plasmids into the supercompetent cells, was not provided by the kit:
10 g/L NZ amine, 5 g/L yeast extract and 5 g/L NaCl were dissolved in de-ionized water, pH adjusted to 7.5 and autoclaved. Autoclaved media was cooled to 60°C before the addition of 12.5 mM autoclaved $MgCl_2$, 12.5 mM autoclaved $MgSO_4$ and 20 mM filter-sterile glucose.

2.3.3: Protein manipulations

2.3.3 (i) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Proteins were separated by SDS–PAGE [based on the method described by Schagger and van Jagow, 1987) using a minigel apparatus (Bio-Rad) and the buffer system: anode buffer [0.2 M Tris (pH 8.9)]; cathode buffer [0.1 M Tris, 0.1 M tricine and 0.1%

SDS (pH 8.25)]. The principles of this method are that, by coating the proteins in SDS, their acquired net negative charge directs their migration towards the anode.

Gels are biphasic so that, upon loading, proteins are initially stacked in a 4% gel before progressing into the 7–10% resolving gel. The acrylamide : bis-acrylamide ratio in the acrylamide stock solution (Ultra Pure ProtoGel) was 37.5:1. For resolving gels, acrylamide was mixed with 33% gel buffer [3M Tris.Cl (pH 8.45), 0.3% SDS], 10.6% glycerol and water as required. Polymerization was initiated by the addition of 0.05% fresh APS and 0.05% TEMED. For 4% stacking gels, acrylamide was mixed with 20.7% gel buffer, and polymerization was initiated by addition of 0.08% APS and 0.08% TEMED. Samples were boiled for 10 min in Laemmeli sample buffer immediately before loading on the gel. 5 µl of Rainbow molecular weight markers (Sigma) were run alongside samples to facilitate the estimation of protein molecular weights. Gels were run at 50 V until the proteins had stacked. The voltage was then increased to 150 V.

2.3.3 (ii) Electro-transfer of proteins to nitrocellulose

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes was performed according to the method described by Towbin (Towbin, 1979). Six gel-sized sheets of Whatman 3MM filter paper were soaked in transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol, 0.037% SDS) and placed on the anode (wet with transfer buffer) of a horizontal, semi-dry electrophoretic transfer apparatus (Bio-Rad). One sheet of pre-soaked (in transfer buffer) Hybond ECL nitrocellulose membrane (Amersham) was placed on top of this, followed by the gel, which had been pre-soaked in transfer buffer for 20 min. The 'sandwich' was completed with six more pre-soaked sheets of Whatman 3MM paper and the cathode (wet with transfer buffer) was then placed in contact with this layer. Transfer was conducted at 21–23 V for 45–120 min, depending on the size of the protein to be transferred. Once transferred, the membrane was blocked at 4°C overnight in TBST (TBS/0.1% Tween 20) containing 5% low fat dried milk powder (or 5% BSA if the membrane was to be analysed for the presence of phosphoproteins), before being used for the immunodetection of proteins.

2.3.3 (iii) Immunodetection of proteins bound to nitrocellulose membrane

The method used for the immunodetection of proteins immobilized on nitrocellulose membranes exploited the catalytic activity of HRP. The theory behind this method is that a primary antibody is initially bound to the protein under investigation on the membrane. A secondary antibody, coupled to HRP, then recognizes the bound primary antibody. The membrane is then exposed to a mixture of reagents in which luminol is degraded by an oxidizing reagent. In response to this oxidative degradation, the HRP

bound to the membrane catalyzes light emission, at a wavelength of 428 nm, which is detected by exposure to chemiluminescence-sensitive film.

The blocked membrane (see Electro-transfer of proteins to nitrocellulose) was incubated with an appropriate dilution of primary antibody in TBST supplemented with 5% low fat dried milk powder (or 5% BSA) for 1 h while agitating at room temperature. The membrane was washed four times (4 x 10 min) with TBST, and then incubated, by agitating for 1 h at room temperature, with a 1:1000 dilution of the HRP-coupled secondary antibody in TBST. The membrane was then washed four times with TBST, and once with de-ionized H_2O. The membrane was then covered with a 1:1 mix of ECL chemiluminescent detection reagents (Western Blot Chemiluminescence Reagent Plus; NEN™ Life Science Products) for 2 min, and membane-bound HRP-linked antibody complexes were detected by exposure to chemiluminescence-sensitive film.

After exposure, if the blot needed to be re-probed, the membrane was incubated at 60°C for 50 min in blot-strip buffer [62.5 mM Tris.Cl (pH 6.7), 2% SDS] supplemented with 0.8% fresh β-mercaptoethanol. The membrane was then washed several times with de-ionized H_2O, and four times with TBST, before being blocked at 4°C overnight.

2.3.3 (iv) Expression and purification of glutathione S-transferase (GST) fusion proteins

The method for expression and purification of GST fusion proteins was based on that described by Smith *et al.* (Smith *et al.*, 1988). The construct encoding the GST fusion protein was transformed into DH5α. 5 ml LB–Amp was inoculated with a single colony resulting from this transformation and the culture was grown at 37°C, with agitation at 225 rpm, overnight. The following day, 2 ml of this culture was diluted 1:75 into 150 ml LB–Amp and grown at 37°C, with agitation at 225 rpm, until the $OD_{600} = 0.6$. Expression of the fusion protein was then induced by addition of 0.2 mM isopropyl β-D-thiogalactoside (IPTG) and grown for a further 5 h. Cells were pelleted by centrifugation at 8000 rpm for 15 min and then lysed on ice for 20 min in 5 ml lysis buffer [100 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA] containing 5 mg lysozyme, 1 mM DTT and protease inhibitor cocktail as desired (Sigma). After 20 min, 4 mg deoxycholate (DOC; 40 mg/ml stock in lysis buffer) was added, and the mixture incubated at room temperature for 20 min. DNAse (10 mg/ml) and 500 µl 1M $MgCl_2$ were then added, and the mixture incubated for a further 20 min at room temperature. Insoluble debris was pelleted by centrifugation at 8000 rpm, 4°C for 10 min. The cell supernatant was then added to 0.5 ml glutathione-sepharose beads (Glutathione Sepharose® 4B; Pharmacia Biotech) in PBS (50% v/v), which had previously been washed twice in 5 ml PBS (1000 rpm, 4°C, 1 min). The beads and supernatant were incubated with rotation overnight at 4°C. The following day, the beads were washed

(1000 rpm, 4°C, 1 min) twice with PBS/0.1% TritonX100/1 mM DTT, and twice with PBS/1 mM DTT. 1 vol PBS was then added to the beads. An aliquot of this mixture was taken for estimation of protein concentration. 20% glycerol was added to the remainder and this was aliquoted and stored at –80°C. The amount of protein immobilized on the beads was estimated by running varying volumes of the beads alongside known concentrations (0.1–5.0 μg) of bovine serum albumin (BSA) on a polyacrylamide gel. The gels were stained with coomassie Brilliant Blue (45% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue®) for 30 mins, and then destained (45% methanol, 10% acetic acid) as required.

2.3.4: Bacterial cell culture

2.3.4 (i) Luria-Bertani medium (LB)

Bacto-tryptone (10 g/L), Bacto-yeast extract (5 g/L) and NaCl (10 g/L) were dissolved in de-ionized water, pH adjusted to 7.0 and autoclaved.

For solid media, Bacto-agar (15 g/L) was added to LB before autoclaving. Autoclaved LB–agar was cooled to 55°C, poured into sterile petri dishes and allowed to set before storage at 4°C. For selection of bacteria containing ampicillin-resistant plasmids, ampicillin was added to the cooled, molten, autoclaved LB–agar to a final concentration of 100 μg/ml.

2.3.4 (ii) Maintenance of Escherichia coli DH5α

DH5α [genotype F⁻ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_K^- m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 λ⁻ *gyr*A96 *rel*A1] were routinely grown in liquid LB, or on LB–agar plates, at 37°C. DH5α transformed with ampicillin-resistant plasmids were grown in LB media supplemented with 100 μg/ml ampicillin.

2.3.4 (iii) Preparation of transformation-competent DH5α

5 ml LB was inoculated with a single colony of DH5α and the culture grown at 37°C, with agitation at 225 rpm, overnight. The following day, 500 μl of this culture was diluted 1:100 into 50 ml LB and grown at 37°C, with agitation, until an OD_{600} of 0.2 was reached. Cells were pelleted at 2200 rpm, 4°C, for 10 min. The cell pellet was resuspended in 5 ml ice-cold, filter-sterile transformation buffer (10% w/v PEG 1500, 30 mM $MgCl_2$, 5% fresh DMSO in LB), and chilled on ice for 20 min. Cells were then aliquoted into prechilled microcentifuge tubes and, if not used immediately, flash-frozen in a dry ice–ethanol bath for storage at –70°C.

2.3.4 (iv) Transformation of plasmid DNA into DH5α

100 pg ampicillin-resistant plasmid was mixed with 100 μl competent DH5α and incubated on ice for 10 min, at room temperature for 10 min, and on ice for a further

10 min. 900 µl LB was then added to the cells. In the case of ligation reactions (this step is optional if an intact plasmid is being retransformed), this culture was then grown at 37°C, with agitation at 225 rpm, for 1 h. 200 µl of the culture was then plated out onto LB-Amp (100 µg/ml) plates and grown at 37°C overnight.

2.3.5: Mammalian cell culture

2.3.5 (i) PAE cell medium

PAE cells were routinely cultured in Ham's nutrient mix F12 medium with Glutamax-I, supplemented with 10% (v/v) fetal calf serum (FCS), 100 µg/ml streptomycin and 100 units/ml penicillin. For cell lines in which neomycin-resistant constructs had been stably transfected, this media was supplemented with G418 sulfate (0.4 mg/ml).

2.3.5 (ii) Maintenance of PAE cell lines

PAE cells were routinely cultured as monolayers in 75 cm^2 T75 plastic tissue culture flasks. Upon approaching confluence, cells were split by trypsinization. Cells were washed once with PBS, and 1 ml trypsin–EDTA (0.25% trypsin, 1 mM EDTA) was then added, and the cells incubated at 37°C for 5 min. 9 ml media was then added and the cells either split at a ratio of 1:15, or counted (10 µl was applied to haemocytometer) for use in experiments.

2.3.5 (iii) Freezing down cells for storage

Cells were frozen in liquid nitrogen for long-term storage. Cells approaching confluence in a 75 cm^2 T75 flask were washed once with PBS, trypsinized in a minimal volume of trypsin and resuspended in 1 ml FCS. An equal volume of 20% DMSO (in normal growth media) was added dropwise to the cells. Cells were aliquoted and frozen slowly by placing in an insulated box at –70°C for 24 h before transfer to liquid nitrogen.

2.3.5 (iv) Recovery of frozen cells

Cells were thawed quickly at 37°C, and added to 10 ml medium in a 75 cm^2 T75 flask. Once cells had adhered, medium was replaced with fresh medium to remove traces of DMSO.

2.3.5 (v) Whole cell lysate preparations for SDS–PAGE

Cells were seeded at a density of 5 x 10^4 per well of a 24-well plate, and allowed to attach overnight. If no stimulation was required, the following day, cells were washed once with PBS before the addition of 40 µl hot (95°C) 1.5 x Laemelli sample buffer (If analysing phosphoproteins, 100 mM NaF and 100 mM Na_3VO_4 were added to the sample buffer.) The samples were left to stand at room temperature for 2 min. Cell lysates were then transferred to microcentrifuge tubes, mixed with 100 mM DTT, and

boiled for 10 min before loading onto an SDS–PAGE gel. If stimulation was required, the day after seeding, cells were incubated in serum-free medium for 16–24 h, before stimulation. Stimulation was performed as described in the individual chapters.

2.3.5 (vi) Purification of proteins from cell lysates using GST fusion proteins

Cells were seeded at a density of 7.2 x 10^5 cells per 6 cm diameter petri dish and allowed to attach overnight. The following day, cells were washed three times with PBS and then incubated in serum-free medium for 16–24 h. Medium was replaced with fresh serum-free media 1 h before beginning the experiment. Cells were stimulated as described in the individual chapters. Following stimulation, cells were washed twice with ice-cold PBS and lysed for 10 min at 4°C in lysis buffer [1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EGTA, 25 mM benzamidine] containing 100 mM NaF, 100 mM Na_3VO_4 and protease inhibitor cocktail as required (Sigma). Cell debris was removed by centrifugation at 13 000 rpm, 4°C for 5 min, and lysates were incubated with 5 µg GST-fusion-coupled beads with rotation at 4°C overnight. The following day, the beads were pelleted by centrifugation at 5000 rpm, 4°C for 2 min, and then washed four times with TBS/0.1% Triton X-100. Bound protein was eluted from the beads by boiling for 10 min in 35 µl Laemmeli sample buffer, freshly supplemented with 100 mM DTT. The preheated samples were analysed immediately by SDS–PAGE.

2.3.6: Yeast two-hybrid assays

2.3.6 (i) YPD medium

Yeast Nitrogen Base without amino acids (10 g/L) and Bactopeptone (20 g/L) were dissolved in de-ionized water, pH adjusted to 5.8–6.0 and autoclaved. Autoclaved media was cooled to 60°C and filter-sterile glucose was added to a final concentration of 100 mM.

For solid media, Bacto-agar (18 g/L) was added to YPD before autoclaving. After glucose addition, the YPD–agar was poured into sterile petri dishes and allowed to set before storage at 4°C.

2.3.6 (ii) Drop-out media (DOM)

Drop-out mixes comprise the majority of the constituents shown below, although there are specific omissions depending on the particular DOM: DOM1 lacks leucine, tryptophan and histidine, and DOM2 lacks leucine and tryptophan.

Drop-out mixes were mixed thoroughly by end-over-end rotation for several minutes, and stored at room temperature.

Adenine	0.5 g	Leucine	4.0 g
Alanine	2.0 g	Lysine	2.0 g
Arginine	2.0 g	Methionine	2.0 g
Asparagine	2.0 g	p-Aminobenzoic acid	0.2 g
Aspartic acid	2.0 g	Phenylalanine	2.0 g
Cysteine	2.0 g	Proline	2.0 g
Glutamine	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Glycine	2.0 g	Tryptophan	2.0 g
Histidine	2.0 g	Tyrosine	2.0 g
Inositol	2.0 g	Uracil	2.0 g
Isoleucine	2.0 g	Valine	2.0 g

For drop-out medium, Yeast Nitrogen Base without amino acids (6.7 g/L) and drop-out mix (2 g/L) were dissolved in de-ionized water, pH adjusted to 5.8–6.0 and autoclaved. Autoclaved media was cooled to 60°C and filter-sterile glucose was then added to a final concentration of 100 mM. Filter sterile 3-amino-1,2,4-triazole (3-AT) was also added to the media to a final concentration of 30 mM [see section 3.3.3 (ii)].

For solid media, Bacto-agar (18 g/L) was added to the DOM before autoclaving. After glucose addition, the DOM–agar was poured into sterile petri dishes and allowed to set before storage at 4°C.

2.3.6 (iii) X-gal medium

Yeast Nitrogen Base without amino acids (6.67 g/L), DOM2 (0.7 g/L) and Bactoagar (20 g/L) were dissolved in 600 ml de-ionized water and autoclaved. Autoclaved media was cooled to 60°C, mixed with 100 ml filter-sterile 10 x BU (261 mM $Na_2HPO_4.7H_2O$, 220 mM KH_2PO_4, pH 7.0), 100 mM filter-sterile glucose and 100 mg X-gal (Melford Laboratories). [X-gal stock: 20 mg/ml in N,N-dimethylformamide (DMF)]. The X-gal–agar was then poured into sterile petri dishes and allowed to set before storage at 4°C.

2.3.6 (iv) Maintenance of Saccharomyces cerevisiae L40

Untransformed L40 [genotype *MAT*a *his*3Δ200 *trp1-901 leu2-3112 ade2 LYS2*::(4*lex*Aop-*HIS3*) *URA3*::(8*lex*Aop-*lacZ*) *GAL4*] were routinely grown in liquid YPD, or on YPD–agar plates, at 30°C. Once transformed with the appropriate plasmids (in this case pVP16 and pBTM116), yeast were maintained in DOM2 media.

2.3.6 (v) Preparation of transformation-competent L40

5 ml YPD was inoculated with a single colony of L40 and the culture grown at 30°C, with agitation at 250 rpm, overnight. The following day, 100 µl of this culture was diluted 1:500 into 50 ml YPD and grown for ~12 h (250 rpm, 30°C), until the OD_{600} was 1–2. Cells were pelleted at 2500 rpm, 4°C for 5 min, washed with 50 ml 0.1 M LiOAc in TE and repelleted. The cells were resuspended in 1 ml 0.1 M LiOAc in TE, shaken at 30°C, 250 rpm for 1 h, and immediately transformed.

2.3.6 (vi) Transformation of plasmid DNA into competent L40

100 µl yeast were added to a microcentrifuge tube already containing 1 µg of each of the appropriate bait and prey plasmids. 400 µl 50% PEG 3350 in TE was added, and the solutions mixed by inversion. Transformations were incubated at 30°C for 30 min, followed by heat-shock at 42°C for 20 min. The yeast were then pelleted by centrifugation for 30 sec at 13 000 rpm, and the supernatant aspirated. After a few seconds, any remaining liquid was re-aspirated and the pellet was resuspended in 100 µl filter-sterile PBS. The entire mixture was then plated out onto DOM2 plates and incubated for 3 days at 30°C.

2.3.6 (vii) Preparations of yeast whole cell lysates for SDS–PAGE

2.5 ml DOM2 media was inoculated with a single colony of transformed L40, and the culture grown at 30°C, with agitation at 250 rpm, overnight. The following day, cells were pelleted at 2600 rpm, 4°C for 5 min. The pellet was resuspended in 1 ml 0.25 M NaOH/1% β-mercaptoethanol and incubated on ice for 10 min. 160 µl 50% tricholoacetic acid was added and the mixture incubated on ice for a further 10 min. The solution was pelleted at 14 000 rpm for 10 min, and the pellet resuspended in 1 ml ice-cold acetone by vortexing vigourously. The cells were repelleted at 14 000 rpm for 10 min and, after air-drying, were resuspended in 200 µl 2 x Laemmeli sample buffer, boiled for 5 min and subjected to SDS–PAGE.

2.3.6 (viii) Yeast two-hybrid growth assay

Single transformants were streaked onto DOM2 plates and allowed to grow at 30°C for 3 days. The individual transformants were then streaked onto DOM1 plates, and their growth was observed over the following week.

2.3.6 (ix) Yeast two-hybrid colorimetric assay: X-gal plates

Single transformants were streaked onto DOM2 plates and allowed to grow at 30°C for 3 days. The individual transformants were then streaked onto X-gal plates, and the growth of blue colonies was observed over the following week.

2.3.6 (x) Yeast two-hybrid colorimetric assay: β-galactosidase filter assay

Single transformants were streaked onto DOM2 plates and allowed to grow at 30°C for 3 days. The individual transformants were then streaked onto grade 50, 9 cm diameter, Whatman® filter papers (Whatman International), overlying the media in DOM2 plates. Yeast were allowed to grow on the surface of the filters at 30°C overnight. The following day, the filter was submerged for 10 sec in liquid nitrogen to permeabilize the cells. Meanwhile, a Whatman grade 3 filter (9 cm diameter) was placed in a petri dish and saturated with fresh X-gal substrate solution [0.27 % β-mercaptoethanol, 1.67% X-gal in autoclaved Z-buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl and 1 mM $MgSO_4.7H_2O$; pH 7.0)]. The frozen filter was then placed, colony side up, on top of this saturated filter, and incubated at 30°C with constant observation of colour changes.

2.3.6 (xi) Yeast two-hybrid colorimetric assay: β-galactosidase solution assay

1 ml DOM2 media was inoculated with a single colony of transformed L40 and the culture grown at 30°C, with agitation at 250 rpm, overnight. The following day, cells were pelleted at 13 000 rpm for 5 min, resuspended in 500 µl Z buffer and repelleted. Cells were resuspended in 80 µl Z buffer by vortexing, and flash frozen in liquid nitrogen. Cells were then thawed at 37°C and pelleted at 13 000 rpm for 5 min. 15.4 µl supernatant was added to one well of each of two 96-well plates. The samples in one plate were subjected to a protein assay (see below). To the samples in the second plate, 106 µl Z buffer/0.27% β-mercaptoethanol was added. 24.6 µl ONPG (5 mg/ml in Z buffer) was then added, and the time taken for development of a yellow colour was recorded. Once yellow, 52.3 µl Na_2CO_3 was added to stop the reaction. Absorbance at OD_{405} was then read, and β-galactosidase activity calculated according to the following equation:

$$\text{Activity [nmol/(min x mg)]} = \frac{OD_{405} \text{ x total vol (ml)}}{0.0045 \text{ x [protein] (mg/ml) x extract vol (ml) x time (min)}}$$

2.3.6 (xii) Protein assay

15.4 µl supernatant (derived for the yeast two-hybrid solution assay; see above) was added to one well of a 96-well plate. 85 µl water was then added, followed by 100 µl mixed PIERCE reagent. (Protein standards were treated similarly for comparison. Typically 2, 5, 10, 15 and 20 µl of a 2mg/ml BSA solution were used.) The plate was then incubated at 37°C for 30 min and the absorbance at OD_{630} was read and used to calculate the transcriptional activity of the yeast two-hybrid solutions according to the equation above.

Chapter 3

Chapter 3: Screening yeast two-hybrid cDNA libraries for novel effectors of VEGF receptors

Chapter 3: Screening yeast two-hybrid cDNA libraries for novel effectors of VEGF receptors

3.1: Introduction

3.1.1: Effectors implicated in VEGF-induced signalling cascades

Stimulation of endothelial cells with VEGF has been shown to induce the phosphorylation of many well-known signalling components, including PLCγ, PI3K, GAP, Akt, ERK1/2 and SHPs 1 and 2, as well as of proteins found at intercellular junctions, such as VE-cadherin, occludin, FAK and paxillin (see section 1.6.3 for references and a more detailed discussion). However, despite this wealth of information, several observations have suggested the existence of additional, as yet uncharacterized, effectors. For example, the activation of ERK1/2 upon VEGF stimulation of primary endothelial cells does not appear to require the prototypical phosphorylation of Shc (Seetharam *et al.*, 1995). Similarly, the anti-apoptotic survival mechanism of VEGF, but not that of bFGF, requires VE-cadherin, thereby implying that VEGF-induced responses might proceed via mechanisms distinct from those employed by other RTKs (Carmeliet *et al.*, 1999a). In support of this hypothesis, VEGF-induced activation of ERK1/2 was much more efficient in VEGFR-2-expressing PAE cells (Kroll and Waltenberger, 1997) than in VEGFR-2-expressing NIH3T3 fibroblasts (Takahashi and Shibuya, 1997), and VEGF induced the phosphorylation of more proteins in PAE cells than in NIH3T3 cells (Takahashi and Shibuya, 1997; Yamane *et al.*, 1994). Unless the ability of VEGF to activate VEGFR-2 expressed by these two cell types differs; for example by the cell-type-specific expression of a co-receptor [ligand binding to VEGFR-2 has been shown to be enhanced in HUVECs because of the presence of the co-receptor neuropilin-1 (Soker *et al.*, 1998)], this discrepancy probably reflects the use of different effector proteins. Evidence in favour of the existence of unknown VEGF effectors was presented by Ito *et al.* (Ito *et al.*, 1998). In this study, three previously uncharacterized proteins were shown to bind VEGFR-1-derived peptides in a phosphorylation-dependent manner. One of these proteins, identified as a Grb2- and Grap-like protein, was shown to be expressed in endothelial, but not in fibroblast or malignant epithelial, cell lines

To search for such potential effectors, our objective was to screen cDNA libraries, in particular an endothelial cell library, for proteins able to interact with the VEGF receptors. Because expression of RTK intracellular domains in the absence of extracellular domains has previously been associated with constitutive tyrosine kinase activity (Dougher-Vermazen *et al.*, 1994; Mohammadi *et al.*, 1996; Hsu *et al.*, 1990), we decided to screen yeast two-hybrid expression libraries with the VEGFR intracellular domains.

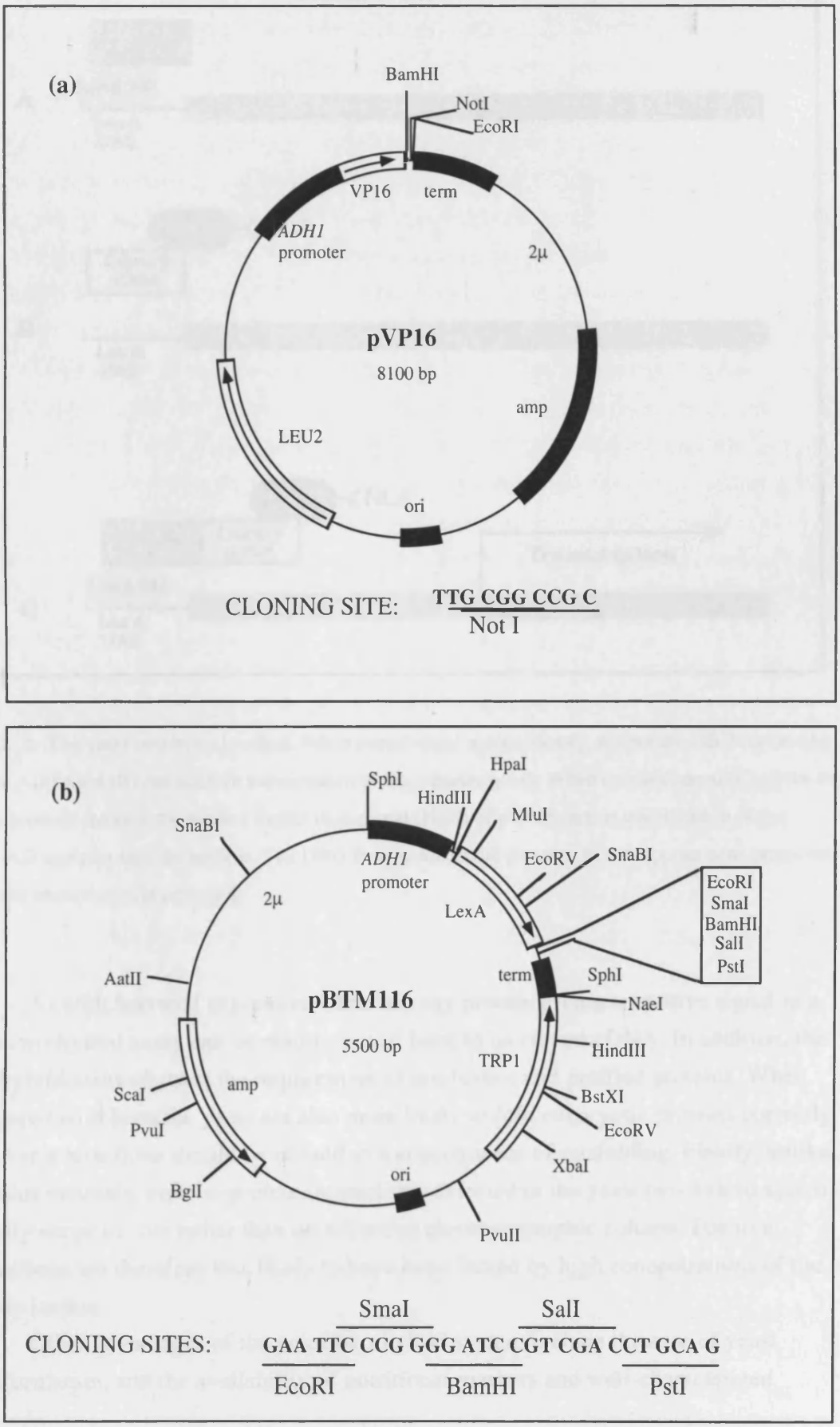
3.1.2: Screening for protein–protein interactions: the yeast two-hybrid system

Protein–protein interactions constitute the fundamental components of most signal transduction pathways. Such interactions have traditionally been studied with biochemical techniques such as cross-linking, co-immunoprecipitation and cofractionation. However, although these methods can identify protein–protein interactions, obtaining the cloned genes that encode the interacting proteins is often cumbersome. Bacterial expression libraries have alleviated this problem because the polypeptide sequence responsible for a positive interaction can be readily traced back to the corresponding cDNA. However, this process requires both a purified source of bait protein and good quality anti-bait antibodies. In addition, interactions could be missed in bacterial expression systems as a consequence of improper protein folding and lack of glycosylation.

In 1989, Fields and Song developed a powerful yeast-based genetic assay for the detection of protein–protein interactions: the yeast two-hybrid system (Fields and Song, 1989; reviewed in Fields and Sternglanz, 1994). This system exploits the modular nature of transcription factors, which allows the physical separation of DNA-binding and activation domains (DBDs and ADs, respectively) while retaining their functional activity. A physically distinct DBD can still interact with its target DNA sequence and, likewise, if recruited to a promoter sequence, an independent AD can still activate transcription.

To analyse the ability of two proteins to interact in the yeast two-hybrid system, their cDNA sequences are subcloned, one downstream of a DBD and the other downstream of a compatible AD, to generate in-frame fusion proteins. The most frequently used combinations of domains are either: (1) the DBD and AD of the yeast protein Gal4p; or (2) the DBD of the *E. coli* lexA repressor and the AD of VP16, a transcription factor from *Herpes* virus (Fig. 3.1). Both AD and DBD plasmids contain markers for selection in yeast (LEU2 and TRP1 in pVP16 and pBTM116, respectively) and bacteria (amp). They also have both yeast (2μ) and bacterial (ori) origins of replication. The two plasmids are cotransformed into an auxotrophic yeast strain and the nutritional markers on the plasmids enable the selection of colonies that contain both plasmids. Such yeast are then subjected to appropriate reporter assays to analyse whether the two fusion proteins interact with each other. The reporter genes, integrated into the yeast genome, are under the control of a minimal GAL1 promoter that is fused to multiple binding sites for the DBD encoded by the bait construct. The DBD construct is therefore recruited to these promoters. The AD construct will only be recruited to the promoter if the AD and DBD fusion proteins interact. Once at the promoter, the AD can then initiate reporter gene transcription (Fig. 3.2).

Fig. 3.1. Maps of (a) DNA activation domain and (b) DNA binding domain vectors



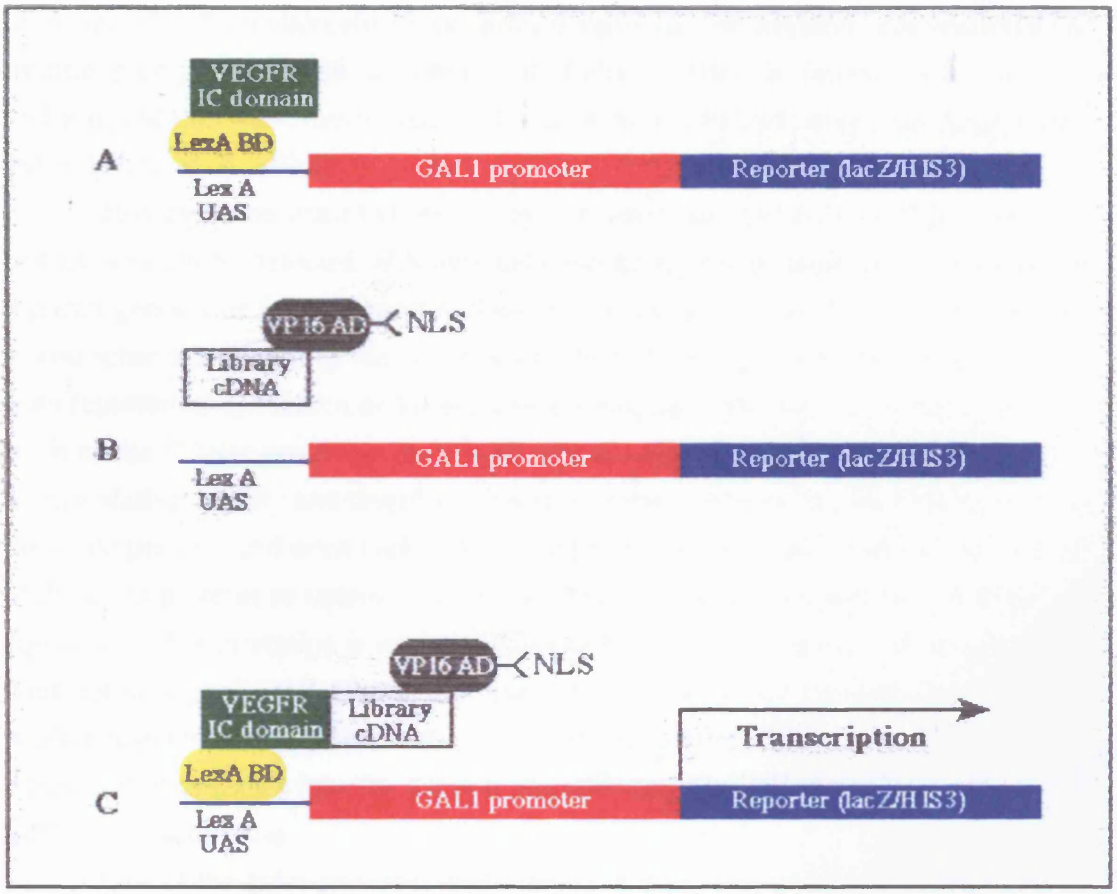


Fig. 3.2: The yeast two-hybrid system. When transformed independently, neither the DBD fusion (A) nor the AD fusion (B) can activate transcription of the reporter genes. When cotransformed (C), if the two fusion proteins interact, the nuclear localization signal (NLS) of pVP16 directs translocation of the DBD–AD complex into the nucleus. The DBD then localizes the complex to the reporter gene promoters and their transcription is activated.

As with bacterial expression libraries, any protein giving a positive signal in a yeast two-hybrid assay can be readily traced back to its cloned cDNA. In addition, the two-hybrid assays bypass the requirement of antibodies and purified proteins. When compared with bacteria, yeast are also more likely to fold eukaryotic proteins correctly, so fewer interactions should be missed as a consequence of misfolding. Finally, unlike previous methods, protein–protein interactions detected in the yeast two-hybrid system actually occur *in vivo* rather than on a filter or chromatographic column. Positive interactions are therefore less likely to have been forced by high concentrations of the protein factors.

Other advantages of the yeast two-hybrid system include the ease of yeast transformation, and the availability of nutritional markers and well-characterized

reporter genes. In addition, studies have suggested that the two-hybrid system might be more sensitive than alternative biochemical methods. For example, conventional co-immunoprecipitation methods consistently failed to detect an interaction between Ras and Raf, but this was clearly identified in a yeast two-hybrid assay (van Aelst *et al.*, 1993; White *et al.*, 1995).

However, the yeast two-hybrid system does have limitations. First, false positives might be detected, although their frequency can be minimized by using two reporter genes, one for a colorimetric assay (e.g. the *lacZ* gene from *E. coli*) and one for auxotrophic selection (e.g. the *HIS3* gene). Selecting only clones that are positive in both reporter assays increases the screening stringency, thereby significantly reducing the number of false positives (e.g. clones encoding His3 analogues), and also allowing a higher plating density and therefore a more extensive screen. Second, unless mutants of the same protein (and even then mutants without large structural differences), the ability of different proteins to interact with a particular bait protein cannot be quantitatively compared. This limitation is imposed because a weaker activation of the reporter genes does not necessarily reflect a weaker interaction between two proteins. Instead, a weaker response could reflect differences between the two proteins in, for example, the efficiency of transport into the nucleus, the efficiency of folding or the stability of the mRNA and/or protein.

One of the most powerful applications of yeast two-hybrid technology is the identification of interacting partners for a specified target protein by library screening. For such purposes, the protein of interest, or bait, is usually fused to the DBD, whereas a library of cDNAs is fused to the ADs. This orientation is favoured because random protein fragments (i.e. from the library) are more likely to have transcriptional activity than they are DNA-binding ability. With respect to RTK signalling, screening yeast two-hybrid libraries with receptor intracellular domains has become a favoured approach by which to identify phosphorylation-dependent interactions that might occur downstream of RTK activation (for examples see O'Neill *et al.*, 1994; Bourette *et al.*, 1997). However, for all interactions, it is important to remember that the yeast two-hybrid system can only be used as a preliminary indication of a potential interaction. For a positive interaction to have potential relevance in mammalian cells, the two proteins must also exhibit overlapping expression patterns and the protein sites responsible for mediating the interaction must be accessible *in vivo*.

3.1.3: Enhancing the tyrosine phosphorylation of VEGFR-1

Many of the pathways mediated through RTKs are initiated by the binding of effector molecules to specific phosphotyrosine residues within the cytoplasmic domains of the receptor. Because VEGF-induced phosphorylation of VEGFR-1, both in intact cells and *in vitro*, appears to be very weak (see section 1.6.2), steps were taken to ensure that the

intracellular domain of this receptor was sufficiently phosphorylated for use as bait in yeast two-hybrid library screens. Ret/ptc2 is a papillary thyroid cancer oncogene that encodes a constitutively active version of the c-Ret RTK (Lanzi *et al.*, 1992). In this oncogene, the N-terminal two-thirds (236 amino acids) of the type I regulatory subunit (RIα) of cAMP-dependent protein kinase (PKA) are fused to the tyrosine kinase domain of c-Ret. It was subsequently shown that fusion of just the N-terminal dimerization domain of RIα (residues 1–84) to the tyrosine kinase domain of c-Ret (and to that of the EGFR) was sufficient to confer constitutive activity upon the protein (Durick *et al.*, 1995). Furthermore, an RIα–Ret bait identified more phosphorylation-dependent interactions in yeast two-hybrid library screens than did the receptor's native intracellular domain (Kyle Durick, personal communication). It was therefore decided to incorporate the RIα-derived sequence into the VEGFR-1 bait construct in an attempt to enhance phosphorylation of the recetor's intracellular domain.

3.2: Materials and methods

Human dermal microvascular endothelial cell (hMVEC) lysates were kindly provided by James R. Feramisco [University of California–San Diego (UCSD), CA, USA]. The pVP16 mouse embryo library was constructed by Stan Hollenberg (Fred Hutchinson Cancer Research Center, Seattle, WA, USA), and the pBTM116 vector was constructed by Paul Bartel and Stan Fields (State University of New York, Stony Brook, NY, USA). RET–PTC2/pBTM116 was obtained from Susan Taylor (UCSD) and Lamin/pBTM116 from Linda van Aelst (Cold Spring Harbor).

3.2.1: Construction of a hMVEC cDNA library in the yeast two-hybrid pVP16 vector

3.2.1 (i) DMPC-treating solutions

All solutions for the preparation and analysis of RNA were made up in dimethyl pyrocarbonate (DMPC)-treated water, to inactivate RNases. A 1% stock solution of DMPC in 50% ethanol was diluted 1:10 to give a working concentration of 0.1% DMPC. The solution was incubated at room temperature for 30 min, before autoclaving to remove residual DMPC.

3.2.1 (ii) Extraction of total RNA from hMVEC lysates

Aliquots of hMVEC lysate, stored at –80°C, were defrosted on ice and homogenized to shear high molecular weight genomic DNA. Homogenization was performed using a biopolymer shredding system (QIAshredder; QIAGEN), in accordance with the protocol for monolayer cultures provided with the kit. Total RNA was isolated from the homogenate using the RNeasy Midi kit (QIAGEN), following the protocol for isolation of total RNA from animal cells. This isolation excluded transcripts <200 bases in length (i.e. tRNAs and small rRNAs) via the selective binding properties of a silica gel-based membrane. The absorbance of the total RNA isolated was measured at OD_{260}, and the RNA concentration was calculated assuming a solution with $OD_{260} = 1$ has a concentration of 40 µg/ml.

3.2.1 (iii) Separation of RNA by formaldehyde–agarose (FA) gel electrophoresis

Before casting the gel, the plastic gel tray and comb were washed in 0.1 M NaOH to remove RNases. A 1% FA gel, consisting of 1% agarose in 2.2 M formaldehyde and 1 x filter-sterile formaldehyde running buffer [5 x: 0.1 M MOPS (pH 7.0), 40 mM autoclaved NaAc, 5 mM autoclaved EDTA (pH 8.0)] was then poured and allowed to set.

Before loading, 2 µg of RNA sample was dried in a speedivac at 1000 rpm, 38°C for 15 min, and the RNA redissolved in 4.5 µl DMPC-treated H_2O. 2 µl 5 x filter-sterile formaldehyde running buffer, 3.5 µl formaldehyde and 10 µl formamide were

then added, the mixture incubated at 65°C for 15 min, and then placed on ice. Contents were pelleted, and 1 µl EtBr (1mg/ml) and 2 µl DMPC-treated loading buffer were added. The gel was pre-run at 60 V in a 10 cm tank for 5 min. Samples were then loaded and the gel was run at 55 V for 1.5 h.

3.2.1 (iv) Purification of mRNA from total RNA

mRNA was purified from total RNA using the DYNABEADS Oligo (dT)25 mRNA Purification Kit (DYNAL). The methodology employed by this kit involves the selective capture of mRNAs by the Dynabeads through base pairing between their 3' poly(A)$^+$ nucleotides and the oligo(dT) nucleotides covalently coupled to the surface of the beads. The magnetic beads, and the attached mRNAs, are then collected and washed by a Magnetic Particle Concentrator. mRNA is eluted in a buffer that destabilizes the dT:rA hybrid. mRNA purification was conducted in accordance with the manufacturer's protocol, with the addition of a final wash in the 1st strand buffer from the Two-Hybrid cDNA Library Constuction Kit (Clontech), to prepare for the downstream enzymatic conversion of mRNA into cDNA.

3.2.1 (v) Reverse transcription of mRNA into cDNA

mRNA was reverse transcribed into cDNA using a modified version of the Two-Hybrid cDNA Library Constuction Kit protocol (Clontech). Only 300 ng poly A$^+$ RNA template was used. In addition, first strand cDNA synthesis was initiated exclusively by random primers, and the primer concentration was doubled to favour the generation of shorter cDNA fragments. The second cDNA strand was then synthesized according to the manufacturer's protocol.

3.2.1 (vi) Amplification of cDNAs

Resultant double-stranded cDNAs were treated with T4 DNA Polymerase (as described in the Two-Hybrid cDNA Library Constuction Kit; Clontech) to create blunt ends, onto which *Not* I phosphorylated adaptors were then ligated. Using a primer complementary to the *Not* I adaptor, the cDNAs were then amplified by PCR. (The conditions for this reaction were essentially the same as those described in section 2.3.2 (xii), with the exception that one reaction was only conducted for 12 cycles. 70 ng DNA was used as template.)

3.2.1 (vii) Ethidium bromide estimation of DNA concentration

For samples containing insufficient DNA to be visualized by agarose gel electrophoresis, the DNA concentration was estimated by directly mixing the DNA with ethidium bromide, and viewing the mixture under ultraviolet light. 5 µl of a 1:5 dilution of the DNA sample was spotted onto Nescofilm. For comparison, DNA of a known

concentration was diluted, in aliquots of 5 µl, to provide a range of standards containing 5–100 ng. These were also spotted onto Nescofilm. 5 µl ethidium bromide (2 ng/µl) was then mixed with the samples on the Nescofilm, and the spots were viewed under ultraviolet light.

3.2.1 (viii) Electroporation of the pVP16 library into ELECTROMAX DH12S™ competent cells

Before electroporation, library cDNAs were precipitated using glycogen carrier as described in the Two-Hybrid cDNA Library Constuction Kit (Clontech). The precipitated ligation mixtures were then centrifuged at 15 000 rpm for 20 min. Pellets were air dried, resuspended in 5 µl DMPC-treated de-ionized H_2O, and divided into 2.5 µl aliquots.

Electroporation was performed using 0.1 cm electrode gap gene pulsar cuvettes (Bio-Rad) and a Bio-Rad Gene Pulsar™ electroporation chamber. SOC medium, cuvettes, microcentrifuge tubes and 15 ml polypropylene tubes containing 800 µl SOC, were prechilled on ice. ELECTROMAX DH12S™ competent cells [genotype ϕ80d*lac*ZΔM15 *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *ara*D139 Δ(*ara*, *leu*)7697 Δ*lac*X74 *gal*U *gal*K *rps*L *deo*R *nup*G *rec*A1/F' *pro*AB$^+$ *lac*I^q ZΔM15; Gibco BRL] were thawed on ice and divided into 50 µl aliquots. Each 2.5 µl DNA aliquot was then added to an aliquot of cells and mixed with a pipette tip. The mixture was transferred to a cuvette and electroporated at 1.8 kV. Immediately after the pulse had been delivered, 150 µl SOC [see section 3.2.1 (ix) for composition] was added to the mixture in the cuvette to facilitate transfer to a SOC-containing polypropylene tube. The cuvette was then washed with a further 50 µl SOC. The electroporation mixtures were incubated at 37°C, with shaking at 225 rpm, for 1 h.

10 µl of one electroporation mixture was then mixed with 40 µl LB, and plated out onto a 9 cm diameter LB–Amp (50 µg/ml) plate (for estimation of electroporation efficiency). The rest of this mixture, and all other electroporation mixtures, were plated out onto 22 cm^2 LB–Amp (50 µg/ml) plates, and incubated at 37°C overnight.

3.2.1 (ix) SOB medium

Bacto-tryptone (20 g/L), Bacto-yeast extract (5 g/L) and NaCl (0.5 g/L) were dissolved in de-ionized water. 2.5 mM KCl was then added, the pH adjusted to 7.0, and the media autoclaved. For SOC media, autoclaved SOB was cooled to 60°C before the addition of 20 mM filter-sterile glucose. 10 mM autoclaved $MgCl_2$ was then added just before use.

3.2.1 (x) Harvesting library DNA

Cells from the 22 cm^2 LB–Amp plates were scraped into 30 ml LB, and the plates rinsed with a further 25 ml LB. Cells from the 25 ml rinse, as well as half of those from

the 30 ml scrape, were pelleted at 8000 rpm for 15 min. The DNA from these cells was then isolated according to the PEG method [see section 2.3.2 (x)], with the exception that, after ethanol precipitation (before the addition of PEG), the DNA was resuspended in 2 ml H_2O instead of 1 ml TE. Also, the final DNA pellets were resuspended in 250 µl, instead of 100 µl, TE.

Cells from the second half of the 30 ml scrape were mixed with glycerol to produce 25% glycerol stocks. These were aliquoted, flash frozen in a dry-ice–ethanol bath and stored at –80°C.

3.2.2: Construction of bait plasmids for yeast two-hybrid assays

3.2.2 (i) Bait construction primers

KDR.F (forward): 5'–CCTAGGTCGACTTAAGCGGGCCAATGGAGGG–3'
KDR.R (reverse): 5'–CTGCCTCGAGTTAAACAGGAGGAGAGCTCAG–3'
FLT.F (forward): 5'–CTCCGGATCCGAAAAATGAAAAGGTCTTCT–3'
FLT.R (reverse): 5'–GTATCTGCAGCTAGATGGGTGGGGTGGAGTA–3'
RI.F (forward): 5'–GCAGGAATTCATGGAGTCTGGCAGTACC–3'
RI.R (reverse): 5'–CTTTGGGATCCCCATGAGGATTCTTCTATA–3'

3.2.2 (ii) Bait mutagenesis primers

K861A.F (forward): 5'–GGACTGTGGCTGTGGCCATGCTGAAAGAGGG–3'
K861A.R (reverse): 5'–CCCTCTTTCAGCATGGCCACAGCCACAGTCC–3'
Y794F.F (forward): 5'–GAAATAAAGACTGACTTCCTATCAATTATAATGGACCC–3'
Y794F.R (reverse): 5'–GGGTCCATTATAATTGATAGGAAGTCAGTCTTTATTTC–3'
Y1169F.F (forward): 5'–GCAGGATGGTAAAGACTTCATCCCAATCAATGCC–3'
Y1169F.R (reverse): 5'–GGCATTGATTGGGATGAAGTCTTTACCATCCTGC–3'
Y1213F.F (forward): 5'–GGAAGCTCTGATGATGTCAGATTCGTAAATGCTTTCAAG–3'
Y1213F.R (reverse): 5'–CTTGAAAGCATTTACGAATCTGACATCATCAGAGCTTCC–3'

3.2.3: Screening yeast two-hybrid cDNA libraries

3.2.3 (i) Library screen transformation

The method used for a library screen transformation was essentially a scale-up of the method used for a small-scale transformation [(section 2.3.6 (vi)], with the exception that transformed yeast were plated onto plates lacking histidine as well as tryptophan and leucine.

A 500 ml yeast culture was routinely used for library screens. Cells were pelleted, washed and incubated for 1 h, as described for a small-scale transformation. The amounts of yeast, DNA and reagents used at each subsequent step were doubled in comparison with those used for small-scale transformations. The efficiency of transformation, and therefore the percentage of library screened, was estimated by

plating 20 µl of one transformation mixture onto a 9 cm diameter DOM2 plate. The rest of the mixure, and all the other mixtures, were then plated onto 15 cm diameter DOM1 plates containing 30 mM 3-AT.

3.2.3 (ii) Isolation of plasmid DNA from positive transformants

Yeast colonies testing positive for reporter transactivation were scraped off into microcentrifuge tubes containing 100 µl yeast lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris/HCl (pH 8.0), 1 mM EDTA] and 200 µl phenol : chloroform : isoamyl alcohol (25:24:1). A small spatula of 425–600 µm, acid-washed glass beads (Sigma) was added, and the tube vigorously vortexed for 2 min, and then centrifuged for 5 min. DNA was isolated from the aqueous phase by ethanol precipitation. After air-drying, the pellet was resuspended in 30 µl de-ionized H_2O.

3.2.3 (iii) Preparation of HB101 electrocompetent cells

50 ml SOB was inoculated with a single colony of HB101 (genotype F^- *mcr*B *mrr* *hsd*S20(r_B^- m_B^-) *rec*A13 *leu ara*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^r) *sup*E44 λ^-), and grown at 37°C, with agitation at 225 rpm, overnight. 0.5 ml of this culture was diluted 1:1000 into 500 ml SOB and grown until the OD_{550} reached 0.8. The cells were harvested by centrifugation at 5000 rpm for 15 min. The pellet was washed three times with 500 ml ice-cold, autoclaved, wash buffer (10% redistilled glycerol) and then resuspended in 2 ml wash buffer. Unless used immediately, the cells were flash frozen in a dry-ice–ethanol bath and stored at –80°C.

3.2.3 (iv) Electroporation of recovered plasmids into electrocompetent HB101

The method used for electroporation into HB101 was essentially the same as that described for electroporation into ELECTROMAX DH12S™ [see section 3.2.1 (viii)]. Modifications to this protocol were that 1 µl DNA was electroporated into a 20 µl aliquot of HB101, and that, after the recovery step, only 100 µl of the electroporation mixtures were plated-out, and this was onto 9 cm diameter LB–Amp (100 µg/ml) plates.

3.2.3 (v) M9 medium

410 mM Na_2HPO_4, 220 mM KH_2PO_4, 86 mM NaCl and 187 mM NH_4Cl were dissolved in de-ionized H_2O, the pH adjusted to 7.2–7.6, and the media autoclaved. Autoclaved media was cooled to 60°C before the addition of 4 mg/L thiamine (2 mg/ml stock). Bactoagar (20 g/L) was then added, and the media re-autoclaved. When cooled to ~60°C, 0.2% filter-sterile glucose, 100 nM autoclaved $CaCl_2$, 1mM autoclaved $MgSO_4$ and 2 mg thiamine were added, the plates poured and allowed to set.

3.2.3 (vi) Isolation of DNA from library plasmids in HB101 cells growing on M9 media
Individual HB101 colonies growing on M9 plates were used to inoculate 5 ml LB–Amp (100 µg/ml), and allowed to grow at 37°C, with shaking at 225 rpm, overnight. The following day, 1.5 ml of these mini-cultures were pelleted, and the cells resuspended by vortexing in 100 µl cold solution I. The mixture was incubated at room temperature for 5 min. 200 µl solution II was then added, the tubes inverted 10 times, and incubated at room temperature for a further 5 min. 300 µl solution III was then added, the tubes vortexed and incubated at room temperature for 5 min. 600 µl 5 M LiCl was added and the solution centrifuged, at 13 000 rpm, for 5 min at room temperature. 1 ml of the supernatant was transferred into a fresh microcentrifuge tube containing 600 µl isopropanol. The tubes were vigorously vortexed and centrifuged for 5 min at room temperature. The pellet was washed with 1 ml cold 70% ethanol, air-dried for 5 min and resuspended in 40 µl TE.

3.2.3 (vii): Sequencing primers
pVP16.F (forward): 5'–GAGTTTGAGCAGATGTTTA–3'
pVP16.R (reverse): 5'–TGTAAAACGACGGCCAGT–3'

3.3: Results

3.3.1: Construction of a cDNA library from hMVECs

3.3.1 (i) Extraction of total cellular RNA

Human microvascular endothelial cell lysates were homogenized and the total cellular RNA purified (Fig. 3.3).

3.3.1 (ii) Isolation of mRNA from total cellular RNA

Approximately 300 ng mRNA template was needed for construction of the hMVEC cDNA library. mRNA constitutes 1–5% of the total cellular RNA. Therefore, assuming that mRNA constitutes only 1% of total RNA, at least 30 μg total cellular RNA was required to ensure the isolation of 300 ng mRNA. An aliquot containing 32 μg total RNA was used as the source for mRNA purification. The mRNA was purified from this aliquot by capture with immobilized oligo dT, and ~600 ng mRNA was obtained. Agarose gel electrophoresis of 50 ng of this mRNA sample showed that the sample no longer contained ribosomal RNA (data not shown).

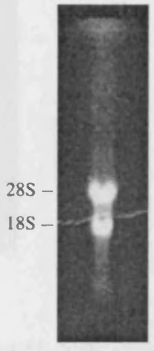


Fig. 3.3: Isolation of total cellular RNA from hMVEC lysates. Total cellular RNA was purified from cell lysates and visualized by agarose gel electrophoresis. The two prominent bands represent the 18S and 28S rRNAs. mRNAs and degraded RNAs are visible as smearing.

3.3.1 (iii) Transcription of mRNA into double-stranded cDNA

Approximately 300 ng mRNA was used as a template for cDNA synthesis. [Although the cDNA library construction kit advised using 5 μg mRNA, Hollenberg *et al.* had constructed a representative cDNA library from only 100 ng mRNA (Hollenberg *et al.*, 1995).] Deviating from the kit's protocol, first strand cDNA synthesis was initiated exclusively by random primers, rather than by random and oligo(dT) primers, to

prevent over-representation of 3′ sequences, and the primer concentration was doubled to favour the generation of shorter cDNA fragments. *Not* I adaptors, supplied in the kit, were ligated onto the resultant cDNA ends, and the products were run on a 1.7% agarose gel. cDNAs 350–700 bps in size were purified from this gel; such size selection was enforced so that resultant polypeptides would be of optimal length to fold into independent domains such as SH2 and PTB domains.

The purified, size-selected cDNAs were then amplified by PCR using a primer complementary to the *Not* I adaptor, for either 19 or 12 cycles, and the products analysed by agarose gel electrophoresis (Fig. 3.4). To minimize mutations generated by excessive PCR cycles, and the potential loss of representation, the product resulting from 12 cycles of amplification was chosen for subcloning into the pVP16 vector.

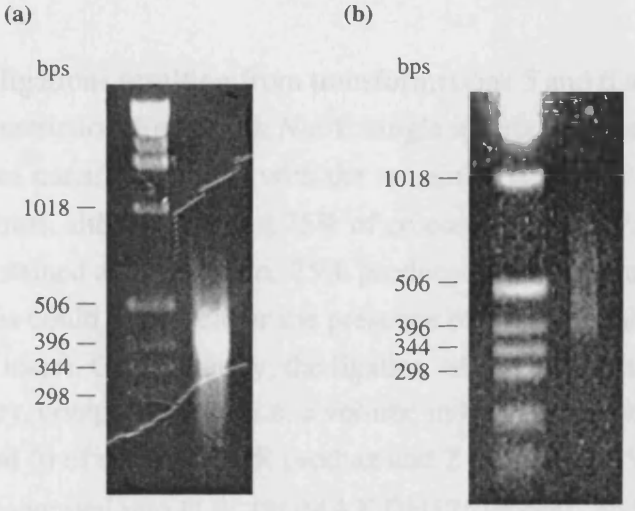


Fig. 3.4: Amplification of cDNA library inserts. Size-selected (350–700 bps) cDNAs, ligated to *Not* I adaptors, were amplified by PCR for (a) 19 cycles and (b) 12 cycles.

3.3.1 (iv) Ligation of cDNAs into pVP16 and transformation into DH5α

The PCR product was purified, digested with *Not* I and repurified. Similarly, the pVP16 vector (Fig. 3.1) was digested with *Not* I, treated with alkaline phosphatase and purified. Variable volumes of the digested PCR product were then ligated with 2 μl (~0.15 μg) digested vector, and the ligations were transformed into DH5α. The number of colonies resulting from these transformations are shown in Table 3.1.

Transformation	PCR product (μl)	Number of colonies
1	0.0	5
2	0.1	14
3	0.5	37
4	1.0	78
5	2.0	128
6	5.0	156
7	10.0	168

Table 3.1: Optimization of vector : insert ligation ratio before library construction. Variable volumes of digested PCR products were ligated with a constant amount (~0.15 μg) of digested pVP16 vector, the ligations transformed into DH5α and the number of resultant colonies counted.

The products of the ligations resulting from transformations 5 and 6 were analysed for insert presence by restriction digest with *Not* I. Single inserts were present in 75% of colonies derived from transformation 5, with the remaining 25% having no inserts (data not shown). By contrast, although at least 75% of colonies resulting from transformation 6 contained a single insert, 25% produced two bands upon digestion (data not shown); this could reflect either the presence of two original inserts or cleavage of a single insert. Consequently, the ligation whose products were to be used to generate the library, comprised 3 μl (i.e. a volume in between those used in transformations 5 and 6) of digested PCR product and 2 μl digesed pVP16. Six such ligations were electroporated into ELECTROMAX DH12S™ cells, and these generated ~2.46 x 10^6 colonies in total. [The mouse embryo library produced by Hollenberg *et al.*, which was considered representative, contained 5 x 10^6 clones (Hollenberg *et al.*, 1995).] *Not* I restriction analysis of representative colonies from this electroporation demonstrated that ~90% of colonies contained a single insert, with the remaining 10% having no insert (Fig. 3.5). These cells were harvested and the library DNA was isolated.

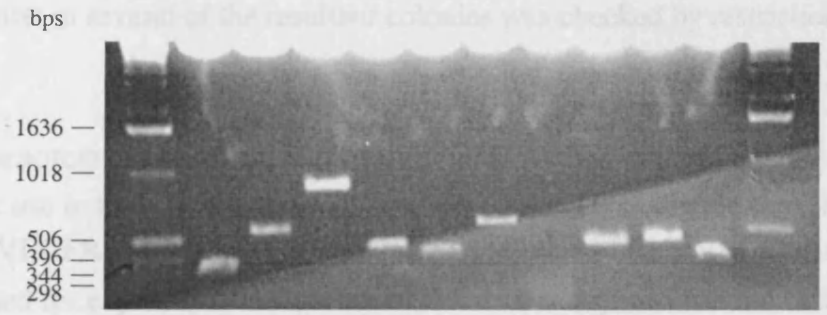


Fig. 3.5: Restriction analysis of library inserts. A ligation 'optimized' to insert one library cDNA per pVP16 vector was electroporated into ELECTROMAX DH12S™ cells. Ten representative colonies from this electroporation were analysed for insert presence by *Not* I restriction digest followed by agarose gel electrophoresis.

3.3.2: Construction of bait proteins

The cytoplasmic domain of VEGFR-1 (residues 781–1338) was amplified by PCR using primers incorporating a 5′ *Bam* HI site (FLT.F) and a 3′ *Pst* I site (FLT.R). [The conditions for this reaction were similar to those described in section 2.3.2 (xii) with the exceptions that annealing was conducted for 1 min at 54°C, elongation was permitted for 2.5 min per cycle, and Pfu polymerase was used instead of Taq. 500 ng DNA was used as template.] The PCR products were run on a 1% agarose gel, and a fragment of the expected size was band purified. Following digestion with *Bam* HI and *Pst* I, this fragment was ligated into the corresponding sites of the pBTM116 vector (Fig. 3.1) to generate in-frame fusions with the LexA DBD. Similarly, the cytoplasmic domain of VEGFR-2 (residues 788–1357) was amplified by PCR with primers incorporating a 5′ *Sal* I site (KDR.F) and a 3′ *Xho* I site (KDR.R). [Conditions were as described in section 2.3.2 (xii). 300 ng DNA was used as template.] The resultant fragment was again band purified from a 1% agarose gel, digested with *Sal* I and *Xho* I, and ligated into the *Sal* I site of pBTM116.

The N-terminal 236 amino acids of RIα were amplified by PCR using primers incorporating 5′ *Eco* RI (RI.F) and 3′ *Bam* HI sites (RI.R). [Conditions were as described in section 2.3.2 (xii). 300 ng DNA was used as template.] The PCR products were run on an agarose gel, and the resultant fragment band purified. Following digestion with *Eco* RI and *Bam* HI, this fragment was ligated into the corresponding sites of both pBTM116 and VEGFR-1/pBTM116. In the case of VEGFR-1/pBTM116, this ligation placed the RIα sequence, in-frame, inbetween the LexA and VEGFR-1 sequences. All four of these ligations were transformed into DH5α, and insert presence

and orientation in several of the resultant colonies was checked by restriction enzyme analysis.

3.3.3: Characterization of bait proteins

Before their use in library screens, the fusion proteins encoded by the three bait constructs, VEGFR-1/pBTM116, VEGFR-2/pBTM116 and RIα–VEGFR-1/pBTM116, were analysed for expression, autophosphorylation and any intrinsic transactivation activity. (Although library screening results for VEGFR-2 are not included in this thesis, the VEGFR-2/pBTM116 characterization data are as this construct was used for experiments described in chapters 5 and 6.)

3.3.3 (i) Fusion protein expression and phosphorylation

Each of the three bait constructs were cotransformed into yeast with the pVP16 vector. Whole cell lysates of the resultant transformants were analysed by western blotting. Membranes were probed with antibodies recognizing either LexA or phosphotyrosine to detect expression and phosphorylation of the fusion proteins, respectively. As can be seen from Fig. 3.6, fusion proteins corresponding to all three constructs are expressed. However, although autophosphorylation of all three proteins can be observed, that of VEGFR-1 appears to be enhanced by incorporation of the RIα sequence.

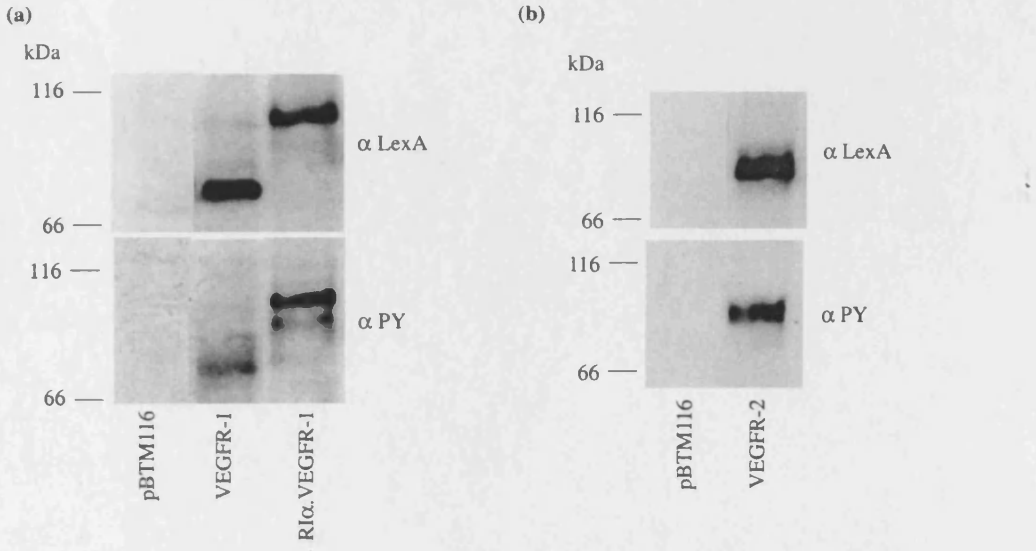


Fig. 3.6: Expression and phosphorylation of bait proteins. Yeast were cotransformed with the pVP16 vector and (a) either VEGFR-1 or RIα–VEGFR-1, or (b) VEGFR-2. Whole cell lysates from representative colonies were then subjected to western blotting and probed with antibodies to LexA (top panels) and phosphotyrosine (bottom panels) to detect expression and phosphorylation of the fusion proteins, respectively.

3.3.3 (ii) Intrinsic transactivation properties of bait proteins

Each of the three bait constructs were cotransformed into yeast with either pVP16 (as a negative control) or a VP16-fusion protein known to interact with the bait (as a positive control). For VEGFRs 1 and 2, these positive controls were Nedd4 and Sck, respectively, both of which (described in chapters 5 and 6) had been previously isolated as VEGFR-binding proteins from screens of a mouse embryo cDNA library (Warner *et al.*, 2000 and unpublished). Comparisons of the transactivation properties of these negative and positive controls would detect any intrinsic activity associated with the bait and, if evident, also determine whether this was of sufficient strength to hinder the selection of true positives from a library screen.

Preliminary experiments demonstrated that the positive and negative controls could be clearly distinguished in β-galactosidase assays (Fig. 3.7). However, the growth of positive and negative control transformants on DOM1 media was indistinguishable (data not shown). Because the *HIS3* promoter allows a basal level of expression, we tested the effect of adding 3-AT, a competitive inhibitor of the *HIS3*-encoded enzyme imidazoleglycerol-phosphate dehyratase, to the DOM1 media. A concentration of 30 mM 3-AT was found to sufficiently suppress basal HIS3 activity while preserving that resulting from activated transcription (Fig. 3.7).

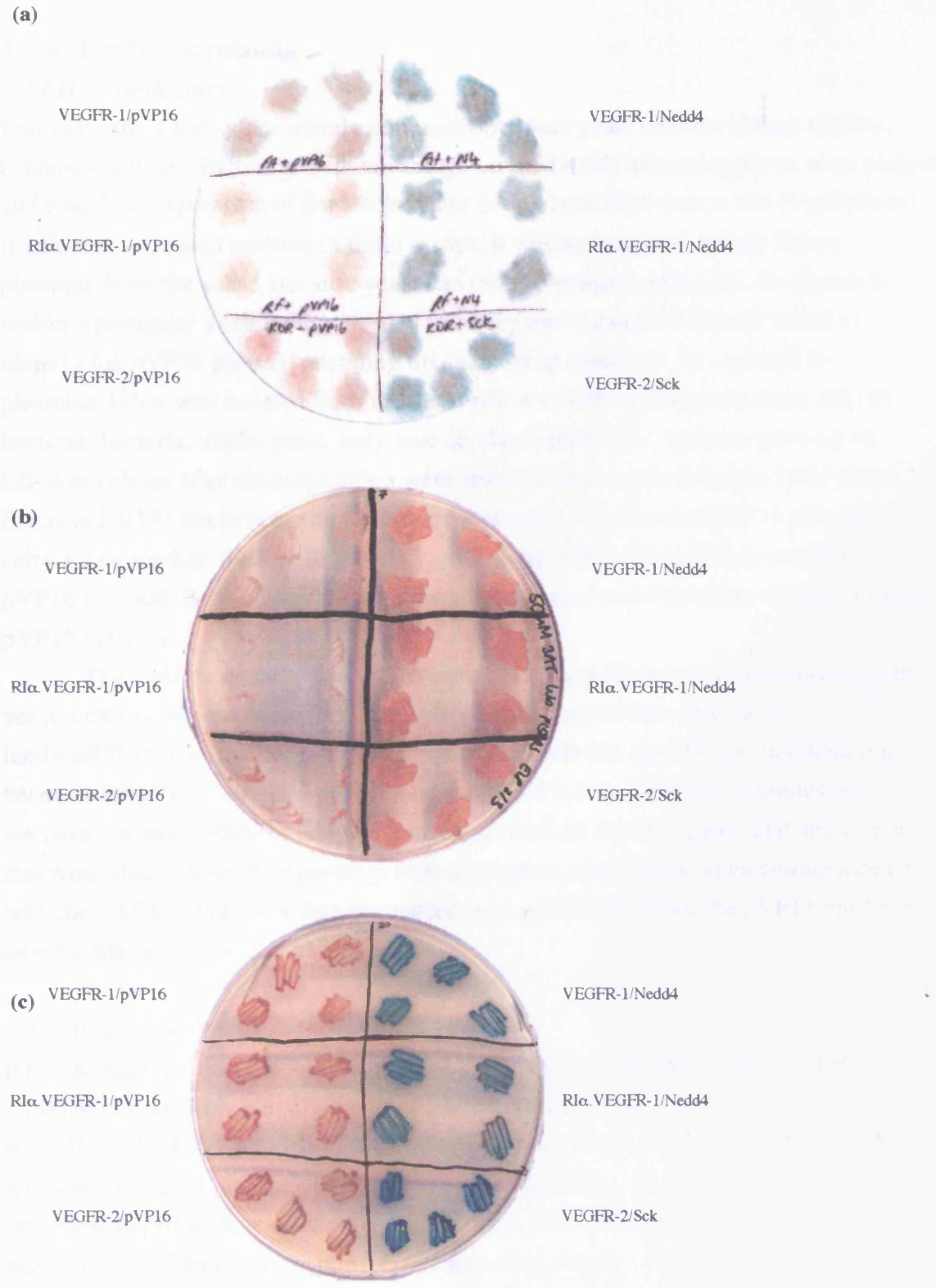


Fig. 3.7. Intrinsic transactivation properties of bait proteins. The VEGFR-1, RIα.VEGFR-1 and VEGFR-2 pBTM116 constructs were cotransformed into yeast with either an empty pVP16 vector, or a construct encoding a protein known to interact with the receptors: Nedd4 and Sck for VEGFRs 1 and 2, respectively. Resultant colonies were analysed for activation of reporter genes by (a) β-galactosidase filter assays, (b) growth on plates lacking histidine and (c) growth on X-gal plates.

3.3.4: Library screening

3.3.4 (i) Introduction

Bait plasmids were independently cotransformed into yeast with the library cDNAs. Colonies with an obvious growth advantage on the DOM1 screening plates were picked and tested for expression of β-galactosidase (using both filter assays and X-gal plates). If colonies were also positive in these assays, it was attempted to isolate library plasmids from the yeast. Because yeast can take up multiple plasmids, the plasmids within a particular yeast colony needed to be separated and individually tested to identify the pVP16 plasmid encoding the interacting sequence. To separate the plasmids, DNA was isolated from the yeast colonies and electroporated into HB101 bacteria: bacteria, unlike yeast, only take up single plasmids. Colonies growing on LB–Amp plates after electroporation were streaked onto minimal media (M9) plates. Because HB101 bacteria are auxotrophic for leucine, and because pVP16 plasmids carry a Leu marker, these cells can only grow on minimal media if they contain a pVP16 plasmid. Each colony growing on an M9 plate should therefore contain a single pVP16 plasmid.

The DNA from up to five M9 colonies per yeast clone was retransformed into yeast with the original bait. They were also cotransformed into yeast with lamin/pBTM116 to control for any activation that was not specific for an interaction between the pVP16-encoded protein and the VEGFR bait. Resultant colonies were assessed for their ability to activate transcription of the reporter genes and any clones that were able to activate reporter gene transcription specifically when cotransformed with the VEGFR bait were then sequenced with primers flanking the pVP16 multiple cloning site (pVP16.F and pVP16.R).

3.4.4 (ii) Screening with RIα–VEGFR-1

[Only a small fraction (24%) of the hMVEC library was screened with VEGFR-2/pBTM116, and this identified only artefacts; consequently, only the results of screens with RIα–VEGFR-1/pBTM116 are discussed in this thesis.] RIα–VEGFR-1/pBTM116 was used as bait in preference to VEGFR-1/pBTM116 because it appeared to autophosphorylate more efficiently in yeast (Fig. 3.6). In addition, an RIα–Ret bait had been shown to identify more phosphorylation-independent interactions in yeast two-hybrid library screens than the receptor's native intracellular domain (Kyle Durick, personnal communication). Approximately 3.9 x 10^5 colonies, representing 16%, of the hMVEC cDNA library was screened with RIα–VEGFR-1/pBTM116. 16 colonies had both an obvious growth advantage on DOM1 media, and also significant β-galactosidase activity. Once separated into individual pVP16 plasmids, three of the recovered plasmids maintained the ability to interact specifically with RIα–VEGFR-1. These clones were sequenced and examined for homology to any known cDNAs using

the BLAST algorithm (Altschul *et al.*, 1990). As can be seen from Table 3.2, these clones were identical to regions of AKAP250 [an A kinase (protein kinase A)-anchoring protein], Grb2 and a poly-A tract.

In addition to the hMVEC library, 2 x 10^6 colonies of a mouse embryo library, representing 40%, was screened with RIα–VEGFR-1/pBTM116. It was by screening this mouse embryo library that the Nedd4 and Sck proteins had been previously identified as VEGFR-1- and VEGFR-2-binding partners, respectively (Warner *et al.*, 2000 and unpublished). Thirty colonies from these screens grew efficiently in the absence of histidine. These colonies were assayed for β-galactosidase activity and, as a result, seven were selected for further investigation. Once separated into individual pVP16 plasmids, six plasmids were able to interact specifically with RIα–VEGFR-1. When sequenced and subjected to BLAST searches, these were found to be identical to two distinct clones of PLCγ (clones 2 and 5), AKAP 220, DNA methyltransferase 1 (DNMT1), dual-specificity AKAP 1 (D-AKAP 1) and a cDNA isolated from male testis (accession number AK019735).

Screen	**Positive library cDNA**
RIα–VEGFR-1 (hMVEC library)	AKAP 250 (residues 1381–1632) Poly-A tract Grb2 (residues 58–152)
RIα–VEGFR-1 (mouse embryo library)	PLCγ1 (clone 2; residues 531–660) PLCγ1 (clone 5; residues 637–773) AKAP 220 (residues 558–611) DNA methyltransferase 1 (residues 669–787) Dual-specificity AKAP 1 (residues 301–399) Male testis cDNA (nucleotides 480–658, accession number AK019735)

Table 3.2: Clones identified from screening yeast two-hybrid cDNA libraries with RI α–VEGFR-1.

3.3.4 (iii) Further characterization of identified clones

Because of the possibility that the clones isolated from screens with RIα–VEGFR-1/pBTM116 could be interacting with either the RIα or the VEGFR-1 sequence, the eight clones (i.e. all but that encoding the poly-A tract) were retransformed with either an RIα/pBTM116 or a VEGFR-1/pBTM116 bait construct. Only three of these clones,

DNMT1 and the two PLCγ clones, were able to interact with VEGFR-1 in the absence of the RIα sequence; these three clones also failed to bind the RIα sequence encoded by RIα/pBTM116. By contrast, AKAP250, AKAP 220, D-AKAP 1 and the male testis cDNA, all activated transcription when transformed with RIα/pBTM116, but not when transformed with VEGFR-1/pBTM116. Although no protein product has, as yet, been assigned to the male testis cDNA, these data suggest that this putative polypeptide could be a novel member of the AKAP family. To investigate this possibility, the nucleotide sequence of this clone was translated using the ExPASy translate tool, and the open reading frame shown below was obtained (Fig. 3.8).

This polypeptide sequence was then used as the query in a BLAST search. Although the most homologous protein [*Homo sapiens* KIAA1678 (accession number AB051465), 69% identity] was uncharacterized, seven out of the top ten related sequences were AKAPs (the region with homology to proteins identified in the search are shown in bold in Fig. 3.8). Because the most related sequence to this polypeptide is an uncharacterized human protein, the male testis cDNA could encode a novel murine AKAP whose human counterpart is also uncharacterized.

5′-M N R V M D E S M N L E D I P D S V S T F A N E V A A K I M N L T E
F S M V D G V W Q G Q S C S R T R L L G G D R W N R L K A S S C E S I
P E E D S E A R V F V N S L G L M S T L S Q P V S R A S S V S K Q S S
C E S I T D E F S R F M V K Q M E N E G R G F E L L L D Y Y A G K N A
S S I M S S A M Q Q A C Q K N D H L N V R P S C P S K Q S S T E S I T
E E F Y R Y M L R D I A K E S K D G A S S R R S S H D W T T G L L S P
S T R S P L C Y R Q S S M P D S R S P C S R L T V N A P V K A N S L D
G F A Q N C P Q D S V N V Q P V S R A S S S G L C K S D S C L Y R R S
G T D Q I T N M L I H E T W A S S I E A L M R K N K I I A D D S E A A
N A S P G P V S S G S P L Q V E K N A N R L A T S K G H R G P T L L V
Q E S V D Y Q R K D A V T E G N H S P V S S P G K T A P V K K P S D F
D P R R E T S A C H N A A G L N S P R R S L C S R D V P L I Q I E T D
Q K E E C I G E P G P F L S Q S G S L E E T E G H Q P E E T I P D V A
R N E D T A P S T C Q S S R D S L E T S G E V E V E V L K E D I P R D
E S R N P P S S S E E S T G S W S Q **L A N E E D I P D D T S S F L Q L**
S E R S M S N G N S S G T S S L G I M D L D I Y Q E S I P S S P M I N
E L V E E K E I L K E Q S E S I K E H A S G L P G R A A S P Q R S L L
V I N F D L E P E C P D A E L R A T L Q W I A A S E L G I P T I Y F K
K S Q E S R I E K F L D V V K L V Q Q K S W K V G D I F H A V V Q Y C
K L H A E Q K E R T P S L F D W L L E L G-3′

Fig. 3.8: Putative translation product of the male testis cDNA clone isolated during a yeast two-hybrid library screen of the mouse embryo library using RIα–VEGFR-1 as bait. Residues highlighted in bold are those with homology to proteins identified in the BLAST searches.

3.3.4 (iii) Further characterization of identified clones: AKAPs

Interactions between AKAPs and PKA are usually mediated by the type II (RII) PKA regulatory subunits; however, RI subunits have also been shown to bind AKAPs, albeit

at a reduced affinity (Carr *et al.*, 1992; Burton *et al.*, 1997). It has been suggested that RI and RII subunits bind the same regions on AKAPs (Huang *et al.*, 1997). Consequently, the AKAP regions identified on the basis of their RI-binding ability in these screens were compared with the regions already reported to be RII-binding sites:
(1) The RII-binding region of AKAP250 has previously been localized to residues 1540–1553 (Nauert *et al.*, 1997), a sequence covered by the clone (1380–1632) isolated from our library screen.
(2) The RII-binding site of AKAP220 has not been definitively determined. However, it has been noted that the region encompassing residues 905–918 shares limited homology with the RII-binding regions of other AKAPs (Lester *et al.*, 1996). The clone identified as an RI-binding partner in our library screens encodes residues 557–610. It remains to be determined whether either of these regions are truely responsible for mediating the interaction of AKAP220 with PKA.
(3) The residues (300–398) encoded by the D-AKAP-1 clone isolated from our library screens encompass the site (317–338) already determined as the RII-binding region in this AKAP (Huang *et al.*, 1997).

3.3.4 (iii) Further characterization of identified clones: PLCγ

The sequences encoded by Clones 2 and 5 coincide with the N- and C-terminal SH2 domains of PLCγ, respectively (Fig. 3.9).

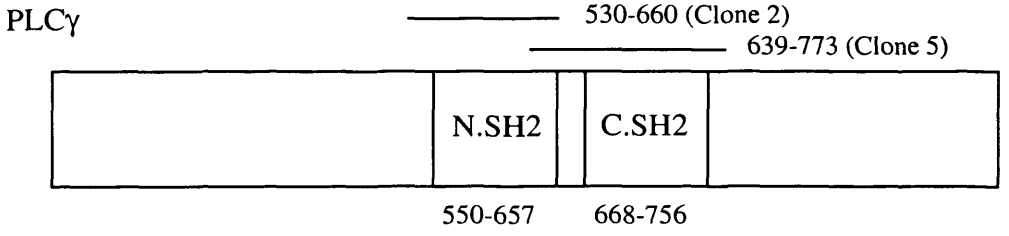


Fig. 3.9: Schematic representation of PLC γ clones isolated from screening the mouse embryo library with RIα–VEGFR-1. Clones 2 (residues 530–660) and 5 (residues 639–773) cover the entire N- and C-terminal SH2 domains of PLCγ, respectively.

In an attempt to determine the residues of the receptor responsible for mediating the interaction of VEGFR-1 with Clones 2 and 5, site-directed mutagenesis was performed on the VEGFR-1 sequence within the VEGFR-1/pBTM116 bait construct. Point mutations were introduced to generate a kinase-inactive receptor (K861A) and four tyrosine mutants: Y794F, Y1169F, Y1213F and the double mutant Y794/1169F. These mutations were chosen because previous mutagenesis of the VEGFR-1

intracellular domain had identified Y1169 and Y794 as major PLCγ-binding sites (Sawano *et al.*, 1997; Cunningham *et al.*, 1997). In addition, Y1213, a site similar to the low-affinity PLCγ-binding motif of c-Met, had been suggested to function as a minor PLCγ binding site (Cunningham *et al.*, 1997). Products of the mutagenesis reactions were verified by DNA sequencing using primers derived from the VEGFR-1 sequence, and mutated constructs were cotransformed individually into yeast with a pVP16 vector to test for their expression and, in the case of K861A, phosphorylation. Western blots of whole cell lysates from these yeast showed that all the mutants were expressed and that mutating the K861 residue to alanine ablated phosphorylation of the VEGFR-1 intracellular domain (Fig. 3.10).

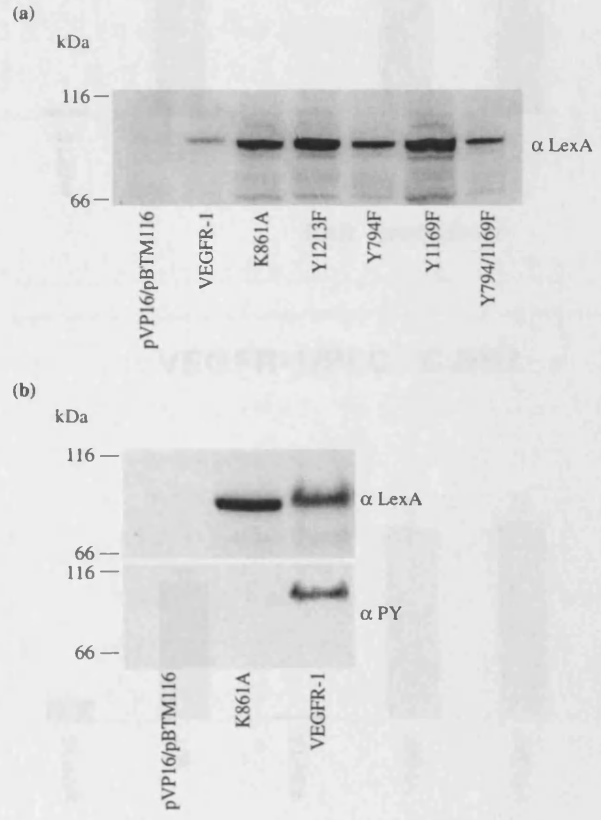


Fig. 3.10: Expression and phosphorylation of VEGFR-1 mutant bait proteins. The VEGFR-1/ pBTM116 construct was mutated by site-directed mutagenesis to generate a kinase-inactive receptor (K861A), three constructs mutated at single tyrosine residues (Y1213F, Y794F and Y11679F) and one double mutant (Y794/1169F). These mutants were individually transformed into yeast with pVP16. (a) Whole cell lysates from transformed yeast were analysed for expression of the mutant proteins by SDS–PAGE; expression was detected by probing with an anti-LexA antibody. Cotransformation of pVP16 and pBTM116 are shown for comparison. (b) The K861A mutant was analysed for expression (top panel) and phosphorylation (bottom panel), by probing the whole cell lysates with anti-LexA and anti-phosphotyrosine antibodies, respectively.

Once expression had been verified, the mutants were cotransformed individually into yeast with the PLCγ clones and interactions between the proteins were assessed in β-galactosidase solution assays (Fig. 3.11).

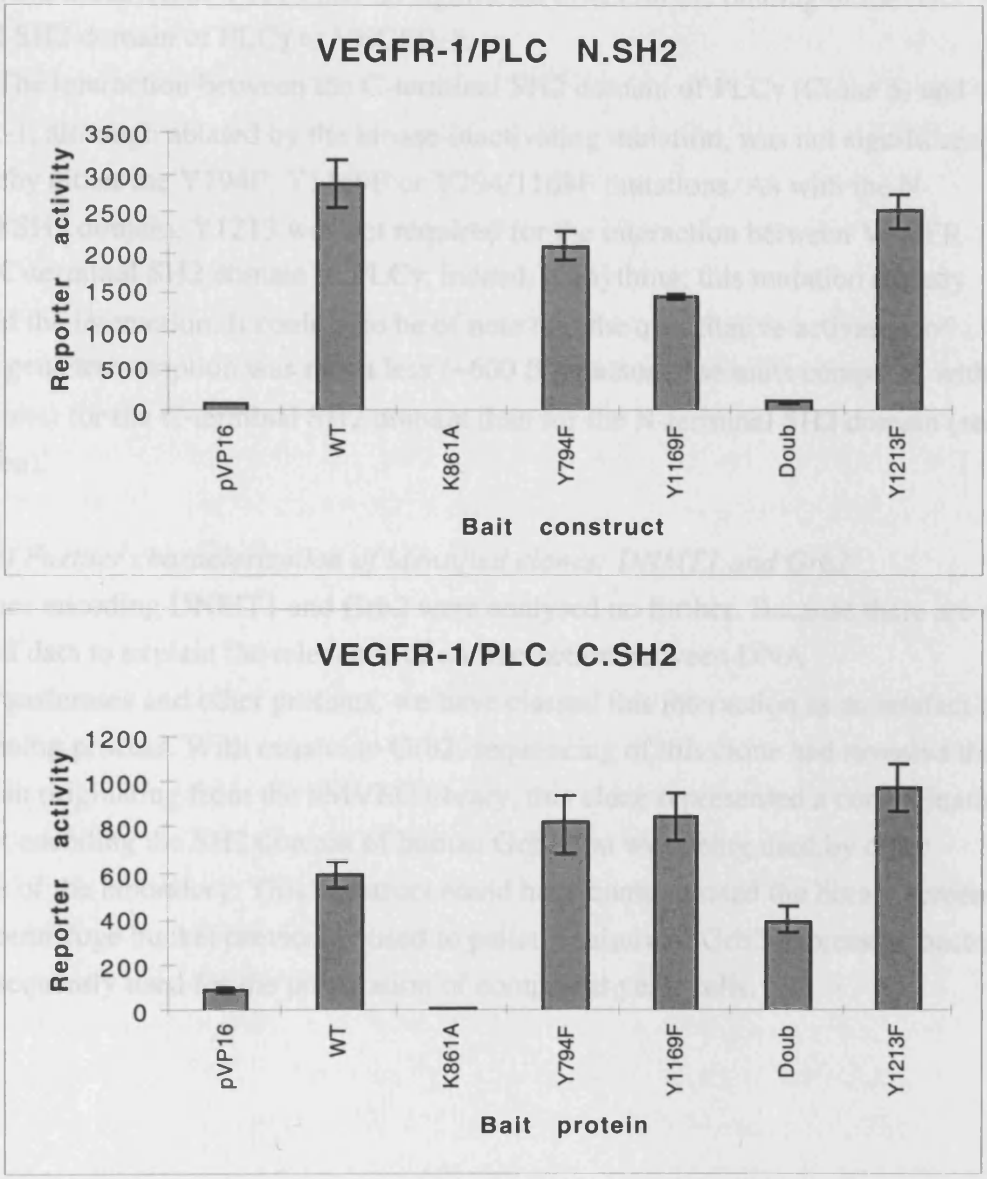


Fig. 3.11: VEGFR-1 residues responsible for the interaction betweeen this receptor and PLCγ in yeast. The two PLCγ clones isolated from library screens with RIα–VEGFR-1 were independently cotransformed into yeast with either wild-type VEGFR-1 or one of the VEGFR-1 mutants (K861A, Y794F, Y1169F, Y794/1169F (doub) or Y1213F). A transformation of pVP16 and wild-type VEGFR-1/pBTM116 is also shown (pVP16) to provide a reference for the basal transcriptional activity. Representative colonies were analysed for transactivation of reporter genes using the β-galactosidase solution assay. (Top) Clone 2, encoding the N-terminal SH2 domain of PLCγ and (bottom) clone 5, encoding the C-terminal SH2 domain. Data are mean ± SE, and are representative of five experiments.

As can be seen in Fig. 3.11, the interaction of both PLCγ clones with VEGFR-1 was completely dependent on the receptor's kinase activity. Mutation of the Y1169 and Y794 residues decreased the binding of PLCγ's N-terminal SH2 domain (Clone 2) by ~50% and ~30%, respectively. Mutating both residues virtually ablated the interaction. By contrast, mutation of Y1213 had no significant effect on the binding of the N-terminal SH2 domain of PLCγ to VEGFR-1.

The interaction between the C-terminal SH2 domain of PLCγ (Clone 5) and VEGFR-1, although ablated by the kinase-inactivating mutation, was not significantly affected by either the Y794F, Y1169F or Y794/1169F mutations. As with the N-terminal SH2 domain, Y1213 was not required for the interaction between VEGFR-1 and the C-terminal SH2 domain of PLCγ; indeed, if anything, this mutation slightly enhanced the interaction. It could also be of note that the quantitative activation of reporter gene transcription was much less (~600 β-galactosidase units compared with ~3000 units) for the C-terminal SH2 domain than for the N-terminal SH2 domain (see discussion).

3.3.4 (iii) Further characterization of identified clones: DNMT1 and Grb2

The clones encoding DNMT1 and Grb2 were analysed no further. Because there are no published data to explain the relevance of an interaction between DNA methyltransferases and other proteins, we have classed this interaction as an artefact of the screening process. With respect to Grb2, sequencing of this clone had revealed that, rather than originating from the hMVEC library, this clone represented a contaminating construct encoding the SH2 domain of human Grb2 that was being used by other members of the laboratory. This construct could have contaminated the library screen when a centrifuge bucket previously used to pellet a culture of Grb2-expressing bacteria was subsequently used for the preparation of competent yeast cells.

3.4: Discussion

3.4.1: Summary of library screening results

A hMVEC library was constructed to screen for endothelial cell effectors of the VEGFRs. Screening ~16% of this library with RIα–VEGFR-1 identified one artefact (a polyA tract), and two 'real' interactions: AKAP250 and Grb2, although the latter was a contaminant. Further characterization of the RIα–VEGFR-1–AKAP250 interaction revealed that this clone was specific for the RIα sequence rather than for VEGFR-1. The failure to identify authentic, VEGFR-specific signalling components in these screens might indicate that their cDNAs are under-represented in this library. Alternatively, it could be a consequence of inexhaustive library screening.

In case signalling components were under-represented in this library, a mouse embryo library, which had previously been used by others to identify proteins interacting with RTKs (Weidner *et al.*, 1996; Durick *et al.*, 1996; Bourette, *et al.*, 1997), was also screened. Forty percent of this library was screened with RIα–VEGFR-1, resulting in the isolation of six interacting polypeptides: two (possibly three) AKAPs, DNMT1 and two independent clones of PLCγ.

3.4.2: AKAPs

All AKAPs have an anchoring domain that binds PKA and a targeting domain that binds a particular protein or subcellular structure (reviewed in Dell'Acqua and Scott, 1997). By virtue of the targeting domain, AKAPs are thought to localize PKA to distinct subcellular compartments, thereby regulating the enzyme's substrate specificity. A subclass of AKAPs, the multivalent AKAPs, are believed to generate an additional level of signalling control by providing a scaffold onto which signalling complexes assemble. AKAP250, for example, can bind both PKA and PKC (Nauert *et al.*, 1997).

AKAPs are generally believed to interact with PKA via the enzyme's RII subunits (Carr *et al.*, 1992). However, RI subunits can also form physiological interactions with AKAPs, albeit with a 500-fold lower affinity than RII subunits (Burton *et al.*, 1997). For example, in RII knockout mice, RI subunits successfully compensate for the lack of RII subunits in processes that require AKAP-mediated PKA localization (Burton *et al.*, 1997). In addition, the affinity of RI subunits for AKAPs was of sufficient strength to identify an AKAP (dAKAP1 – a splice variant of sAKAP84) as an RI-binding partner in a yeast two-hybrid library screen (Huang *et al.*, 1997).

An RIα-derived sequence was incorporated into the VEGFR-1/pBTM116 bait construct to maximize receptor phosphorylation [as had been described for c-Ret and EGFR (Durick *et al.*, 1995)]. As a consequence, ~50% of the clones isolated from screens of the endothelial cell and mouse embryo libraries with RIα–VEGFR-1

represented members of the AKAP family, and interacted with the bait construct via the RIα sequence. Indeed, it is highly probable that screening with RIα–VEGFR-1 identified RIα-specific binding partners at the expense of those specific for VEGFR-1.

The RIα–VEGFR-1 construct encoded residues 1–236 of RIα. Mutagenesis studies had shown that only residues 1–84 of RI were required to confer constitutive activation upon the kinase fusions (Durick *et al.*, 1995). However, this region also houses the AKAP-interaction domain, and so a VEGFR-1 fusion to just the first 84 residues of RIα would not have prevented the bait's interaction with AKAPs.

With hindsight, the proteins that specifically bound VEGFR-1 in a phosphorylation-dependent manner (e.g. the two PLCγ clones) did so even in the absense of the RIα sequence. Although this demonstrates that the phosphorylation associated with the VEGFR-1/pBTM116 construct was sufficient for such interactions, RIα-independent phosphorylation might not have been sufficient for the isolation of interacting clones from library screens.

For AKAP250 and D-AKAP-1, the sequences of the clones identified in our library screens corresponded to regions previously identified as RII-binding sites (Nauert *et al.*, 1997; Huang *et al.*, 1997). The RII-binding site of AKAP220 has not been previously defined, although homology searching has implicated the residues 905–918 (Lester *et al.*, 1996). The clone identified as an RI-binding partner in our library screens encodes residues 557–610. It remains to be determined whether either of these regions are truely responsible for mediating the interaction of AKAP220 with PKA. Finally, our screens might have identified a novel member of the AKAP family: that encoded by the testis cDNA clone. Although a protein product has not yet been characterized for this cDNA, a BLAST search using the putative translation product as query showed significant similarity to several AKAPs.

3.4.3: The VEGFR-1–PLCγ interaction

VEGF-dependent tyrosine phosphorylation of PLCγ has been shown in various primary endothelial cells (Abedi and Zachary, 1997; Seetharam *et al.*, 1995; Guo *et al.*, 1995; Wu *et al.*, 2000). In addition, several independent research groups have demonstrated that PLCγ can bind VEGFR-1 and VEGFR-2 in either yeast, insect cells or when attached to a solid support (Cunningham *et al.*, 1997). Furthermore, both receptors have been shown to bind PLCγ in fibroblasts and VEGFR-2, but not VEGFR-1, bound PLCγ in VEGFR-expressing PAE cells (Sawano *et al.*, 1997; Landgren *et al.*, 1998; Takahashi and Shibuya, 1997) [see section 1.6.5 (i) for details].

The two clones of PLCγ isolated from the mouse embryo library with RIα–VEGFR-1 encode either the complete N- or C-terminal SH2 domain (Fig. 3.11). The N-terminal SH2 domain has previously been shown to bind VEGFR-1 in yeast (Igarashi *et al.*, 1998a; Cunningham *et al.*, 1997); however, only one of these research

groups could demonstrate a similar interaction for the C-terminal SH2 domain (Igarashi *et al.*, 1998a).

To identify sites on VEGFR-1 responsible for mediating the interactions with clones 2 and 5, specific VEGFR-1 mutants were compared with the wild-type receptor for their ability to activate reporter gene transcription in β-galactosidase solution assays. The interaction of both PLCγ clones with VEGFR-1 was completely dependent on the receptor's kinase activity, in agreement with previous results (Sawano *et al.*, 1997). With respect to the N-terminal domain, the interaction with VEGFR-1 was inhibited by 50% if Y1169 was mutated and by 30% if Y794 was mutated. The interaction was virtually ablated by mutating both Y794 and Y1169. These data agree with those of Cunningham *et al.*, who showed that, in yeast, mutating the Y1169 and Y794 residues inhibited the VEGFR-1–PLCγ interaction by 67% and 57%, respectively; the Y794/1169F double mutant decreased binding by 97% (Cunningham *et al.*, 1997). By contrast, mutating Y1213 had no effect on the binding of the N-terminal SH2 domain of PLCγ to VEGFR-1. In other studies, phosphopeptides encompassing Y1213 and Y1333 residues independently precipitated PLCγ from cell lysates (Ito *et al.*, 1998; Sawano *et al.*, 1997). However, these studies then went on to show that the VEGFR-1 intracellular domain precipitated PLCγ from cell lysates equally well whether wild-type, or mutant for either Y1213 or Y1333.

The interaction between VEGFR-1 and the C-terminal SH2 domain, although ablated by the kinase-inactivating mutation, was not significantly affected by Y794F, Y1169F or Y794/1169F mutations. As with the N-terminal SH2 domain, Y1213 was not required for the interaction between VEGFR-1 and the C-terminal SH2 domain of PLCγ. Interestingly, whereas one group agree with our data showing an interaction between the C-terminal SH2 domain of PLCγ and VEGFR-1 (Igarashi *et al.*, 1998), another failed to detect such an interaction, both in yeast and in co-immunoprecipitation experiments (Cunningham *et al.*, 1997). Assuming the levels of functional protein are comparable (see section 3.1.2), it might, therefore, be of relevance that the reporter gene activation induced by the C.SH2–VEGFR-1 interaction was much weaker (~600 β-galactosidase units compared with ~3000 units) than that induced by the N.SH2–VEGFR-1 interaction. Indeed, these data could indicate that the C.SH2–VEGFR-1 interaction does not occur in more physiological circumstances. By contrast, the N-terminal SH2 domain could mediate the involvement of PLCγ in the VEGF-induced mitogenesis that has been observed in endotheial cells (Xia *et al.*, 1996; Wellner *et al.*, 1999; Wu *et al.*, 2000).

3.4.4: Significance of the VEGFR-1 interactions with Grb2 and DNMT1

Although Grb2 was isolated during screening of the endothelial cell library as a VEGFR-1-interacting protein, the Grb2 plasmid had contaminated the library screen. Consequently, this construct was probably of much higher abundance than clones originating from the library. It is possible, therefore, that the observed VEGFR-1–Grb2 interaction would not occur in more physiological circumstances. The interactions between Grb2 and the VEGFRs are analysed further in section 5.3.

The partial cDNA clone of DNMT1 identified from screens of the mouse embryo library with RIα–VEGFR-1 had previously been isolated by screening this same library with a former VEGFR-1 construct. However, as yet, there are no published reports that could lead us to suggest a role for such an interaction, and no reported evidence that DNA methyltransferases can methylate proteins; consequently, we have classed the isolation of this protein as an artefact of the screening process. However, VEGFR-2 has been shown to translocate to the nucleus upon VEGF stimulation (Feng *et al.*, 1999b). If a similar movement of VEGFR-1 was demonstrated, the interaction between this receptor and a DNA methyltransferase could become more significant.

3.4.5: Future screening potential

Screening was abandoned before the libraries had been screened exhaustively primarily because it appeared that RIα-interacting clones were being isolated at the expense of VEGFR-1-interacting clones. With the hindsight that phosphorylation-dependent interactions could be detected in the absence of the RIα sequence, it would be worth returning to these screens using just the VEGFR-1/pBTM116 construct as bait. In addition, the chance of identifying phosphorylation-dependent, VEGFR-1-specific interactions from library screens might be increased if the libraries were first enriched for SH2 domain-containing proteins. For example, if a degenerate SH2 target could be designed, this could then be used to screen the libraries, isolating SH2 domain-containing proteins that could then be viewed as an SH2-enriched library, and screened with VEGFR-1 bait.

Chapter 4

Construction and characterization of cell lines expressing chimeric VEGF receptors

Chapter 4: Construction and characterization of cell lines expressing chimeric VEGF receptors

4.1: Introduction

4.1.1: Limitations of current systems for studying VEGF signalling pathways

Research into signalling cascades induced by VEGF has been consistently plagued by the inability to demonstrate ligand-induced activation of VEGFR-1 both *in vivo* and *in vitro* (Park *et al.*, 1994; Seetharam *et al.*, 1995; Waltenberger *et al.*, 1994). The tyrosine kinase domain of this receptor does have significant intrinsic kinase activity, however, as when expressed in yeast and insect cells, the intracellular domain of VEGFR-1, similar to that of other RTKs, is constitutively phosphorylated (Cunningham *et al.*, 1995; Sawano *et al.*, 1995; Ito *et al.*, 1998). It is possible, therefore, that the weak kinase activity associated with full-length VEGFR-1 reflects inadequate activation of the receptor, possibly because cell-surface proteoglycans and/or co-receptors that are essential for this process are absent from the experimental systems used.

The vast majority of VEGF-induced cellular responses (see section 1.6 for a more detailed discussion) have been attributed to VEGFR-2, although this might be a consequence of poor VEGFR-1 phosphorylation. Attempts to define the signalling capabilities of the individual VEGFRs have generally been conducted in PAE or NIH3T3 cells (i.e. cell types that do not express endogenous VEGFRs) to prevent activation of endogenous receptors by VEGF. However, results have been cell-type specific, possibly reflecting the existence of cell-type-specific signalling proteins or cell-surface proteoglycans. In addition, these cells could lack intracellular components that are required for physiological VEGF signalling cascades. To obtain a more accurate picture of VEGF signalling therefore, experiments should ideally be conducted in primary endothelial cells.

Although VEGF-induced responses can be and have been studied in primary endothelial cells (see section 1.6), attributing these responses to a specific receptor was not possible until relatively recently because the available tools did not allow specific activation of one particular type of VEGFR. The exploitation of receptor-specific ligands, along with the development of receptor-specific inhibitors and antisense-knockdown approaches, have provided a means by which a VEGF-induced response can be definitively attributed to a particular receptor, even when expressed endogenously in a native endothelial background (for examples see Bernatchez *et al.*, 1999; Wu *et al.*, 2000a; Keyt *et al.*, 1996; Rousseau *et al.*, 2000; Gerber *et al.*, 1998a). However, as yet there has been no publication of a system in which significant ligand-induced activation of VEGFR-1 can be reproducibly observed.

That VEGFR-1 tyrosine kinase activity is important *in vivo* has been demonstrated using mice in which the expressed VEGFR-1 encodes only the extracellular and transmembrane domains of the receptor (Hiratsuka *et al.*, 1998). Unlike their wild-type counterparts, monocytes derived from these mice were unable to migrate in response to VEGF or PlGF. However, the VEGF-induced proliferation and permeability of endothelial cells derived from these mice were comparable to the responses of wild-type endothelial cells. Therefore, at least in this case, it seems that the tyrosine kinase activity of VEGFR-1 is required for signalling in monocytes but not in endothelial cells. Such a discrepancy could reflect the expression of cell-type specific cell-surface molecules, with those expressed by monocytes facilitating ligand-induced activation of VEGFR-1, whereas those associated with endothelial cells do not. Interestingly, targeted deletion of the VEGFR-1-specific ligand PlGF had no effect on embryonic angiogenesis, but did impair angiogenesis associated with pathological conditions such as ischaemia and wound healing (Carmeliet *et al.*, 2001). This could indicate that the angiogenic signalling capabilities of VEGFR-1 are restricted to pathological conditions, which would make signalling induced by this receptor a desirable target for anti-angiogenic therapy.

4.1.2: Using chimeras to study receptor-specific signalling cascades

Chimeras are often used to facilitate the study of signalling cascades mediated by orphan receptors. They are also being increasingly used to ensure the specific activation of a particular receptor. In these chimeric constructs, the extracellular domain of the receptor being investigated is replaced by that of a second receptor for which a functional growth factor has been characterized (for examples see Seedorf *et al.*, 1991; Prigent and Gullick, 1994). Binding of ligand to the heterologous extracellular domain activates the desired intracellular kinase domain, enabling analysis of downstream signalling cascades. Chimeras of receptors for which ligands are already known are useful because they can be selectively activated in native cell types without activating endogenous receptors. However, there is a possibility that the growth factors required to activate such chimeras might also activate their physiological receptors if these are present in the cell-type being used. This problem has now been circumvented by the development of systems in which chimera activation can be controlled by small, non-mammalian molecules.

In these systems, bivalent, membrane-permeable compounds bind to cognate drug-binding domains that are fused to intracellular signalling molecules. This binding induces dimerization, and thereby activation, of the fusion proteins. Two such ligands, FK1012 and coumermycin A_1, dimerize FKBP12 (an immunophilin) and the B subunit of bacterial DNA gyrase (GyrB), respectively (Spencer *et al.*, 1993; Farrar *et al.*, 1996). FK1012 is a synthetic dimer of the natural monomeric FK506, whereas

coumermycin A_1 is a natural dimeric antibiotic. Chimeras of Janus kinase-2, Raf-1, Stat3 and the intracellular domains of receptors for insulin, PDGF and G-CSF, have all been successfully, and selectively, activated upon addition of one of these two dimerizing agents (Farrar *et al.*, 1996; Mohi *et al.*, 1998; Kume *et al.*, 1999; Yang *et al.*, 1998; O'Farrell *et al.*, 1998).

With respect to analysis of VEGF-induced signalling cascades, chimeras could have two main uses. First, individual chimeric VEGFRs could be selectively activated in the background of native endothelial cells without activating endogenous receptors. This would enable the allocation of various cellular responses, in cells that are most likely to contain the relevant physiological effectors, to specific VEGFRs. (Such determination has already been deemed feasible with the use of receptor-selective ligands and inhibitors.) Second, unlike alternative approaches, a chimeric version of VEGFR-1 might also bypass the weak ligand-induced activation of the full-length receptor. Seeing as, to the best of our knowledge, no method to accomplish this has yet been published, the alleviation of this bottleneck is probably where chimeric VEGFRs would be most useful. It was decided to develop chimeric VEGFRs, based on the coumermycin A_1–GyrB system, with the hope that by fusing the intracellular domains of the VEGFRs to the coumermycin-binding domain of GyrB, their kinase activities, in particular that of VEGFR-1, would be significantly activated in response to coumermycin A_1 (Fig. 4.1). By using such a chimeric system, it was hoped to be able to assess the relative involvement of the individual VEGFRs in VEGF-induced signalling pathways.

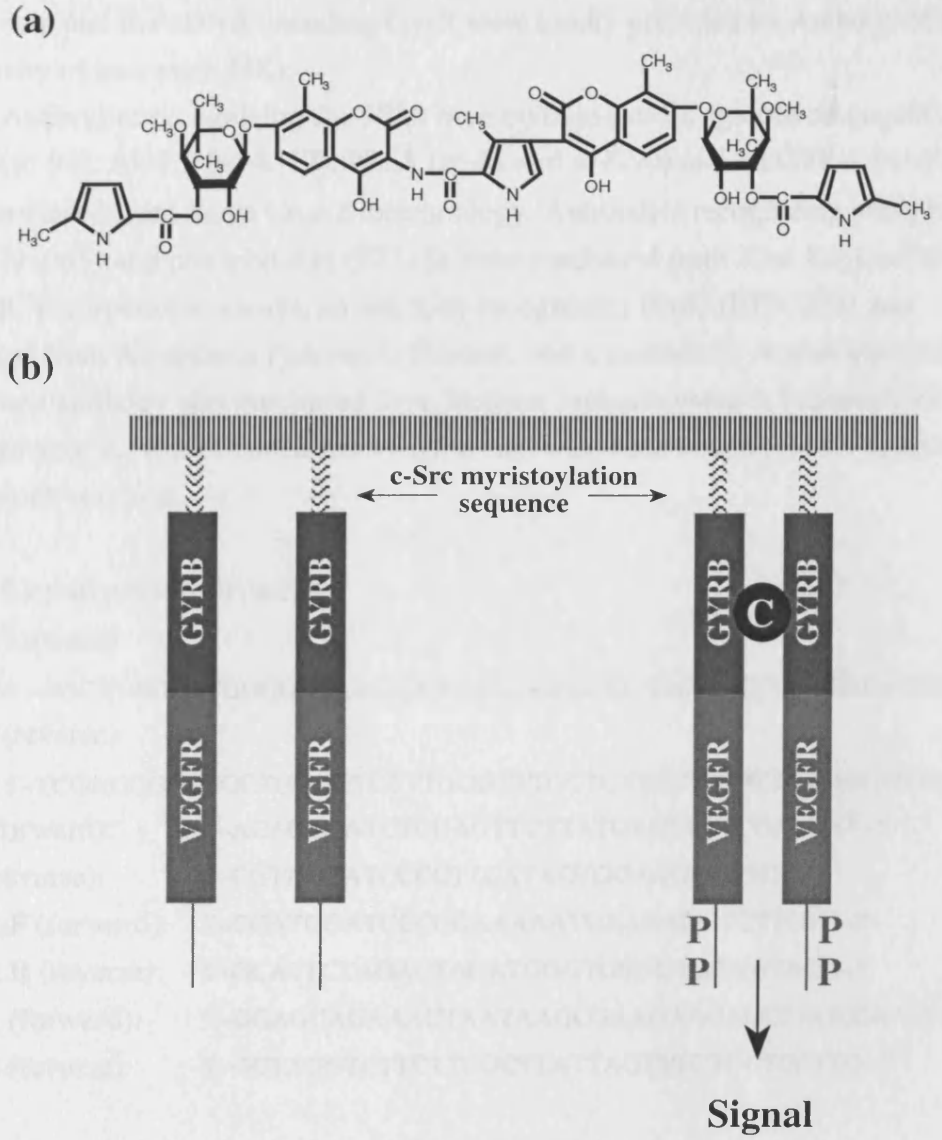


Fig. 4.1: Strategy to dimerize VEGFRs using coumermycin-regulatable chimeras. (a) Chemical structure of coumermycin A_1. (b) Intracellular domains of VEGFRs are expressed as fusions to the coumermycin-binding domain of GyrB. The chimeric receptors are anchored to the plasma membrane by a myristoylation sequence from c-Src. Upon addition of coumermycin (C), chimeric receptors dimerize, and their cytoplasmic domains transautophosphorylate (P) and induce downstream signalling cascades.

4.2: Material and methods

Novobiocin and the cDNA encoding GyrB were kindly provided by Anthony Maxwell (University of Leicester, UK).

Antibodies recognizing the 9E10 Myc epitope (sc-40, agarose conjugate), Erk1/2 (sc-94), Akt1/2 (sc-8312), PLCγ (sc-81 and sc7290) and VEGFR-2 (sc-504) were purchased from Santa Cruz Biotechnology. Antibodies recognizing phospho-Erk1/2 (9106S) and phospho-Akt (9271S) were purchased from New England BioLabs. For BrdU incorporation assays, an antibody recognizing BrdU (RPN 202) was purchased from Amersham Pharmacia Biotech, and a secondary, rhodamine RedX-conjugated antibody was purchased from Jackson Immunoresearch Laboratories. Coumermycin A_1 was obtained from Sigma, and was dissolved in DMSO to give a 5 mM stock solution.

4.2.1: Construction primers

c-src1 (forward):

5'–AGCTTACCATGGGGAGCAGCAAGAGCAAGCCCAAGGACCCCAGCCAGCGC–3'

c-src2: (reverse)

5'–TCGAGCGCTGGCTGGGGTCCTTGGGCTTGCTCTTGCTGCTCCCCATGGTA–3'

Gyr1 (forward): 5'–ACAGCGATCTCGAGTTCTTATGACTCCTCCAGTAT–3'

Gyr2 (reverse): 5'–CGTAGGATCCCCTTCATAGTGGAAGTGGTC–3'

Gyr.Flt.F (forward): 5'–CGATGGATCCCCGAAAAATGAAAAGGTCTTCTG–3'

Gyr.Flt.R (reverse): 5'–GCATTCTAGACTAGATGGGTGGGGTGGAGTACA–3'

9E10.1 (forward): 5'–GGAGCAGAAACTAATAAGCGAAGAAGACCTAGCCAA–3'

9E10.2 (reverse): 5'–GCTAGGTCTTCTTCGCTTATTAGTTTCTGCTCCTTG–3'

4.2.2: Kinase treatment of myc oligonucleotides

400 ng oligo was mixed with 10 U polynucleotide kinase (Roche), 10 x reaction buffer and 1 mM ATP, in a total volume of 20 µl. The reaction was performed at 37°C for 1 h, after which the kinase was inactivated by heating to 95°C for 5 min, followed by 65°C for 15 min.

4.2.3: Stable transfections

24 h before transfection, PAE cells were seeded at a density of 2.5 x 10^5 cells per well of a 6-well plate. The following day, media was replaced with 2 ml fresh medium. Serum-free Hams F12 was supplemented with 5% Fugene reagent (Roche Diagnostics), and the mixture incubated at room temperature for 5 min. 100 µl of this solution was then mixed with 1.5 µg DNA, and incubated at room temperature for a further 20 min. The mixture was then added, dropwise, to cells, and the cells incubated overnight. 24 h post-transfection, cells were split into six petri dishes (10 cm diameter) at varying

dilutions, and incubated overnight. The following day, media was replaced with selection media containing 0.4 mg/ml G418 (Promega). During selection, medium was changed once per week, until individual colonies were of sufficient size to be isolated. When ready for subculturing, each clone was encircled within a sterile greased ring, washed twice with PBS and transferred, in 100 µl trypsin, to one well of a 24-well plate.

4.2.4: Immunoprecipitation of proteins from PAE cells

Cells were seeded at a density of 7.2 x 10^5 cells per 6 cm petri dish, and allowed to attach overnight. The following day, cells were washed three times with PBS and then incubated in serum-free medium for 16–24 h. Medium was replaced with fresh serum-free media 1 h before beginning the experiment.

2.5 mg protein A-sepharose (for precipitations with the anti-PLCγ sc-81 antibody) or protein G-sepharose (for precipitations with the anti-VEGFR-2 sc-6251 antibody) were washed once with PBS (5000 rpm, 2 min). 2 µg antibody was then pre-bound to the beads by incubating at room temperature, with occasional gentle flicking, for 1–2 h.

Cells were stimulated with coumermycin A_1 as described in the individual figure legends. Following treatment, cells were washed twice with ice-cold PBS and lysed for 10 min at 4°C in lysis buffer [1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EGTA, 25 mM benzamidine] containing 100 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail (Sigma) as required. Cell debris was removed by centrifugation at 13 000 rpm, 4°C for 5 min, and protein was precipitated from the supernatant by incubating with antibody, pre-bound to beads, with rotation at 4°C overnight. For immunoprecipitation of myc-tagged GyrB–VEGFR-1, protein was precipitated with 10 µg of the myc antibody agarose conjugate.

The following day, immunoprecipitates were pelleted by centrifugation at 5000 rpm, 4°C for 2 min, and then washed four times with TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl)/0.1% Triton X-100. Receptor was eluted from the beads by boiling for 10 min in 35 µl Laemmeli sample buffer, freshly supplemented with 100 mM DTT. The preheated samples were analysed immediately by SDS–PAGE.

4.2.5: BrdU incorporation assay

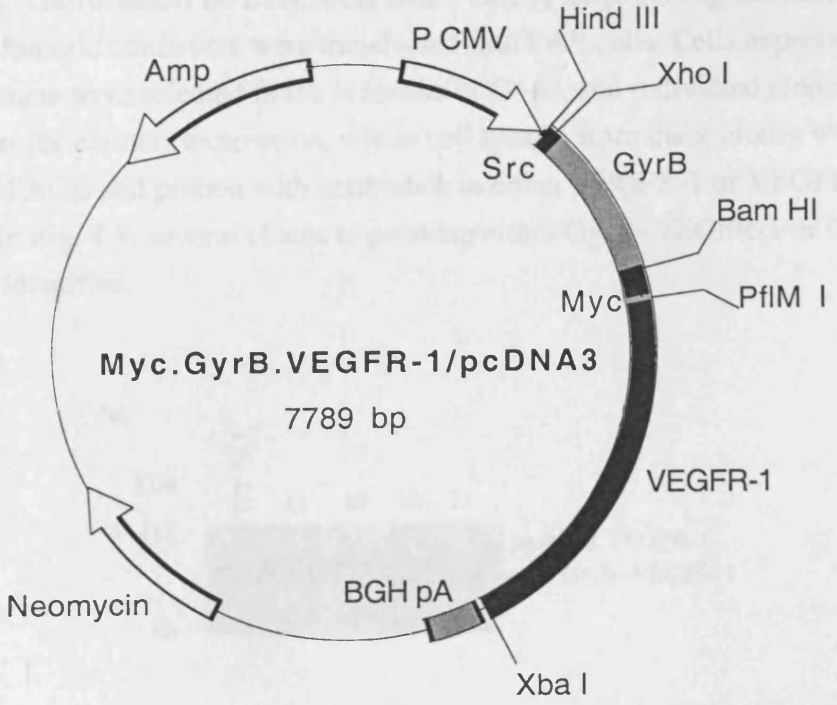
Cells were seeded at a density of 3.0 x 10^5 cells per 6 cm petri dish, and allowed to attach overnight. The following day, cells were washed four times with PBS and then incubated in serum-free medium for 40 h, with the medium being replaced after ~20 h. Cells were then stimulated with coumermycin A_1, at a dose specified in the individual figure legends, for 24 h. After 7 h stimulation, 4 µl of a 1:1000 dilution of BrdU was added to the cells. When stimulation was complete, cells were washed three times with

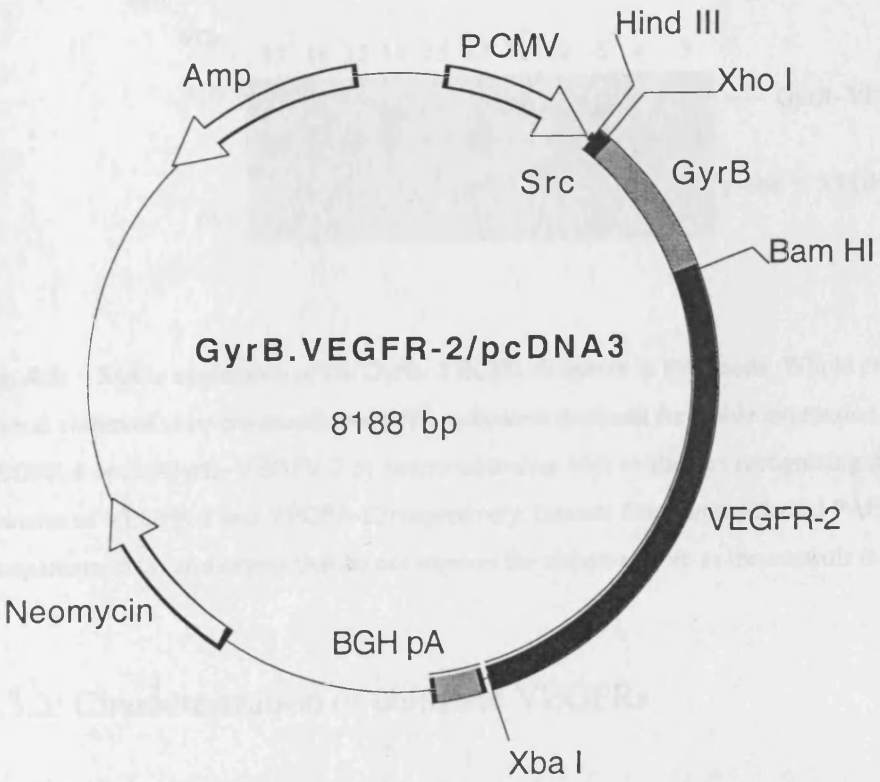
PBS, before fixing in ice-cold, fresh 5% acetic acid in ethanol for 30 min. Cells were then washed twice with PBST, and blocked in 5 mg/ml BSA (in PBST) for 1 h at 37°C. After blocking, an area of ~1 cm diameter was marked out on the base of the dish. 25 µl anti-BrdU antibody was placed on top of the cells enclosed within this area, and incubated at room temperature for 1 h. Cells were then washed three times with PBST, each time leaving the buffer on top of the cells for 5 min. The secondary, rhodamine RedX-conjugated antibody,was diluted 1:25 in 15 mg/ml BSA (in PBST). 25 µl of this diluted antibody was added to the specified area of cells, and incubated at 37°C for 55 min. 1.25 µl of a 1:100 dilution (in PBST) of Hoechst was then added, and the cells were incubated at 37°C for a further 10 min. Cells were then washed three times with PBST and once with PBS, before being mounted in 12 µl 80% glycerol/PBS and viewed by fluorescence microscopy.

4.3: Results

4.3.1: Construction of chimeric receptor constructs

A DNA fragment encoding the 24-kDa, N-terminal sub-domain (residues 2–220) of GyrB was amplified from the full-length GyrB cDNA by PCR, using primers incorporating 5′ *Xho* I (Gyr1) and 3′ *Bam* HI (Gyr2) sites. PCR products were run on an agarose gel and the resultant fragment was band purified, digested with *Xho* I and *Bam* HI, and ligated into the *Xho* I–*Bam* HI sites of pcDNA$_3$/Neo. The cytoplasmic domain of VEGFR-1 (residues 780–1339) was amplified by PCR from the full-length cDNA using primers incorporating 5′ *Bam* HI (Gyr.Flt.F) and 3′ *Xba* I (Gyr.Flt.R) sites. [Conditions were as in section 2.3.2 (xii) with the exceptions that annealing was perfomed at 54°C for 1 min, and elongation was permitted for 2.5 min per cycle. 500 ng DNA was used as template.] PCR products were run on an agarose gel and the resultant fragment was band purified, digested with *Bam* HI and *Xba* I, and ligated into the *Bam* HI–*Xba* I sites of GyrB/pcDNA$_3$/Neo. This ligation placed the VEGFR-1 sequence immediately downstream of GyrB, generating an in-frame fusion between the GyrB and VEGFR-1 sequences. To facilitate immunoprecipitation of the GyrB–VEGFR-1 chimera (commercially available anti-VEGFR-1 antibodies are poorly optimized for receptor immunoprecipitation), a myc epitope tag was inserted into the fusion protein. Complementary oligonucleotides (9E10.1 and 9E10.2) encoding the 9E10 myc epitope tag (Evan *et al.*, 1985) were subcloned into the *Pfl* MI site in the juxtamembrane region of the VEGFR-1 sequence in GyrB–VEGFR-1. (The oligos were phosphorylated and 40 ng oligo was ligated into 500 ng of a cleaved, phosphatased vector.) In addition, because the VEGFR-1 transmembrane domain was absent from the construct, a myristoylation sequence was inserted to anchor the chimera at the cell membrane. Complementary oligonucleotides (c-src1 and c-src2) encoding the myristoylation sequence of c-Src (Pellman *et al.*, 1985) were incorporated, as a *Hind* III–*Xho* I fragment, immediately upstream of the GyrB sequence in GyrB–VEGFR-1/pcDNA$_3$/Neo (Fig. 4.2). Independently, the cytoplasmic domain of VEGFR-2 (residues 808–1358) was subcloned into GyrB/pcDNA$_3$/Neo, and the myristoylation sequence of c-Src again incorporated at the N-terminus of the chimera (Fig. 4.2). After transformation of the ligations into DH5α, insert presence and orientation in several of the resultant colonies was checked by restriction enzyme analysis. In addition, correct insertion of all fragments, in particular of the myc epitope tag and the c-Src myristoylation sequence, were verified by DNA sequencing using primers derived from the receptor sequences.


Hind III
P CMV
Amp
Xho I
Src
GyrB
Bam HI
Myc
PflM I
Myc.GyrB.VEGFR-1/pcDNA3
7789 bp
VEGFR-1
Neomycin
BGH pA
Xba I


Hind III
P CMV
Amp
Xho I
Src
GyrB
Bam HI
GyrB.VEGFR-2/pcDNA3
8188 bp
VEGFR-2
Neomycin
BGH pA
Xba I

4.3.2: Generation of PAE cell lines stably expressing chimeric VEGFRs

The chimeric constructs were transfected into PAE cells. Cells expressing these constructs were selected in the presence of G418, and individual clones were isolated. To test for chimera expression, whole cell lysates from these clones were subjected to SDS–PAGE and probed with antibodies to either VEGFR-1 or VEGFR-2. As can be seen in Fig. 4.3, several clones expressing either GyrB–VEGFR-1 or GyrB–VEGFR-2 were identified.

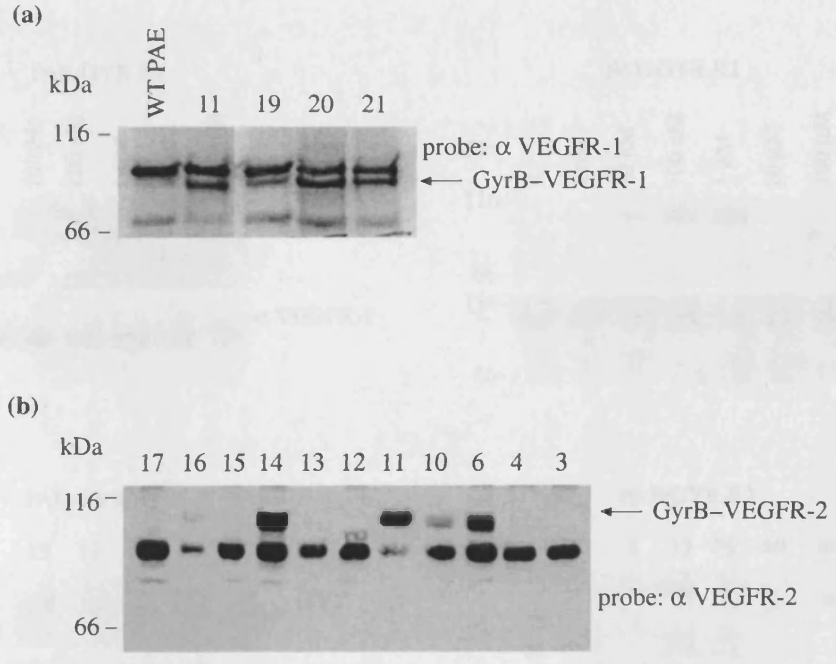


Fig. 4.3: Stable expression of the GyrB–VEGFR chimeras in PAE cells. Whole cell lysates from several clones of chimera-transfected PAE cells were analysed for stable expression of (a) GyrB–VEGFR-1 or (b) GyrB–VEGFR-2 by immunoblotting with antibodies recognizing the cytoplasmic domains of VEGFR-1 and VEGFR-2, respectively. Lysates from untransfected PAE cells are shown for comparison in (a) and clones that do not express the chimera serve as the controls in (b).

4.3.3: Characterization of chimeric VEGFRs

4.3.3 (i) Ligand-induced autophosphorylation

The potential of the chimeric system for use in analysing VEGFR-mediated signalling cascades was dependent on the ability of coumermycin to regulate the activity of the chimeric receptors. To assess this initially, the ability of coumermycin to induce phosphorylation of the chimeric receptors was tested. Following coumermycin

treatment, chimeric receptors were immunoprecipitated from their respective cell lines and the samples subjected to western blot analysis with anti-phosphotyrosine antibodies. As seen in Fig. 4.4, coumermycin induced the phosphorylation of VEGFRs 1 and 2 in both a concentration-dependent and a time-dependent manner. Coumermycin had no effect on phosphorylation of the full-length wild-type receptors expressed in PAE or human embryonic kidney (HEK) 293 cells, indicating that this drug specifically activated the chimeric receptors by binding to the GyrB domain (data not shown, Andrea Warner).

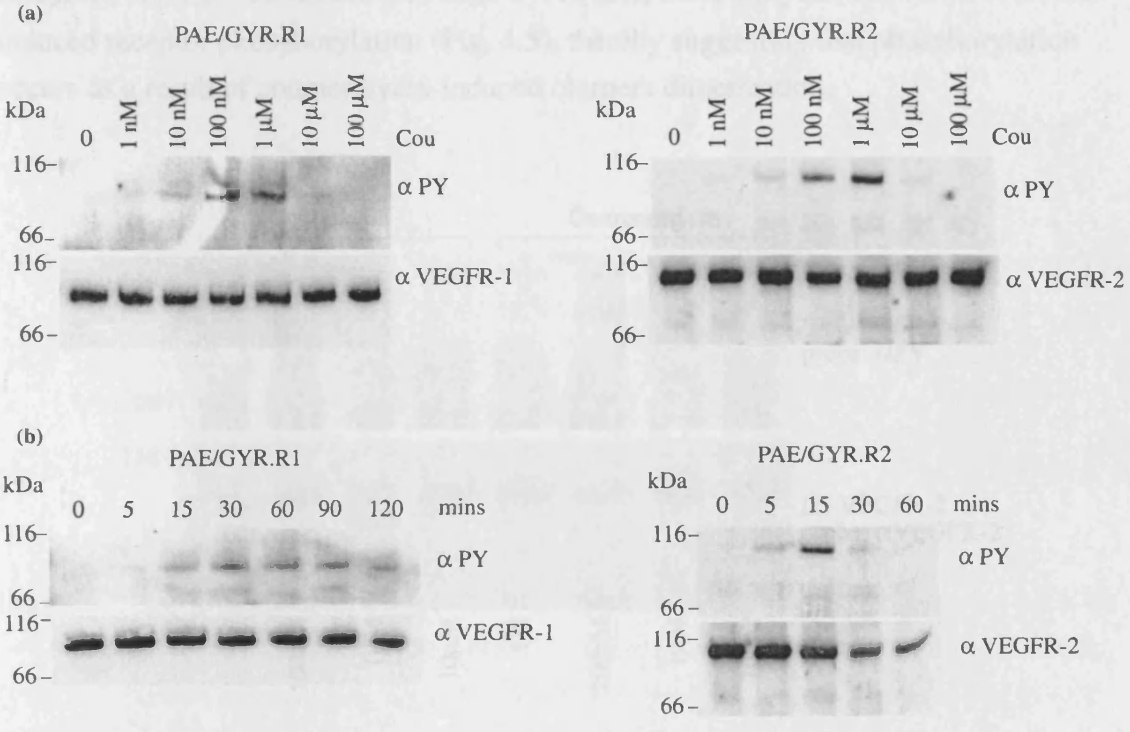


Fig. 4.4: Coumermycin stimulates the phosphorylation of GyrB–VEGFR chimeras in both a time- and concentration-dependent manner. (a) Cells were incubated for 15 mins, at 37°C, with 0–100 µM of coumermycin (Cou). GyrB–VEGFR-1 and GyrB–VEGFR-2 chimeras were immunoprecipitated from cell lysates with anti-myc and anti-VEGFR-2 (sc-6251) antibodies, respectively. Precipitated material was subjected to SDS–PAGE and immunoblotted with antibodies recognizing phosphotyrosine (αPY; top panels). Blots were then stripped and reprobed with antibodies recognizing the corresponding receptor [αVEGFR-1 (sc-316) or αVEGFR-2 (sc-504)] to control for variation in protein levels (bottom panels). Results are representative of experiments performed on two independent clones. (b) Cells were treated with 100 nM coumermycin for varying lengths of time, and chimeric receptors were immunoprecipitated and analysed as for (a).

At higher concentrations (>1 μM), coumermycin inhibited phosphorylation of the receptors, thereby suggesting that, at these doses, it is saturating the expressed chimeric receptors, inhibiting their dimerization and activation. To confirm that the mechanism of coumermycin-induced receptor activation required dimerization (similar to ligand-induced activation of the full-length receptors), experiments were conducted in which GyrB–VEGFR-2-expressing cells were stimulated with either novobiovin or coumermycin. Novobiocin is structurally related to coumermycin, but is monomeric and so binds GyrB in a 1:1, rather than an 1:2, ratio (Farrar *et al.*, 1996). Treatment of cells with novobiocin should therefore not induce dimerization of the chimeric receptors. Over the concentration range 0–100 μM, coumermycin, but not novobiocin, induced receptor phosphorylation (Fig. 4.5), thereby suggesting that phosphorylation occurs as a result of coumermycin-induced chimera dimerization.

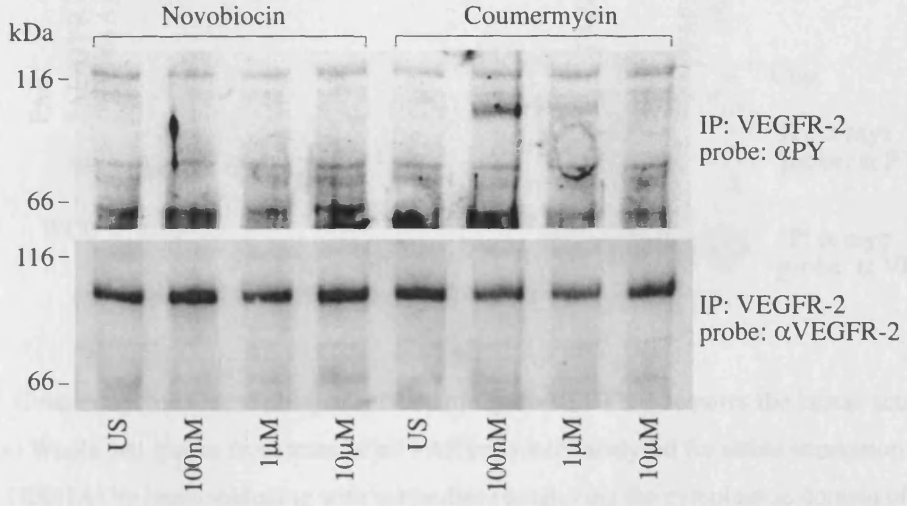


Fig. 4.5: Phosphorylation of GyrB–VEGFR chimeras requires coumermycin-induced receptor dimerization. Cells were incubated for 15 mins, at 37°C, with increasing concentrations (0–10 μM) of novobiocin or coumermycin. GyrB–VEGFR-2 was immunoprecipitated from cell lysates with anti-VEGFR-2 (sc-6251) antibodies, subjected to SDS–PAGE and immunoblotted with antibodies recognizing phosphotyrosine (αPY). Blots were stripped and reprobed with antibodies recognizing VEGFR-2 (sc-504) to control for variations in protein levels.

As can be seen from Fig. 4.4, maximal activation of chimeric VEGFR-2 in response to coumermycin peaked between 5 and 15 mins. Phosphorylation of chimeric VEGFR-1 was somewhat more sustained, still being observed >90 mins after stimulation. Because VEGFR-1 is thought to have a very weak intrinsic kinase activity, it was attempted to determine whether the phosphorylation induced by coumermycin

was dependent on the receptor's intrinsic catalytic activity. Using site-directed mutagenesis, a kinase-inactive GyrB–VEGFR-1 chimera was generated by mutating a lysine residue at the ATP-binding site to alanine (K861A). [The primers for this reaction are described in section 3.2.2 (i)]. Once the mutation had been verified by DNA sequencing, this kinase-inactive receptor was transfected into PAE cells, and cell lines stably expressing the fusion protein were generated [Fig 4.6 (a)]. The effect of coumermycin on these cells was then analysed. As shown in Fig. 4.6 (b), coumermycin failed to induce the phosphorylation of this chimera, thereby confirming that the coumermycin-induced phosphorylation of wild-type GyrB–VEGFR-1 was indeed catalysed by the receptor's intrinsic kinase activity.

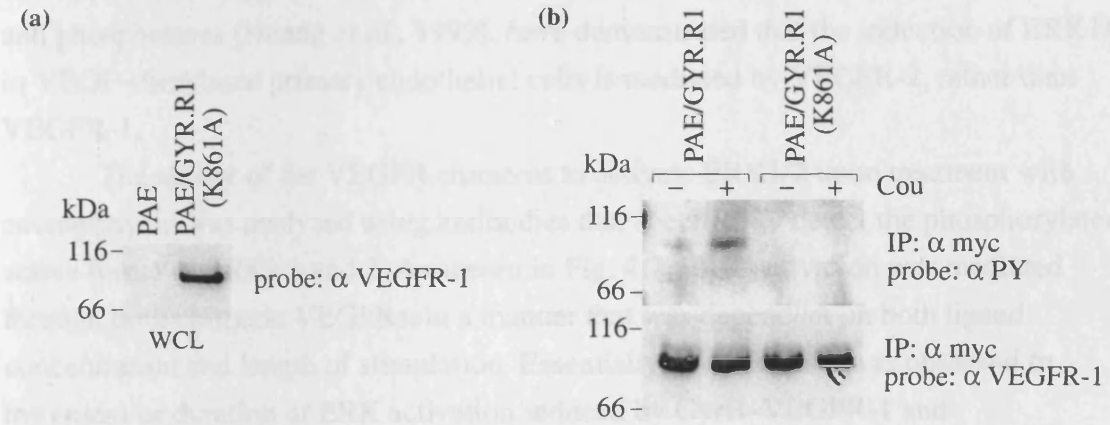


Fig. 4.6: Coumermycin-induced phosphorylation of GyrB–VEGFR-1 requires the kinase activity of the receptor. (a) Whole cell lysates from transfected PAE cells were analysed for stable expression of GyrB–VEGFR-1 (K861A) by immunoblotting with antibodies recognizing the cytoplasmic domain of VEGFR-1. Lysates from untransfected PAE cells are shown for comparison. (b) Cells expressing either wild-type or kinase-inactive GyrB–VEGFR-1 were incubated for 15 mins, at 37°C, with 100 nM coumermycin (Cou). Chimeric receptors were immunoprecipitated from cell lysates with anti-myc antibodies, subjected to SDS–PAGE and immunoblotted with antibodies recognizing phosphotyrosine (αPY). Blots were stripped and reprobed with antibodies recognizing VEGFR-1 (αVEGFR-1) to control for variations in protein levels.

Having developed a system in which both VEGFRs 1 and 2 can be induced to phosphorylate in endothelial cells, the aim was then to study molecular responses downstream of the individual receptors. First, this would determine whether the chimeric VEGFR-2 could mediate responses previously shown to be coupled to the full-length receptor. Second, if the system succeeded in this first respect, data from comparisons of the signalling responses mediated by the chimeric VEGFRs 1 and 2

would be more likely to be relevant to the full-length receptors. The responses chosen for study were activation of ERK1/2, PLCγ and Akt/PKB, because these responses have previously been observed in response to VEGF stimulation of primary endothelial cells (see section 1.6).

4.3.3 (ii) Activation of ERK1/2

In addition to primary endothelial cells (Yu and Sato, 1999, Seetharam *et al.*, 1995, Takahashi and Shibuya, 1997, Wu *et al.*, 2000a, Abedi and Zachary, 1997; Pedram *et al.*, 1998), VEGF has been consistently shown to induce ERK1/2 activation in VEGFR-2-, but not VEGFR-1-, transfected PAE cells (Kroll and Waltenberger, 1997; Rousseau *et al.*, 2000; Landgren *et al.*, 1998). In addition, studies using receptor-selective neutralizing antibodies (Rousseau *et al.*, 2000), ligands (Ogawa *et al.*, 1998) and phosphatases (Huang *et al.*, 1999), have demonstrated that the induction of ERK1/2 in VEGF-stimulated primary endothelial cells is mediated by VEGFR-2, rather than VEGFR-1.

The ability of the VEGFR chimeras to activate ERK1/2 upon treatment with coumermycin was analysed using antibodies that specifically detect the phosphorylated, active forms of ERKs 1 and 2. As shown in Fig. 4.7, ERK activation was mediated through both chimeric VEGFRs, in a manner that was dependent on both ligand concentration and length of stimulation. Essentially no difference was observed in the extent or duration of ERK activation induced by GyrB–VEGFR-1 and GyrB–VEGFR-2.

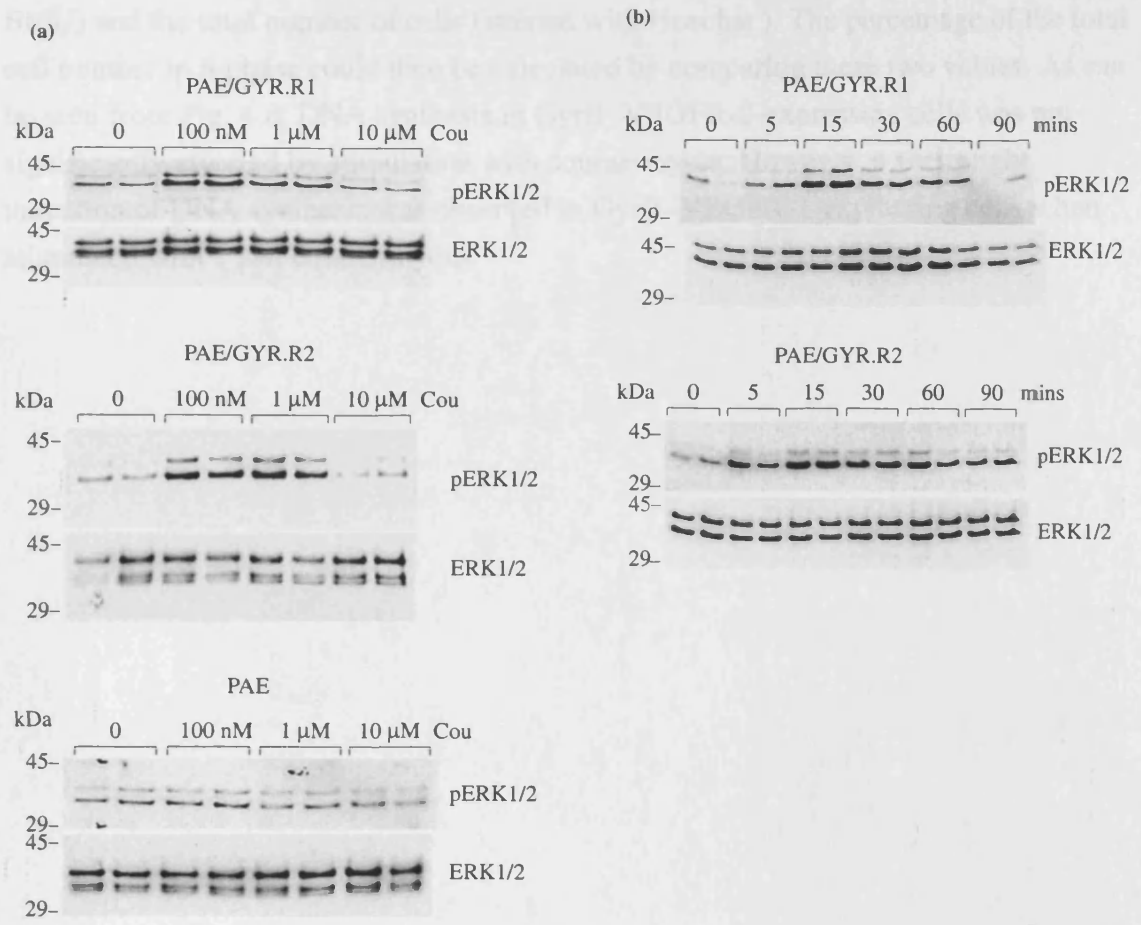


Fig. 4.7: Coumermycin-induced ERK1/2 activation downstream of GyrB–VEGFR chimeras. (a) PAE cells expressing the GyrB–VEGFR chimeras were stimulated with increasing concentrations (0–10 μM) of coumermycin (Cou) for 15 mins at 37°C. Untransfected PAE cells were treated similarly for comparison. Whole cell lysates from duplicate wells were analysed by immunoblotting with antibodies recognizing the phosphorylated forms of ERKs 1 and 2 (pERK1/2). Blots were then stripped and reprobed with antibodies recognizing both phosphorylated and non-phosphorylated forms of ERK (ERK1/2) to control for differences in protein expression. (b) PAE cells expressing the chimeras were stimulated with 100 nM coumermycin for various lengths of time, and whole cell lysates were analysed for the presence of phosphorylated ERKs 1 and 2 (pERK1/2), or total ERKs 1 and 2 (ERK1/2), as in (a).

4.3.3 (iii) Stimulation of cell proliferation

Because ERK1/2 activation is commonly required for cell proliferation, the chimeras were analysed for their ability to induce DNA synthesis. Cells were stimulated with a range of coumermycin concentrations (0–100 nM) and subjected to BrdU incorporation assays. DNA synthesis was quantified using fluorescence microscopy to count the number of cells in S phase (stained by a rhodamine-conjugated secondary antibody to

BrdU) and the total number of cells (stained with Hoechst). The percentage of the total cell number in S phase could then be calculated by comparing these two values. As can be seen from Fig. 4.8, DNA synthesis in GyrB–VEGFR-2-expressing cells was not significantly affected by stimulation with coumermycin. However, a very slight induction of DNA synthesis was observed in GyrB–VEGFR-1-expressing cells when stimulated with 1 pM coumermycin.

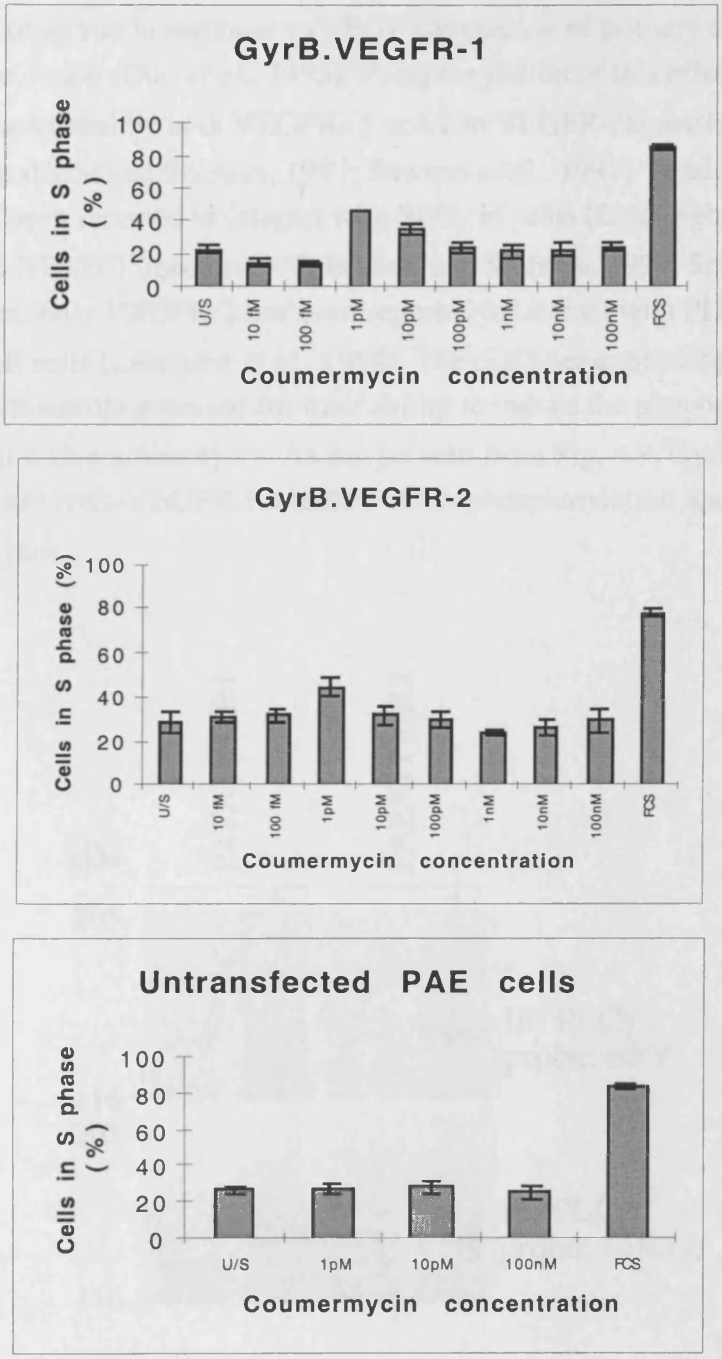


Fig. 4.8: Coumermycin-induced DNA synthesis of GyrB–VEGFR-expressing cells. PAE cells expressing GyrB–VEGFR-1 (top) or GyrB–VEGFR-2 (middle) were stimulated with increasing concentrations (0–100 nM) of coumermycin, or with 20% FCS, in the presence of BrdU. Wild-type PAE cells (bottom) were treated similarly for comparison. Cells were subjected to BrdU incorporation assays and viewed by fluorescence microscopy. The total number of nuclei and the number of nuclei undergoing cell proliferation were visualized by Hoerscht and rhodamine staining, respectively, and counted. The percentage of total nuclei undergoing cell proliferation was then calculated. Data are ±SE, and are averaged from five experiments.

4.3.3 (iv) Activation of PLCγ

Another effect observed in response to VEGF stimulation of primary endothelial cells is PLCγ phosphorylation (Guo *et al.*, 1995). Phosphorylation of this effector has been reported to be mediated by both VEGFRs 1 and 2 in VEGFR-expressing NIH3T3 fibroblasts (Takahashi and Shibuya, 1997; Sawano *et al.*, 1997). In addition, both VEGFRs have been reported to interact with PLCγ in yeast (Cunningham *et al.*, 1997) and transfected NIH3T3 fibroblasts (Takahashi and Shibuya, 1997; Sawano *et al.*, 1997). However, only VEGFR-2 has been reported to interact with PLCγ in VEGFR-transfected PAE cells (Landgren *et al.*, 1998). The cell lines expressing the chimeric VEGFRs were therefore analysed for their ability to induce the phophorylation of PLCγ upon activation with coumermycin. As can be seen from Fig. 4.9, GyrB–VEGFR-2, and to a lesser extent GyrB–VEGFR-1, mediate PLCγ phosphorylation upon stimulation with coumermycin.

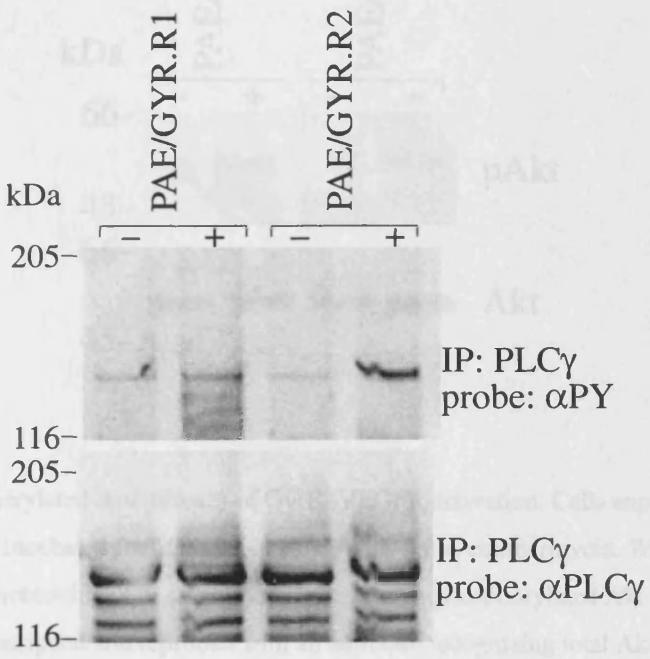


Fig. 4.9: PLCγ is phosphorylated downstream of GyrB–VEGFR activation. Cells expressing the chimeric receptors were incubated for 15 mins, at 37°C, with 1 μM coumermycin. PLCγ was immunoprecipitated from cell lysates with an anti-PLCγ antibody (sc-81) and analysed by western blotting for the presence of phosphotyrosine (PY; top panel). Immunoblots were stripped and reprobed with an anti-PLCγ antibody (sc-7290) to control for the amount of precipitated protein (bottom panel). Results are representative of two experiments.

4.3.3 (v): Activation of Akt/PKB

The PI3K–Akt pathway is used by many growth factors, including VEGF (Thakker *et al.*, 1999; Gerber *et al.*, 1998a), to promote cell survival. Through the use of receptor-selective VEGF mutants (Gerber *et al.*, 1998a) and specific VEGFR-2 inhibitors (Wu *et al.*, 2000a), this response has been attributed to VEGFR-2. No Akt phosphorylation has been observed downstream of stimulation with VEGFR-1-selective ligands (Wu *et al.*, 2000a; Carmeliet *et al.*, 1999a; Gerber *et al.*, 1998a). The abilities of the chimeric receptors to mediate Akt phosphorylation upon activation with coumermycin were investigated. As can be seen from Fig. 4.10, Akt activation was mediated through both GyrB–VEGFR-1 and GyrB–VEGFR-2.

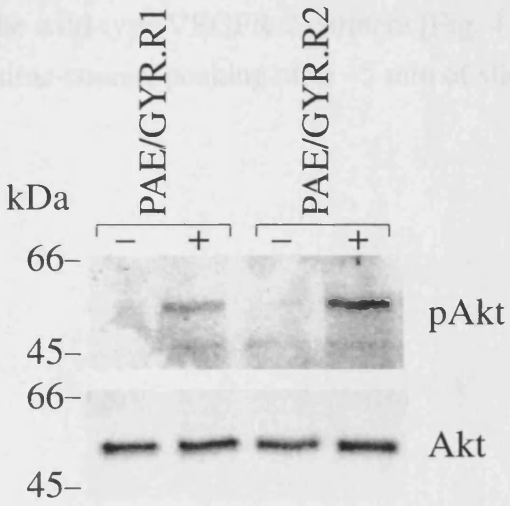


Fig. 4.10: Akt is phosphorylated downstream of GyrB–VEGFR activation. Cells expressing the chimeric receptors were incubated for 15 mins, at 37°C, with 1 μM coumermycin. Whole cell lysates were analysed by immunoblotting with an antibody recognizing phosphorylated Akt (pAkt; top panels). Immunoblots were then stripped and reprobed with an antibody recognizing total Akt (Akt; bottom panels) to control for variations in protein levels.

4.3.3 (vi) Analysis of responses mediated by a GyrB–VEGFR-2(Y1175F) mutant

Having demonstrated that activation of VEGFR chimeras could induce effects similar to those observed downstream of endogenous receptors, it was asked whether the signalling pathways of these two types of receptor were comparable; that is, whether the chimeric receptors also rely on the interaction between specific phosphotyrosine residues and SH2 domains. Alternatively, the chimeras might phosphorylate proteins

promiscuously. To investigate this possibility, the coumermycin-induced phosphorylation of ERK1/2, Akt and PLCγ were analysed downstream of a GyrB–VEGFR-2 chimera containing a Y1175F mutation. The Y1175 site was selected for these studies as it had previously been shown to bind PLCγ (Cunningham *et al.*, 1997) and the Shc-like protein Sck (Igarashi *et al.*, 1998b; Warner *et al.*, 2000), in yeast.

A PAE cell line stably expressing a GyrB–VEGFR-2(Y1175F) mutant was constructed (Knight *et al.*, 2000; Fig. 4.11). Western blot analysis confirmed that the expression levels of the mutant and wild-type GyrB–VEGFR-2 chimeras in their respective PAE cell lines were similar (data not shown). Coumermycin-induced phosphorylation of the Y1175F mutant was also analysed. Maximal phosphorylation of this mutant was induced over the same coumermycin concentration range (10 nM–1 μM) as was the wild-type VEGFR-2 chimera [Fig. 4.11 (a)], and also occurred over a similar time-course, peaking after ~5 min of stimulation [Fig. 4.11 (b)].

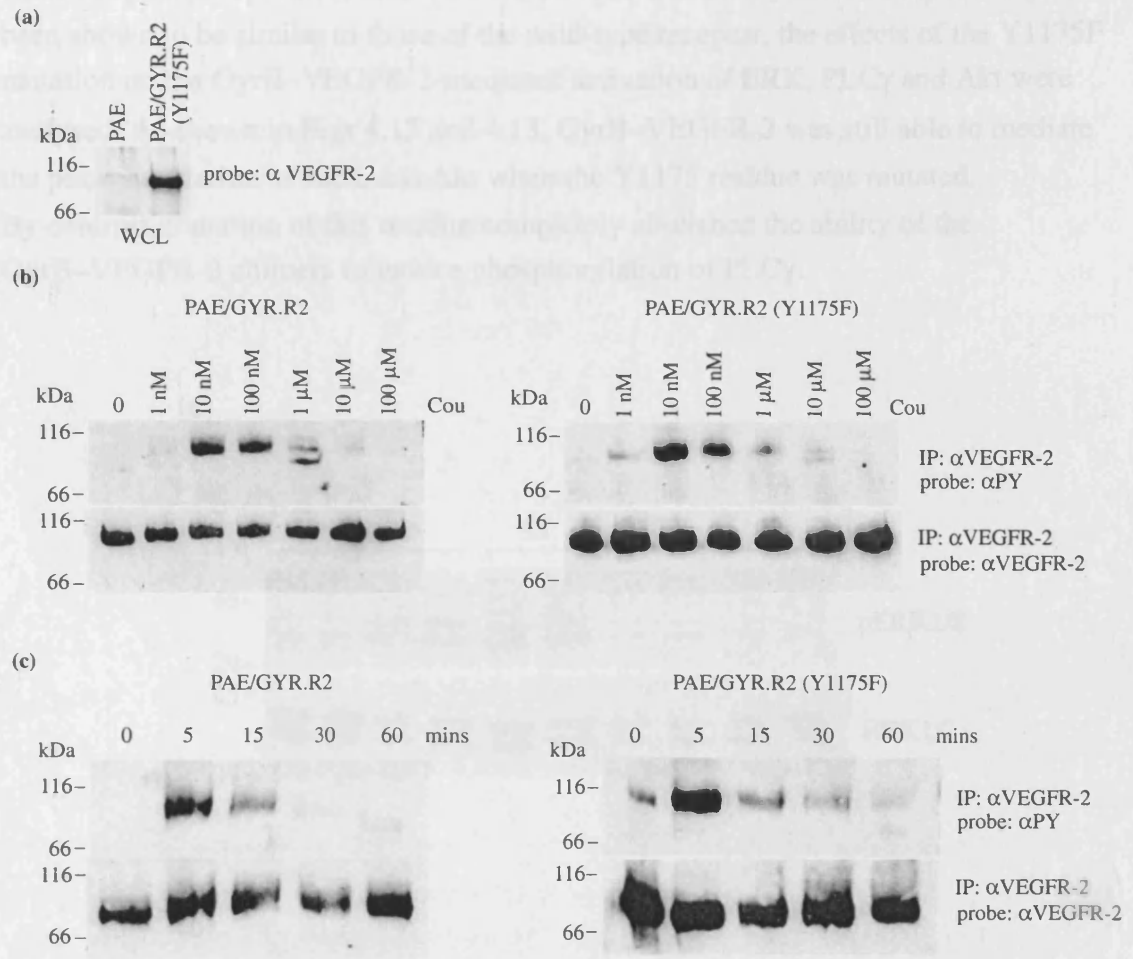


Fig. 4.11: Coumermycin-induced phosphorylation kinetics of a Y1175F GyrB–VEGFR-2 mutant. The coumermycin-induced activation of a Y1175F GyrB–VEGFR-2 mutant is comparable to that of the wild-type receptor. (a) Whole cell lysates from transfected PAE cells were analysed for stable expression of GyrB–VEGFR-2(Y1175F) by immunoblotting with antibodies recognizing the cytoplasmic domain of VEGFR-2 (sc-6251). Lysates from untransfected PAE cells were analysed for comparison. (b) PAE cells expressing either the wild-type GyrB–VEGFR-2 chimera (left), or a GyrB–VEGFR-2(Y1175F) mutant (right), were treated with a range (0–100 μM) of coumermycin concentrations for 15 min. Chimeric receptors were then precipitated from cell lysates with an antibody recognizing VEGFR-2 (sc-6251) and analysed for phosphorylation by immunoblotting with an antiphosphotyrosine antibody (α PY; top panels). To control for the amount of protein precipitated, immunoblots were stripped and reprobed with an anti-VEGFR-2 antibody (sc-504, bottom panels). (c) PAE cells expressing either wild-type GyrB–VEGFR-2 (left) or GyrB–VEGFR-2(Y1175F) (right) were treated with 100 nM coumermycin for the times indicated (0–60 mins). Chimeric receptors were then precipitated and analysed for the presence of phosphotyrosine as in (b).

Once the phosphorylation kinetics of the Y1175F mutant GyrB–VEGFR-2 receptor had been shown to be similar to those of the wild-type receptor, the effects of the Y1175F mutation on the GyrB–VEGFR-2-mediated activation of ERK, PLCγ and Akt were analysed. As shown in Figs 4.12 and 4.13, GyrB–VEGFR-2 was still able to mediate the phosphorylation of ERK and Akt when the Y1175 residue was mutated.
By contrast, mutation of this residue completely abolished the ability of the GyrB–VEGFR-2 chimera to induce phosphorylation of PLCγ.

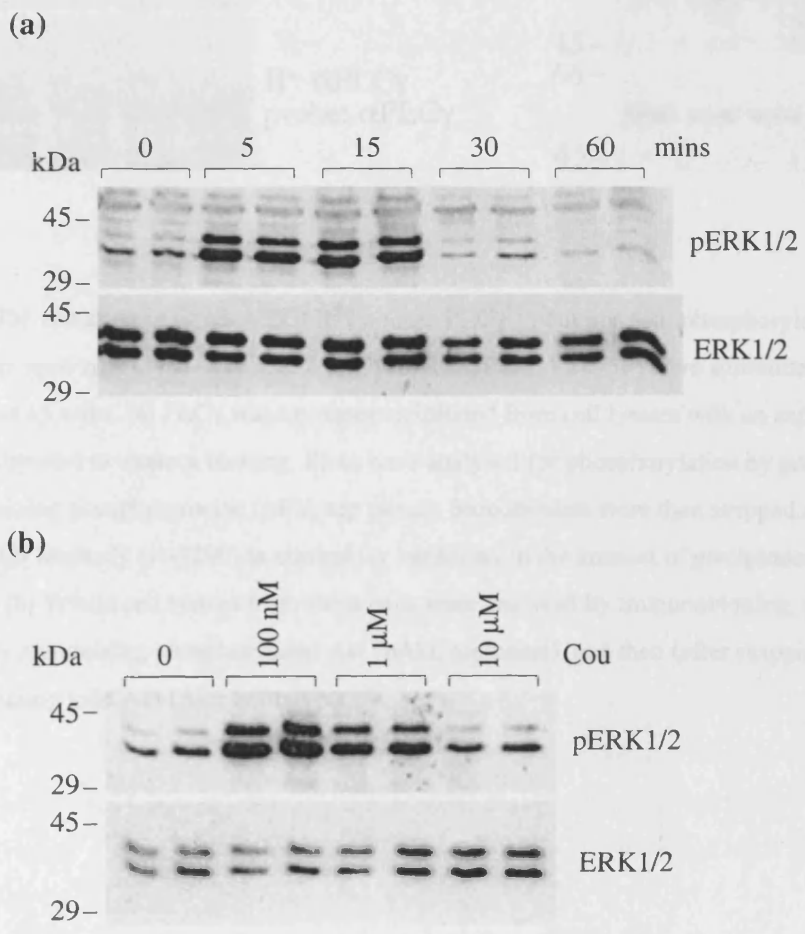


Fig. 4.12: ERK1/2 activation downstream of GyrB–VEGFR-2 is not affected by a Y1175F mutation. PAE cells expressing GyrB–VEGFR-2(Y1175F) were stimulated (a) with 1 μM coumermycin for various lengths of time (0–60 mins) or (b) for 15 mins with a range (0–10 μM) of coumermycin concentrations. Whole cell lysates from duplicate wells were analysed by immunoblotting with antibodies recognizing the phosphorylated forms of ERK1/2 (pERK1/2; top panels). Western blots were then stripped and reprobed with antibodies recognizing the total cellular complement of ERK1/2 (ERK1/2; bottom panels) to control for variations in protein levels.

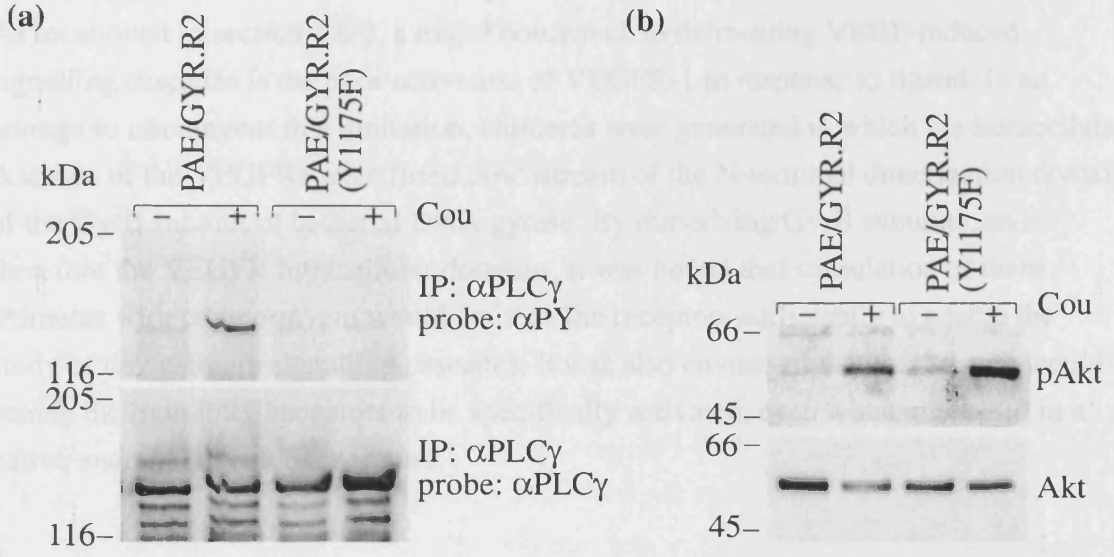


Fig. 4.13: Y1175F mutation in GyrB–VEGFR-2 ablates PLCγ , but not Akt, phosphorylation. PAE cells expressing either wild-type GyrB–VEGFR-2 or GyrB–VEGFR-2(Y1175F) were stimulated with 1 μM coumermycin for 15 mins. (a) PLCγ was immunoprecipitated from cell lysates with an anti-PLCγ (sc-81) antibody and subjected to western blotting. Blots were analysed for phosphorylation by probing with an antibody recognizing phosphotyrosine (αPY, top panel). Immunoblots were then stripped and reprobed with an anti-PLCγ antibody (sc-7290) to control for variations in the amount of precipitated protein (bottom panel). (b) Whole cell lysates from these cells were analysed by immunoblotting, probing first with an antibody recognizing phosphorylated Akt (pAkt; top panel), and then (after stripping) with an antibody recognizing total Akt (Akt; bottom panel).

4.4: Discussion

As mentioned in section 1.6.2, a major bottleneck to delineating VEGF-induced signalling cascades is the poor activation of VEGFR-1 in response to ligand. In an attempt to circumvent this limitation, chimeras were generated in which the intracellular domains of the VEGFRs were fused downstream of the N-terminal dimerization domain of the GyrB subunit of bacterial DNA gyrase. By dimerizing GyrB subunits, and therefore the VEGFR intracellular domains, it was hoped that stimulation of these chimeras with coumermycin would activate the receptors sufficiently to enable the study of downstream signalling cascades. It was also envisaged that this system would enable the individual receptors to be specifically activated, even when expressed in a native endothelial cell background.

4.4.1: Can chimeric VEGFRs be of use for studying signalling cascades downstream of VEGF?

4.4.1 (i) Ligand-induced autophosphorylation

As shown in Fig. 4.4, coumermycin stimulation of cells expressing the GyrB–VEGFR chimeras significantly induced autophosphorylation of the chimeric receptors. (This is of particular significance in the case of VEGFR-1 because of the poor ligand-induced phosphorylation associated with the full-length receptor.) Because the attribution of signalling capabilities to the individual VEGFRs in previous experiments might have been influenced by poor VEGFR-1 phosphorylation, these chimeras could be used to provide a more balanced comparison of receptor-specific responses. However, it must be remembered that chimeras represent an artifical system, and so the data they produce might not be applicable to more physiological situations. Despite this caution, current opinion is that VEGFR-1 is incapable of mediating signalling responses comparable to those mediated by VEGFR-2; at least here, therefore, use of the chimeras should highlight the potential capabilities of VEGFR-1.

Although coumermycin induced the phosphorylation of both chimeras, the phosphorylation of VEGFR-1 was more sustained than that of VEGFR-2: >90 mins and 5–15 mins, respectively. This might reflect the presence of VEGFR-2-specific phosphatases. Indeed, HCPTPA, a tyrosine phosphatase known to be expressed in endothelial cells, has been shown to interact with VEGFR-2 and to inhibit VEGFR-2-mediated cellular responses; by contrast, no interaction has been reported between this phosphatase and VEGFR-1 (Huang *et al.*, 1999). Although this discrepancy seems intriguing, it might not be relevant to more physiological signalling. For example, the mechanism of phosphorylation of the chimeric receptors might differ from that of the full-length receptors, possibly as a consequence of the incorporated myristoylation sequence, which could affect the spatial and temporal relationships between the

receptors and the plasma membrane. In addition, it is probable that the mechanisms regulating the duration of phosphorylation differ between the chimeric and the endogenous receptors.

4.4.1 (ii) Responses mediated by the chimeric VEGFRs

Because of the artificial nature of the chimeric system, it was necessary to investigate whether signalling observed downstream of the full-length VEGFRs could also be mediated by the chimeric receptors. In analysing such responses, it could also be assessed whether they can only be mediated by VEGFR-2, as previously reported using the full-length receptors, or whether VEGFR-1, if sufficiently activated, is also potentially competent in this respect. Coumermycin-induced phosphorylation of ERK1/2, PLCγ and Akt was examined in the chimera-expressing cells because VEGF-induced phosphorylation of these effectors has been consistently demonstrated in endothelial cells, and has also been primarily attributed to VEGFR-2 (see sections 1.6).

As can be seen from Figs 4.7, 4.9 and 4.10, both GyrB–VEGFR-1 and GyrB–VEGFR-2 mediate phosphorylation of ERK1/2, Akt and PLCγ upon stimulation with coumermycin. The receptors appeared to mediate the activation of ERK and Akt with similar efficiency; however, PLCγ phosphorylation was slightly more pronounced downstream of the VEGFR-2 chimera. In addition to demonstrating that coumermycin-activation of GyrB–VEGFR-2 can induce similar downstream responses to VEGF-activation of full-length VEGFR-2, these studies show that GyrB–VEGFR-1 is also able to mediate comparable responses, data not previously ascertained for the full-length receptor.

Having observed ERK activation, which is frequently associated with the induction of cell proliferation downstream of RTKs, the effect of coumermycin on DNA synthesis in chimera-expressing cells was then analysed. A very slight stimulation was observed, which was significant, in GyrB–VEGFR-1-expressing cells at 1pM. At this concentration, although an increase in DNA synthesis was observed in the GyrB–VEGFR-2-expressing cells, this value was not significant. Previous studies have attributed VEGF-induced proliferation of endothelial cells to VEGFR-2 rather than VEGFR-1 [see section 1.6.3 (i)]. However, in these studies, whether VEGFR-2 was expressed in PAE (Kroll and Waltenberger, 1997; Landgren *et al.*, 1998) or NIH3T3 (Takahashi and Shibuya, 1997) cells, proliferation was induced only twofold upon VEGF stimulation. Consequently, no firm conclusions can be drawn from the observation of a weak induction of DNA synthesis in GyrB–VEGFR-1. Indeed, even in primary endothelial cells, the magnitude of the VEGF-induced proliferation response varies dramatically from less than twofold to sevenfold (Wu *et al.*, 2000a; Thakker *et al.*, 1999; Bernatchez *et al.*, 1999), depending on the cell type and the assay used. It is therefore possible that the BrdU incorporation assay was of insufficient sensitivity to

detect any significant coumermycin-induced proliferation. In future experiments, the effects of including variable amounts of FCS in the stimulation media could be assessed. Alternatively, proliferation could be analysed using [^{3}H]thymidine incorporation and SRE-driven transcription assays. In addition, it will be important to analyse VEGF-induced proliferation of VEGFR-2/PAE cells and coumermycin-induced proliferation of GyrB–VEGFR-2/PAE cells in parallel. If a stimulation of proliferation was consistently observed in the chimera-expressing cells, it would then be possible to analyse the involvement of JNK, PI3K, PLCγ and NO [all of which have been implicated in VEGF-induced proliferation; see section 1.6.3 (i)] in this pathway, downstream of the individual receptors.

Recently, a second group reported data from signalling studies using a chimeric VEGFR-2 receptor. This receptor contained the entire transmembrane and cytoplasmic domains of VEGFR-2 fused to the extracellular domain of CSF-1R/c-fms; and was expressed in PAE cells (Rahimi *et al.*, 2000). Ligand stimulation of these receptors activated PLCγ, Akt and ERK, as did the GyrB–VEGFR-2 chimera. In addition, this group also demonstrated activation of PI3K and an approximately three-fold induction of cell proliferation downstream of the VEGFR-2 chimera (Dayanir *et al.*, 2001).

4.4.1 (iii) The mechanisms of chimeric VEGFR signalling

Even though GyrB–VEGFR-2 had been shown to mediate effector phosphorylation similar to that observed downstream of the full-length receptor, it was not known whether the mechanisms leading to these responses were analogous; that is, whether the chimeric receptor recruited SH2-containing effectors to specific phosphotyrosine residues. These mechanisms of effector activation would need to be comparable for the signalling data obtained through using the chimeric system to have any potential relevance to endogenous VEGF signalling pathways. To investigate this, an individual tyrosine residue (Y1175) in the cytoplasmic domain of GyrB–VEGFR-2 was mutated, and the signalling capabilities of this receptor were analysed. This residue was chosen because the Y1175 site had previously been shown to bind PLCγ (Cunningham *et al.*, 1997) and the Shc-like protein Sck (Igarashi *et al.*, 1998b; Warner *et al.*, 2000) in yeast. If the chimeric systems did couple to downstream effectors via phosphotyrosine–SH2 interactions, they could be of use for further defining VEGFR residues important for mediating such interactions *in vivo*.

Phosphorylation of Akt and ERK1/2 was not affected by mutating the Y1175 residue. By contrast, that of PLCγ was completely ablated, thereby suggesting that the chimeric receptors do indeed couple to downstream effectors via specific phosphotyrosine–SH2 interactions.

Phosphorylation of Akt downstream of GyrB–VEGFR-2 was not affected by mutation of the Y1175 residue. Many RTKs couple to the Akt/PKB pathway via the

recruitment, and subsequent phosphorylation, of PI3K (reviewed in Coffer *et al.*, 1998). Therefore, if Akt activation is dependent upon PI3K in the chimeric system, a point which warrants further experimentation, it can be concluded that the Y1175 site is not required for PI3K activation. The interaction of VEGFR-1 with p85 in yeast has been shown to require the Y1213 residue; however, no interaction was detected between VEGFR-2 and p85 (Cunningham *et al.*, 1995). It is possible, therefore, that VEGFR-2 does not couple to Akt activation through the direct binding of PI3K. Alternatively, PI3K might be activated further downstream, for example by src-like kinases (Pleiman *et al.*, 1994), or might interact with VEGFR-2 via VRAP (Wu *et al.*, 2000b). The VEGFR-2 Y951 residue has been shown to be essential for the receptor's interaction with VRAP (Wu *et al.*, 2000b). Therefore, mutating this residue in the chimeric system might further define the mechanism by which VEGFR-2 activates Akt.

It is of note that the chimeric VEGFR-2 construct lacks the juxtamembrane Y801 residue. Therefore, it can be concluded that Y801 is not essential for the Akt phosphorylation mediated by this receptor, although it remains possible that the phosphorylation could be enhanced in the presence of Y801. In addition, the Akt phosphorylation observed downstream of the Y1175F mutant (in affect the Y801/1175F double mutant) was comparable to that mediated by the wild-type (i.e. Y801 mutant) receptor. Data from studies with the CSF-IR–VEGFR-2 chimera demonstrated that a Y801/1175F mutant ablated the ligand-induced interaction with PI3K observed with the wild-type chimera (Dayanir *et al.*, 2001). Therefore, GyrB–VEGFR-2 Akt activation must either be independent of PI3K, or PI3K must be recruited to sites other than Y801 and Y1175.

In contrast to the activation of Akt, PLCγ phosphorylation was completely ablated by the Y1175F mutation, suggesting that Y1175 might be the primary site for recruiting PLCγ *in vivo*. An important role for this residue in PLCγ binding has previously been demonstrated in yeast: mutating the Y1175 residue significantly disrupted the PLCγ–VEGFR-2 interaction, and this interaction was virtually ablated in the Y801/1175F double mutant (Cunningham *et al.*, 1997; Wu *et al.*, 2000a). In addition, Y1175 corresponds to Y1169 in VEGFR-1, a major interaction site for PLCγ in both yeast and mammalian cells (Sawano *et al.*, 1997; Cunningham *et al.*, 1997). Another study in yeast showed that the VEGFR-2 residue Y951 (i.e. the residue that recruits VRAP) was essential for the interaction between VEGFR-2 and PLCγ (Wu *et al.*, 2000b). The results presented here provide the first data linking PLCγ with the Y1175 residue of VEGFR-2 in mammalian cells. The complete disruption of the GyrB–VEGFR-2–PLCγ interaction by Y1175F suggests that if a Y951–VRAP interaction is involved, it is not sufficient to mediate PLCγ recruitment in the absence of the Y1175 residue. In support of a direct association between VEGFR-2 and PLCγ, rather than an indirect interaction via VRAP, mutation of Y951 had no effect on the

interaction of full-length VEGFR-2 with PLCγ in HEK293 cells (Dougher and Terman, 1999). However, the binding of PLCγ to the CSF-IR–VEGFR-1 chimera was not affected by single or double Y801 and Y1175 mutations (Dayanir *et al.*, 2001).

The studies in yeast implicated an involvement of both Y801 and Y1175 in PLCγ recruitment. Because the chimeric VEGFR-2 receptor lacks Y801, the ablation of PLCγ phosphorylation seen using the chimeric system reflects the absence of both Y801 and Y1175. However, the fact that phosphorylation of this effector is seen downstream of 'wild-type' GyrB–VEGFR-2 (i.e. lacking Y801 but containing Y1175), implies that Y801 is not essential for this response; although it remains possible that this residue could have a role in PLCγ phosphorylation. Future experiments need to determine whether the Y1175 mutation alone is capable of ablating VEGFR-2-mediated PLCγ activation, or whether both sites need to be mutated for this effect.

Similar to the phosphorylation of Akt, that of ERK1/2 was not affected by mutation of the Y1175 residue. Because of the additional lack of Y801 in the chimeric VEGFR-2 constructs, it can also be concluded that this residue is not essential for ERK phosphorylation; however, it remains possible that phosphorylation could be enhanced in the presence of Y801. In addition, the ERK1/2 phosphorylation observed downstream of the Y1175F mutant (i.e. the Y801/1175F double mutant) was comparable to that mediated by the wild-type (i.e. Y801 mutant) chimera.

Both PLCγ and PI3K have been implicated in VEGF-induced proliferation [see section 1.6.3 (i)]. Interestingly, because PLCγ phosphorylation was ablated in the Y1175 mutant but ERK1/2 phosphorylation was unaffected by this mutation, at least in the case of chimeric VEGFR-2, it appears that this effector is not involved in the pathway to ERK activation. By contrast, Akt phosphorylation was not affected by this mutation and so, if this phosphorylation is mediated by PI3K as has been suggested (Fujio and Walsh, 1999; Gerber *et al.*, 1998a), a role for PI3K in the activation of ERK remains plausible.

As mentioned previously, the Shc adaptor protein is often involved in the prototypical pathway from RTKs to ERK activation. However, VEGF has been shown to induce strong activation of ERK alongside only a weak phosphorylation of Shc (Seetharam *et al.*, 1995). Relatively recent studies with the Shc-like protein Sck [discussed in more detail in section 5.1.2 (ii)] have led to the suggestion that this adaptor might substitute for Shc in VEGF-induced ERK activation. The interaction between Sck and VEGFR-2 has been shown to require the Y1175 residue in yeast (Igarashi *et al.*, 1998b; Warner *et al.*, 2000). In addition, binding of VEGFR-2 to Sck in PAE cells was competitively inhibited with a Y1175 phosphopeptide (Warner *et al.*, 2000). Therefore, the fact that mutation of this residue did not affect the ability of GyrB–VEGFR-2 to mediate ERK activation suggests that if Sck is required for this response, it must be able to bind at a site other than Y1175.

Other effectors that have been implicated in VEGF-induced ERK activation are NO and JNK. It remains to be determined whether these are involved in the response mediated by the chimeric receptors. In addition to extending the GyrB–VEGFR-2 analysis, for example by mutating other tyrosine residues, similar analyses now need to be conducted on GyrB–VEGFR-1.

4.4.1 (iv) Future use of chimeric receptors

The initial aims for generating these constructs were: (1) to obtain a version of VEGFR-1 that could be sufficiently phosphorylated to enable the study of signalling mediated by this receptor; and (2) to develop a system in which individual VEGFRs could be specifically activated in native endothelial cells without activating endogenous receptors. The GyrB-VEGFR-1 chimera is phosphorylated in response to coumermycin and so should prove invaluable in exploring the signalling capabilities of VEGFR-1. In addition, this activation implies that previous studies, demonstrating only limited VEGFR-1-mediated responses, might have underestimated the signalling potential of this receptor as a consequence of inefficient ligand-induced activation. Furthermore, the results presented here suggest that the apparent inability of VEGFR-1 to activate signalling pathways in endothelial cells is not caused by a deficient intrinsic kinase activity, as has been reported for certain atypical tyrosine kinases such as Ryk and c-erbB3 (Katso *et al.*, 1999; Sierke *et al.*, 1997). Instead, the 'resistance' of full-length VEGFR-1 to ligand-induced activation, both *in vivo* and *in vitro*, might reflect the requirement of as yet unidentified components, such as additional growth factors or co-factors.

The preliminary results described above also imply that mutational analysis of the chimeras might permit the identification of phosphotyrosine residues important for VEGFR-mediated responses. However, there are many ways in which the chimeric receptors could differ from the native full-length receptors, necessitating cautious careful interpretation of any results. Such potential discrepancies include the mechanisms of phosphorylation and regulation of activation, as well as post-translational modifications and expression levels of the two types of receptors.

A second chimeric VEGFR system has been characterized relatively recently (Dayanir *et al.*, 2001). Data generated using this system, in which the entire transmembrane and cytoplasmic domains of VEGFR-2 are fused to the extracellular domain of CSF-IR, contrast with several of the GyrB–VEGFR-2 observations. For example, the association of the chimeric receptors with PLCγ is ablated by a Y1175F mutation in our studies, but unaffected in those of Dayanir *et al.* By contrast, this group showed a complete dependency of PI3K association on this residue, which could cast doubt over how Akt activation is achieved, despite a Y1175F mutation, in our system. It is possible that the mechanisms of receptor activation and phosphorylation differ in

these two chimeric systems. For example, because of the myristoylation sequence, the gyrase chimeras are more likely to be constitutively localized at the plasma membrane than the CSF-IR chimeras. However, the extracellular domain of the latter are more likely to interact with cellular factors, which could influence the signalling response, than are the gyrase domains of the GyrB chimeras.

Finally, coumermycin had no effect on the phosphorylation status of the full-length VEGFRs expressed in transfected PAE cells. This strengthens their proposed potential for studying signalling cascades mediated by the individual VEGFRs expressed in a native endothelial cell background containing physiological effectors. Ultimately, it is hoped that such drug-activatable receptors will be of use in therapeutic angiogenesis, enabling manipulation of VEGF-induced signalling without affecting the physiology of endogenous receptors.

Chapter 5

Chapter 5: Interactions between VEGFR-1 and SH2-domain-containing proteins

Chapter 5: Interactions between VEGFR-1 and SH2-domain-containing proteins

5.1: Introduction

5.1.1: The role of SH2 domains in intracellular signalling cascades

SH2 domains are phosphotyrosine-recognition domains that mediate protein–protein interactions in signalling cascades. As mentioned in section 1.6.1, activation of RTKs results in phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor. Such phosphorylation creates docking sites for downstream targets containing SH2 domains or other phosphotyrosine-recognition domains such as PTB domains (Pawson, 1995). Only SH2 domains are discussed in this chapter.

SH2 domains are ~100 amino acid residues in length, and resemble a noncatalytic region of the c-Src tyrosine kinase (Sadowski *et al.*, 1986). These domains have a hydrophobic core, comprising a large, antiparallel β sheet with two flanking α helices, and numerous charged residues (Cohen *et al.*, 1995). The phosphopeptide-binding site in SH2 domains is bipartite: a conserved pocket lined with basic residues binds the phosphotyrosine, and adjacent residues selectively bind amino acids immediately C-terminal to the phosphotyrosine – Yxxx (Waksman *et al.*, 1993; Eck *et al.*, 1993; Pascal *et al.*, 1994; Lee *et al.*, 1994; Songang *et al.*, 1993).

As the number of proteins harbouring SH2 domains increased, subgroups of SH2 domains began to emerge on the basis of their target specificity. Using a phosphopeptide library to determine the target sequence specificity of various SH2 domains, it was found that SH2 domains can be grouped into four subgroups on the basis of the βD5 residue (fifth residue of the fourth β structure) in the SH2 domain (Songyang *et al.*, 1993; 1995). The structural basis of SH2 substrate specificity is reviewed in Cohen *et al.*, (Cohen *et al.*, 1995).

Many effectors contain SH2 domains, including both nonenzymatic adaptors such as Shc, Grb2 and the p85 subunit of PI3K, as well as effectors with catalytic activity such as PLCγ and SHP 1 and 2 (see examples in Pawson and Gish, 1992). By mediating protein–protein interactions, SH2 domains can influence cellular signalling cascades by recruiting cytosolic enzymes to their substrates [e.g. recruiting GAP to Ras (McCormick, 1989)]. In addition, recruitment of some enzymes to activated RTKs increases their activity; for example PI3K (Backer *et al.*, 1992; Shoelson *et al.*, 1993; Carpenter *et al.*, 1993) and PLCγ1 (Nishibe *et al.*, 1990).

5.1.2: The role of 'novel' effectors in VEGF signalling cascades

Several discrepancies have been observed during studies of VEGF signalling cascades that suggest VEGFRs might mediate responses using effectors distinct from those used by other RTKs. For example, VEGF induces a strong activation of ERK1/2 in SECs in the absence of significant Shc activation (Seetharam *et al.*, 1995), which is required for this response downstream of other RTKs. In addition, when expressed in fibroblasts, EGFR, but not VEGFR-2, is able to induce a strong activation of ERK (Kroll and Waltenberger, 1997; Takahashi and Shibuya, 1997). Because these same researchers showed that VEGFR-2 can mediate this response in endothelial cells, it has been suggested that VEGF might couple to downstream cellular responses using atypical pathways, possibly involving endothelial-cell-specific effectors. Indeed, upon stimulation with VEGF, more cellular proteins were phosphorylated in endothelial cells than in fibroblasts (Takahashi and Shibuya, 1997; Yamane *et al.*, 1994). Recently, novel candidates for these potential VEGF-specific pathways have been identified: three previously unknown proteins were isolated from cell lysates as phosphorylation-dependent binding partners for immobilized VEGFR-1-derived peptides (Ito *et al.*, 1998).

5.1.2 (i) Grb2-like proteins and VEGFR signalling

Grb2 is a ubiquitous adaptor protein with no catalytic function. Because it contains three protein–protein interaction domains: a central SH2 domain flanked by two SH3 domains (Fig. 5.1), Grb2 is well suited to mediating the assembly of multimeric protein aggregates. The most well-studied role for Grb2 is in transducing growth signals from activated RTKs to the ERK1/2 pathway [see section 1.6.1 (i)]. In this context, the SH2 domain of Grb2 mediates docking of the Grb2–Sos complex onto an activated RTK. Sos then activates Ras, which initiates the Ras–ERK phosphorylation cascade. The SH3 domain of Grb2 has also been shown to bind the GTPase dynamin; an interaction that might serve to link Ras signalling to clathrin-mediated endocytosis (Miki *et al.*, 1994; Scaife *et al.*, 1994; Seedorf *et al.*, 1994).

Discrepancies exist in the literature over whether Grb2 has a role in VEGF-induced signalling. Although no interactions have been detected between Grb2 and either VEGFR-1 or VEGFR-2 in yeast two-hybrid assays (Igarashi *et al.*, 1998a), when attached to a solid support, both a VEGFR-1-derived peptide and the intact VEGFR-1 intracellular domain were able to bind Grb2 in a phosphorylation-dependent manner (Ito *et al.*, 1998). In addition, VEGF-activated VEGFR-2 has been shown to associate with Grb2 *in vitro* (Kroll and Waltenberger, 1997). It is also possible that Grb2 could bind the VEGFRs indirectly, via tyrosine phosphorylated SHP-2 (Lechleider *et al.*, 1993; Li *et al.*, 1994). Alternatively, VEGFRs could couple to ERK activation via Crk and Nck, both of which are able to substitute for Grb2 in the Ras–ERK pathway

(Matsuda *et al.*, 1994; Hu *et al.*, 1995). Both of these adaptors have also been shown to bind a VEGFR-1-derived peptide (Ito *et al.*, 1998). In addition, VEGF-activated VEGFR-2 has been shown to associate with Nck *in vitro* (Kroll and Waltenberger, 1997) and VEGF-induced Nck phosphorylation has been detected in BAECs (Guo *et al.*, 1995).

Another possibility is that VEGFRs signal through other members of the Grb2 family; for example, through Grap and/or Gads. Intriguingly, one of the three unknown VEGFR-1-binding proteins identified by Ito *et al.* was characterized as a protein with homology to Grb2 and Grap (characterization of Gads had not been reported at this time) (Ito *et al.*, 1998). Furthermore, this protein was expressed in endothelial cell lines but not in fibroblast nor malignant epithelial cell lines (Ito *et al.*, 1998). Interestingly, the characterization of Gads revealed that expression of this protein was restricted to haematopoietic cells, including T lymphocytes, megakaryocytes, mast cells, natural killer cells and macrophages (Liu and McGlade, 1998; Liu, *et al.*, 1999).

All three of the Grb2-like proteins, Grb2, Grap and Gads, share the SH3–SH2–SH3 structure; however, Gads also has a unique, proline/glutamine-rich, region of 120 residues, located in between the SH2 domain and the C-terminal SH3 domain (Fig. 5.1, Liu and McGlade, 1998). The Gads SH2 domain has a similar substrate specificity to the SH2 domains of Grb2 and Grap, binding Shc and the Bcr-Abl fusion protein (Trub *et al.*, 1997; Feng *et al.*, 1996b; Liu and McGlade, 1998). By contrast, the SH3 domains of Grb2 and Gads have distinct target specificities; for example, Grb2, but not Gads, binds Sos, Sam68 and Cbl, whereas Gads has a higher affinity for SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) than does Grb2 (Liu and McGlade, 1998). In addition, the specificity of Gads, similar to that previously reported for Grap (Trüb *et al.*, 1997), seems to be more restrictive than that of Grb2 (Liu and McGlade, 1998). The similarity of the SH2 specificities and the divergence of the SH3 specificities have led to the suggestion that Grb2 and Gads could couple common upstream factors to distinct downstream effectors.

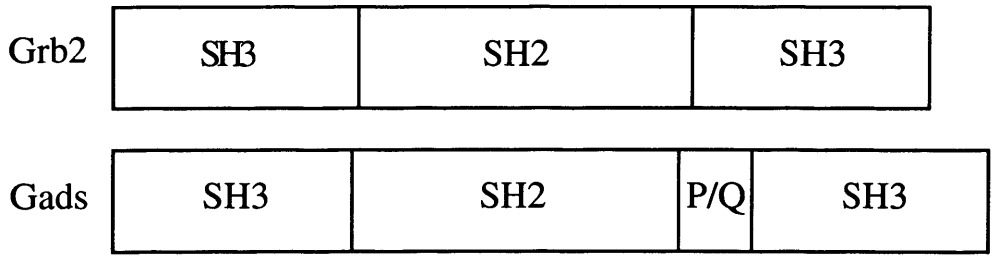


Fig. 5.1: Domain structure of Grb2 and Gads. Both proteins have a central SH2 domain and two terminal SH3 domains. Gads also has a unique, proline/glutamine-rich (P/Q) region of 120 residues.

Signalling roles for Gads reported so far have been restricted to T cells. In particular, upon T-cell receptor (TCR) ligation, Gads recruits SLP-76 to LAT (linker for activated T cells) at the TCR (Liu *et al.*, 1999). This Gads-mediated SLP-76 recruitment enhances ERK activation and transcription from the IL-2 promoter (Liu *et al.*, 1999; Asada *et al.*, 1999), and has also been implicated in the regulation of cytoskeletal reorganization (Bubeck Wardenburg *et al.*, 1998). Although Gads seems to play a significant role in ERK activation (Kikuchi *et al.*, 2001), ablating Gads expression had no effect on cell proliferation (Yoder *et al.*, 2001).

Because of the possibiity that Gads could represent the VEGFR-1-binding protein described by Ito *et al.* (Ito *et al.*, 1998), we decided to analyse Gads in respect to VEGF signalling in more detail.

5.1.2 (ii) Shc-like proteins and VEGFR signalling

Similar to Grb2, Shc is a ubiquitous adaptor protein with no catalytic function. Shc encodes three isoforms of 46, 52 and 66 kDa (Pelicci *et al.*, 1992). The two smaller isoforms are generated through the use of different translation start sites, whereas p66 is produced by alternative splicing. All three isoforms contain an N-terminal PTB domain, followed by a collagen homology (CH) domain and then an SH2 domain (Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994) (Fig. 5.2). In addition, the 66-kDa isoform has an extra CH domain (CH2) (Harun *et al.*, 1997). The prototypical role of Shc is in coupling RTKs to the Ras–ERK pathway via Grb2 [see section 1.6.3 (i)]. Shc has also been implicated in the suppression of apoptosis (Gotoh *et al.*, 1996).

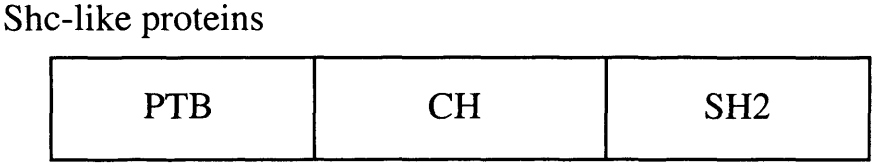


Fig. 5.2: Domain structure of Shc-like proteins. All Shc proteins contain a phosphotyrosine binding domain (PTB), a collagen homology domain (CH) and a src-homolgy (SH) 2 domain.

In addition to the three isoforms of Shc, the Shc-like family also includes Sck (Shc B) and N-Shc (Shc C). These proteins differ in their patterns of expression: N-Shc is restricted to the central nervous system; Sck is primarily expressed in liver, pancreas and prostate, but is also expressed in the brain; and Shc is ubiquitously expressed in all tissues other than brain (Nakamura *et al.*, 1996; 1998b; Kavanaugh and Williams, 1994). In addition, the SH2 domains of these proteins have a related, but distinct, substrate specificity (O'Bryan *et al.*, 1996). For example, although all three SH2

domains bind phosphorylated EGFR in cell lysates, the Sck SH2 domain does so with highest affinity, followed by that of N-Shc and then by that of Shc (Nakamura *et al.*, 1998b). Variations in signalling capabilities were also suggested by the observations that Src activated
N-Shc much more efficiently than Sck, and that Sck bound a phosphoprotein, pp135, much more tightly than did N-Shc (Nakamura *et al.*, 1998b).

In relation to VEGF signalling, VEGFR-2 was shown to mediate Shc phosphorylation *in vitro* (Kroll and Waltenberger, 1997). However, VEGF-induced Shc phosphorylation was barely detectable in intact cells, both in transfected NIH3T3 fibroblasts and in SECs (Seetharam *et al.*, 1995). Because VEGF induces a strong activation of ERK1/2 in SECs despite only weak Shc phosphorylation (Seetharam *et al.*, 1995), it has been suggested that one of the Shc-like proteins might substiute for Shc in this pathway. Indeed, several observations have led to the hypothesis that Sck is more likely a physiological effector of the VEGFRs than Shc. First, VEGFR-2 was precipitated from VEGFR-2/PAE cells with a GST fusion protein encoding the SH2 domain of Sck but not that of Shc (Warner *et al.*, 2000). Second, a VEGF-stimulated interaction between Sck and VEGFR-2 was observed in cotransfected HEK293 cells (Warner *et al.*, 2000); and third, both VEGFR-2 and VEGFR-1 interact with Sck in yeast (Igarashi *et al.*, 1998b; Warner *et al.*, 2000). In support of a potential role for Sck in VEGF signalling, this protein has also been shown to be expressed in HUVECs (Igarashi *et al.*, 1998b). In light of these observations, it was decided to study the ability of Sck to interact with the VEGFRs in more detail.

5.2: Materials and methods

An anti-Gads antibody was kindly provided by Jane McGlade, Hospital for Sick Childern, Toronto, ON, Canada. Three distinct antibodies recognizing VEGFR-1 were used: sc-316 (Santa Cruz), P3H8A9 (from Jan S. Rosenbaum, Procter & Gamble Pharmaceuticals, Mason, OH, USA) and AF-321-PB (R & D Systems). Jurkats were kindly provided by Marianne MacFarlan (Univeristy of Leicester, UK), and white blood cells (polynuclear monocytes and lymphocytes) were kindly provided by Phil Kulhman (University of Leicester, UK).

5.2.1: Maintenance of Jurkat T cells

Jurkat cells were routinely cultured in RPMI 1640 medium with Glutamax-I, supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were cultured in suspension in 75 cm^2 T75 plastic tissue culture flasks.

5.2.2: Isolation of monocytes from a white blood cell preparation

White blood cells (polynuclear monocytes and lymphocytes) had been isolated from human whole blood by Phil Kuhlman. This cell population was added to a tissue culture flask (in Ham's F12 medium with Glutamax-I) for 1 h to allow platelets and monocytes to adhere. Lymphocytes and any red blood cells were then removed by aspirating the media.

5.2.3: Whole cell lysate preparations for SDS–PAGE

5.2.3 (i) Jurkats

Cells approaching confluence were pelleted at 1200 rpm for 10 min and washed once with PBS before the addition of lysis buffer. Subsequent steps were as described for PAE cells [section 3.2.5 (v)].

5.2.3 (ii) Monocytes

Adherent monocytes were washed once with PBS before the addition of lysis buffer. Subsequent steps were as described for PAE cells [section 3.2.5 (v)].

5.3: Results

5.3.1: Interactions of SH2-domain-containing proteins with VEGFRs in yeast

The SH2 domains of Grb2, Gads, Shc and Sck had previously been subcloned into the pVP16 vector in our laboratory. These constructs were individually cotransformed into yeast with either VEGFR-1/pBTM116 or VEGFR-2/pBTM116 and representative colonies were analysed for transactivation of reporter genes by their growth on DOM1 plates, and their colour changes on X-gal plates and in β-galactosidase filter assays. In all assays (only X-gal results are shown), VEGFR-1 interacted with Gads, Grb2, Shc and Sck [Fig. 5.3 (a)]. By contrast, VEGFR-2 only interacted with Sck [Fig. 5.3 (b)].

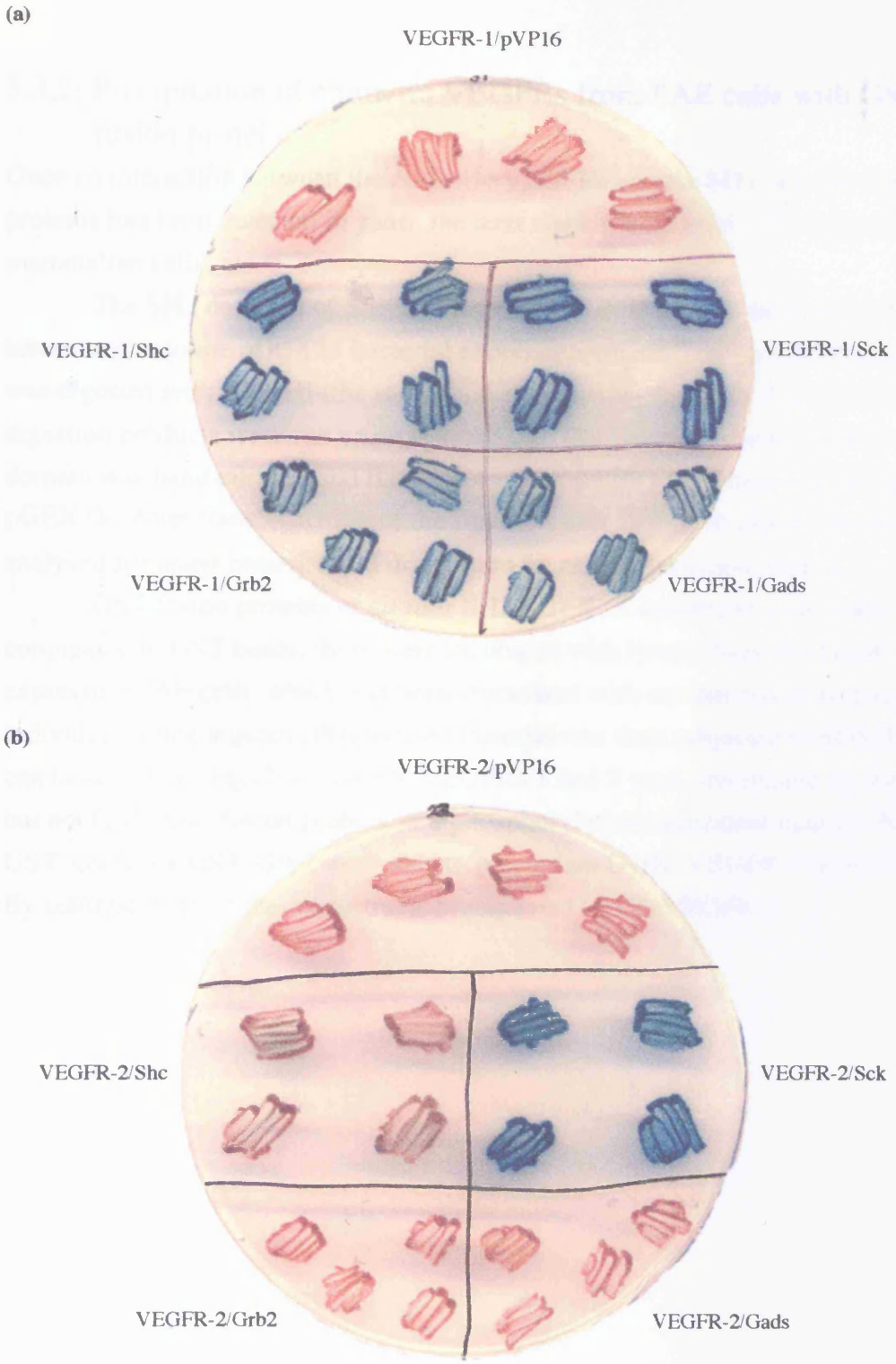


Fig. 5.3: Interaction of VEGFRs with SH2-containing proteins in yeast. (a) VEGFR-1 and (b) VEGFR-2 bait constructs were independently cotransformed into yeast either with pVP16, or with pVP16 constructs encoding the SH2 domains of Shc, Sck, Grb2 or Gads. Representative colonies were analysed for transactivation of reporter gene transcription by colour changes on X-gal plates.

5.3.2: Precipitation of chimeric VEGFRs from PAE cells with GST–SH2 fusion proteins

Once an interaction between the chimeric VEGFRs and the SH2-domain containing proteins had been detected in yeast, the next stage was to verify this interaction in mammalian cells.

The SH2 domains of Shc, Sck and Grb2 had previously been subcloned (in our laboratory) into the pGEX3x bacterial expression vector. The Gads/pVP16 construct was digested with *Bam* HI (the restriction site flanking the Gads SH2 domain) and the digestion products were run on an agarose gel. The fragment corresponding to the SH2 domain was band purified and ligated into the *Bam* HI site of digested, phosphatased pGEX3X. After transformation of the ligations into DH5α, resultant colonies were analysed for insert presence and orientation by restriction digest analysis.

GST fusion proteins of all four SH2/pGEX3X constructs were made. Once conjugated to GST beads, these were incubated with lysates from the GyrB–VEGFR-expressing PAE cells, which had been stimulated with coumermycin as described in the individual figure legends. Precipitated material was then subjected to SDS–PAGE. As can be seen from Figs 5.4, and 5.5, VEGFRs 1 and 2 were precipiated by the GST–Sck, but not GST–Shc, fusion protein, in a phosphorylation-dependent manner. Neither GST–Gads nor GST–Grb2 were able to precipitate GyrB–VEGFR-2 from PAE cells. By contrast, both of these constructs precipitated GyrB–VEGFR-1.

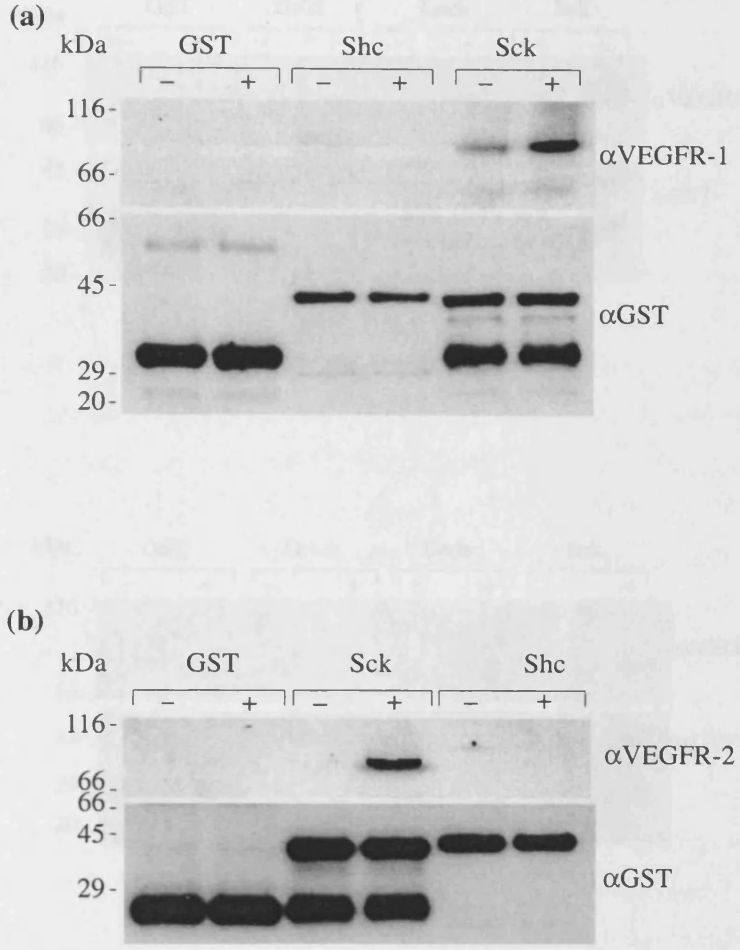


Fig. 5.4: Precipitation of chimeric VEGFRs from PAE cells with GST fusion proteins of Shc-like protein SH2 domains. PAE cells expressing GyrB–VEGFR chimeras were either left untreated or were stimulated with 1 μM coumermycin for 15 min. Lysates were then incubated with GST, GST–Sck or GST–Shc. Precipitates were analysed for the presence of either (a) GyrB–VEGFR-1 or (b) GyrB–VEGFR-2 by immunoblotting with antibodies to the intracellular domains of the receptors (top panels). To control for the amount of GST protein used in the precipitations, immunoblots were stripped and reprobed with an anti-GST antibody (bottom panels).

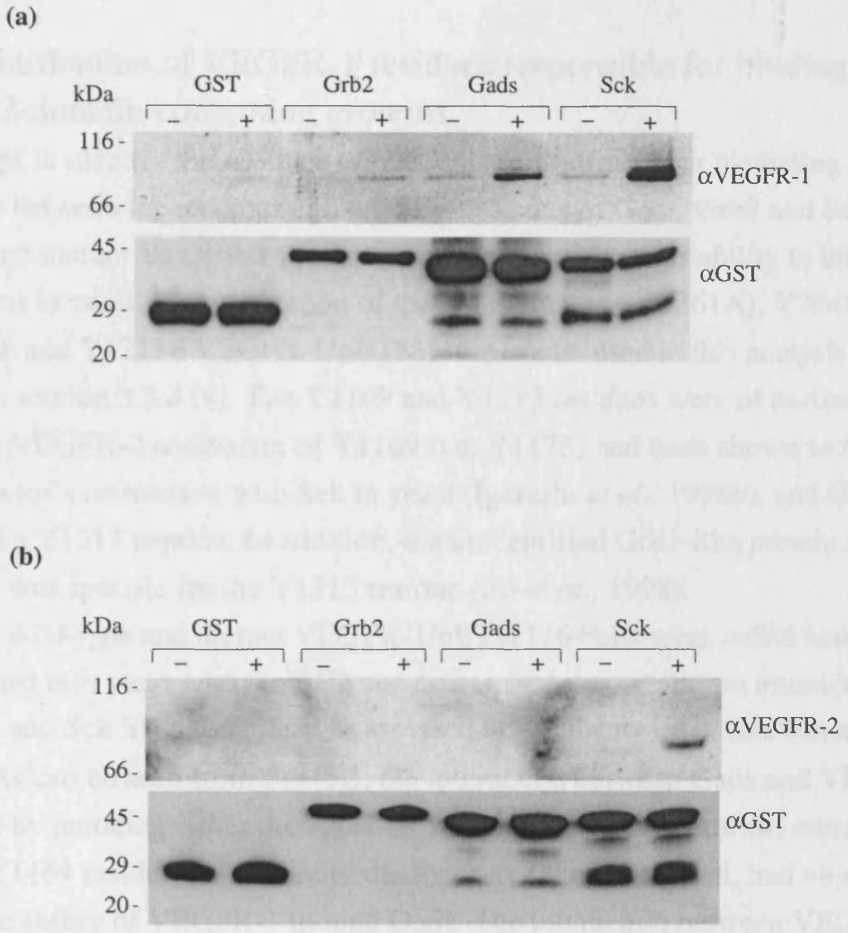


Fig. 5.5: Precipitation of chimeric VEGFRs from PAE cells with GST fusion proteins of Grb2-like protein SH2 domains. PAE cells expressing GyrB–VEGFR chimeras were either left untreated or were stimulated with 1 μM coumermycin for 15 min. Lysates were then incubated with GST, GST–Grb2, GST–Gads or GST–Sck. Precipitates were analysed for the presence of either (a) GyrB–VEGFR-1 or (b) GyrB–VEGFR-2 by immunoblotting with antibodies to the intracellular domains of the receptors (top panels). To control for the amount of GST protein used in the precipitations, immunoblots were stripped and reprobed with an anti-GST antibody (bottom panels).

5.3.3: Identification of VEGFR-1 residues responsible for binding SH2-domain-containing proteins

In an attempt to identify the residues of VEGFR-1 responsible for mediating interactions between the receptor and the SH2 domains of Gads, Grb2 and Sck , wild-type and mutant VEGFR-1 proteins were analysed for their ability to bind these SH2 domains in yeast. The generation of the kinase inactive (K861A), Y794F, Y1169F, Y794/1169F and Y1213F VEGFR-1/pBTM116 mutants used in this analysis is described in section 3.3.4 (v). The Y1169 and Y1213 residues were of particular interest because the VEGFR-2 equivalent of Y1169 (i.e. Y1175) had been shown to be essential for the receptor's interaction with Sck in yeast (Igarashi *et al.*, 1998b), and Grb2 was able to bind a Y1213 peptide. In addition, the unidentified Grb2-like protein identified by Ito *et al.* was specific for the Y1213 residue (Ito *et al.*, 1998).

The wild-type and mutant VEGFR-1/pBTM116 baits were individually cotransformed into yeast with the SH2 constructs, and their ability to interact with the Grb2, Gads and Sck SH2 domains was assessed in β-galactosidase solution assays (Fig. 5.5). As can be seen from Fig. 5.5, the interaction between Gads and VEGFR-1 was ablated by mutating either the K861 or Y1213 residue. By contrast, mutating the Y794 and Y1169 residues, either individually or as a double mutant, had no significant effect on the ability of VEGFR-1 to bind Gads. The interaction between VEGFR-1 and Grb2 was also completely ablated in the K861A and Y1213F mutants. However, the interaction between VEGFR-1 and Grb2 was also significantly decreased (by ~50%) if the Y794 and/or the Y1169 residues were mutated. The interaction between Sck and VEGFR-1 was ablated with the K861A mutation. In addition, the Y794F, Y1169F, Y1213F and Y794/11169F mutants, each inhibited the interaction by ~40–50%.

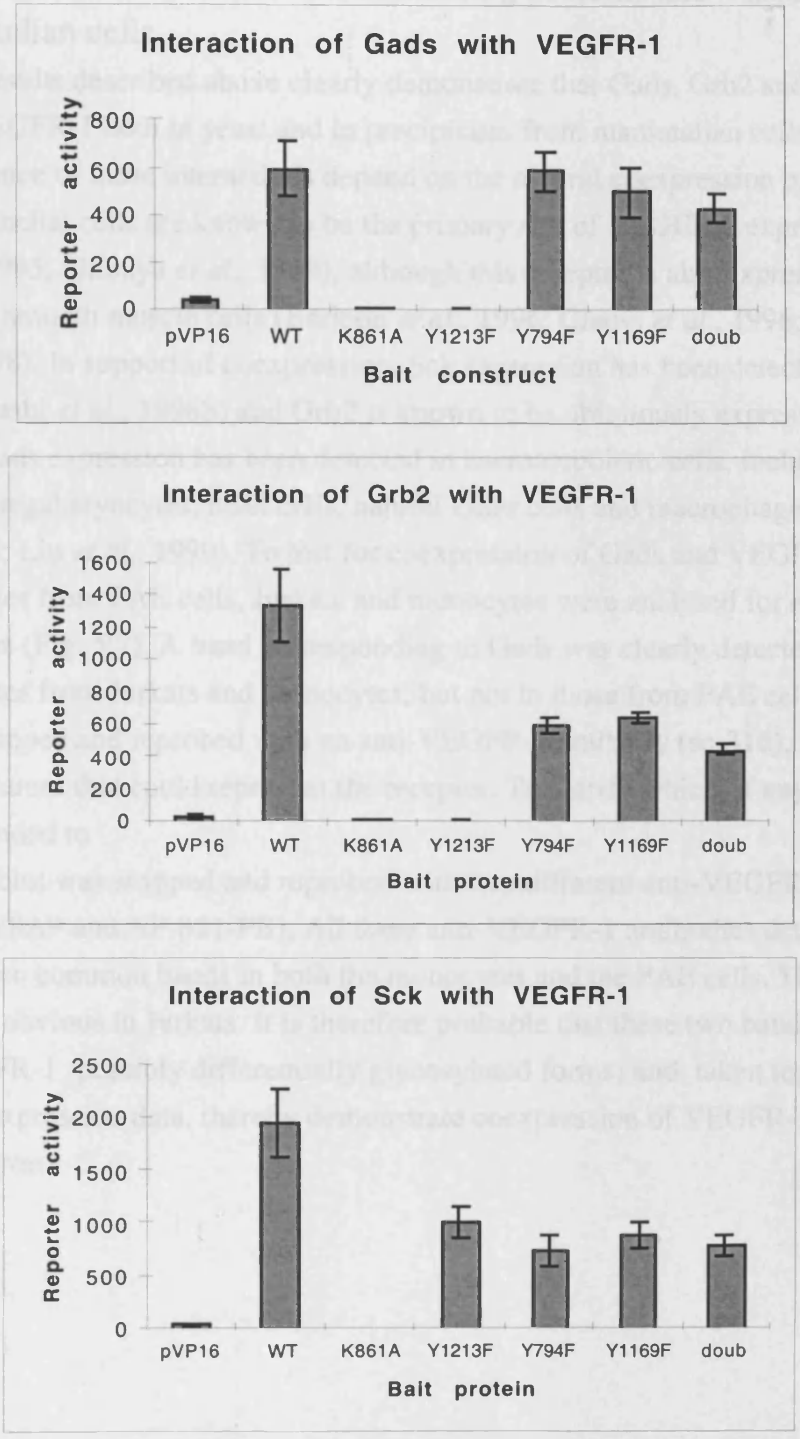


Fig. 5.6: Identification of receptor residues responsible for interactions between Gads, Grb2, Sck and VEGFR-1. Wild-type and mutant (doub represents the Y794/1169F double mutant) VEGFR-1/pBTM116 constructs were individually cotransformed into yeast with either the Gads/pVP16 (top), Grb2/pVP16 (middle) or Sck/pVP16 (bottom) constructs. Wild-type VEGFR-1/pBTM116 was also cotransformed with pVP16 to enable comparison of basal and activated transcription (pVP16). Representative transformants were analysed for transactivation of reporter genes in a β-galactosidase solution assay. Data are ±SE, and are representative of five experiments.

5.3.4: Coexpression of SH2-domain-containing proteins and VEGFR-1 in mammalian cells

Although the results described above clearly demonstrate that Gads, Grb2 and Sck are able to bind VEGFR-1 both in yeast and in precipitates from mammalian cells, any potential relevance of these interactions depend on the natural coexpression of the proteins. Endothelial cells are known to be the primary site of VEGFR-1 expression (Peters *et al.*, 1993; Shibuya *et al.*, 1990), although this receptor is also expressed in monocytes and smooth muscle cells (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Wang and Keiser, 1998). In support of coexpression, Sck expression has been detected in HUVECs (Igarashi *et al.*, 1998b) and Grb2 is known to be ubiqitously expressed (Suen *et al.*, 1993). Gads expression has been detected in haematopoietic cells, including T lymphocytes, megakaryocytes, mast cells, natural killer cells and macrophages (Liu and McGlade, 1998; Liu *et al.*, 1999). To test for coexpression of Gads and VEGFR-1, whole cell lysates from PAE cells, Jurkats and monocytes were analysed for expression of these proteins (Fig. 5.7). A band corresponding to Gads was clearly detected in the whole cell lysates from Jurkats and monocytes, but not in those from PAE cells. When the blot was stripped and reprobed with an anti-VEGFR-1 antibody (sc-316), several bands were apparent that could represent the receptor. To clarify which, if any, of these bands corresponded to

VEGFR-1, the blot was stripped and reprobed with two different anti-VEGFR-1 antibodies (P3H8A9 and AF-321-PB). All three anti-VEGFR-1 antibodies detected, albeit faintly, two common bands in both the monocytes and the PAE cells. These bands were not obvious in Jurkats. It is therefore probable that these two bands represent VEGFR-1 (possibly differentially glycosylated forms) and, taken together with the Gads expression data, thereby demonstrate coexpression of VEGFR-1 and Gads in monocytes.

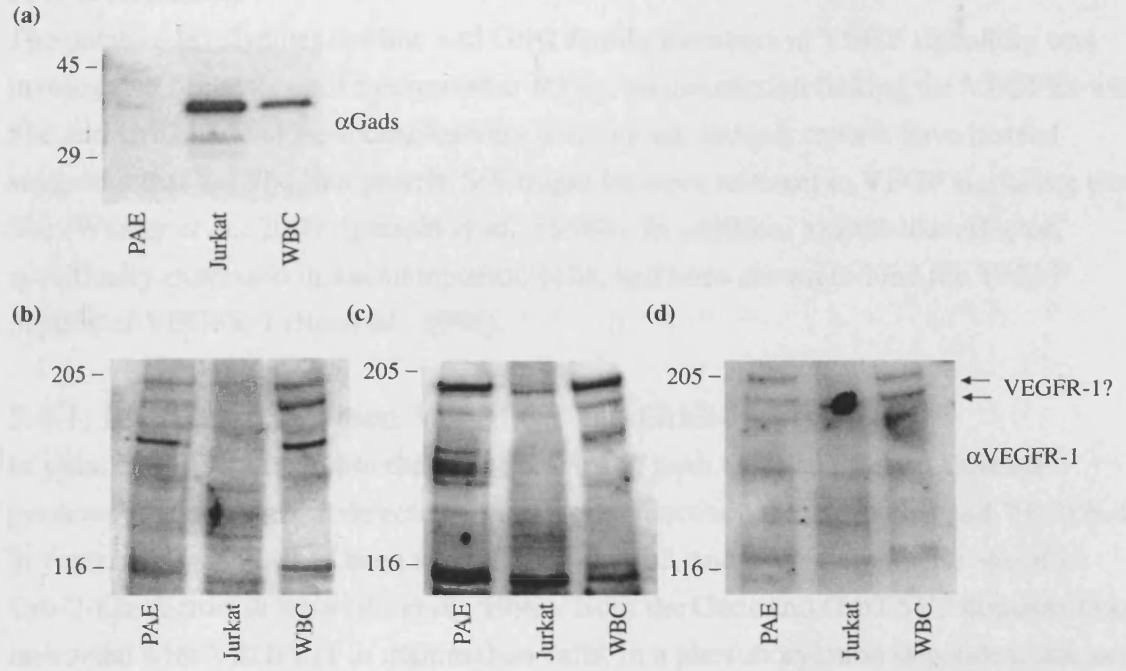


Fig. 5.7: Coexpression of Gads and VEGFR-1 in monocytes. Whole cell lysates from PAE cells, Jurkats and monocytes were subjected to SDS–PAGE and western blotting. (a) Lysates were analysed for the presence of Gads by immunoblotting with an anti-Gads antibody. (b–d) Blots were then stripped and reprobed with three different anti-VEGFR-1 antibodies [sc-316 (a), P3H8A9 (b) and AF-321-PB (c)] to analyse receptor expression.

5.4: Discussion

The putative involvement of Shc and Grb2 family members in VEGF signalling was investigated because, unlike many other RTKs, an association linking the VEGFRs with Shc and Grb2 had not been conclusively determined. Indeed, reports have instead suggested that the Shc-like protein Sck might be more relevant to VEGF signalling than Shc (Warner *et al.*, 2000; Igarashi *et al.*, 1998b). In addition, a Grb2-like effector, specifically expressed in haematopoeitic cells, had been shown to bind the Y1213 peptide of VEGFR-1 (Ito *et al.*, 1998).

5.4.1: Interaction between VEGFR-1 and Grb2-like proteins

In yeast, VEGFR-1 bound to the SH2 domains of both Gads and Grb2. Although previous studies have not detected an interaction between these proteins and VEGFR-1 in yeast, this receptor had been shown to bind Grb2, and a haematopoietic-specific Grb-2-like factor, *in vitro* (Ito *et al.*, 1998). Both the Gads and Grb2 SH2 domains also interacted with VEGFR-1 in mammalian cells, in a phosphorylation-dependent manner. No interactions between this receptor and any Grb-2-like protein have previously been reported in mammalian cells. By contrast, VEGFR-2 did not interact with either the Gads or Grb2 SH2 domain in yeast or mammalian cells. This is in constrast to one report, which demonstrated an interaction between VEGF-activated VEGFR-2 and Grb2 *in vitro* (Kroll and Waltenberger, 1997).

Previous studies had shown that a VEGFR-1-derived Y1213 peptide was able to precipitate both Grb2 and a Grb2-like protein from endothelial cell lysates (Ito *et al.*, 1998). However, mutating this residue in the context of the VEGFR-1 intracellular domain did not affect the receptor's ability to precipitate with Grb2 from insect cell lysates. By contrast, the results presented here show that the binding of both Grb2 and Gads SH2 domains to VEGFR-1 was virtually ablated by mutating the Y1213 residue. This discrepancy might reflect the fact that the whole Grb2 protein was used in the Ito study, compared with only the SH2 domain here. For example, it is possible that in the context of the whole Grb2 protein, when the Y1213 residue is mutated, the remaining contacts between the two proteins are sufficient to maintain an interaction. By contrast, there might be fewer contacts between the isolated SH2 domain and VEGFR-1, such that without Y1213, any remaining contacts are insufficient to maintain the interaction.

Another interesting observation was that whereas the interaction between VEGFR-1 and the Gads SH2 domain did not appear to involve either Y794 or Y1169; both of these residues were important for the interaction with the Grb2 SH2 domain, the individual mutations decreasing the interaction by ~50%. These discrepancies might reflect an increased stringency in the selectivity of the Gads SH2 domain compared with that of Grb2, as has been suggested for the SH3 domains (Liu and McGlade, 1998). In addition, it is possible that the binding of Grb2 to multiple sites implies that

this is a nonspecific, nonphysiological interaction, whereas the specific binding of Gads to Y1213 could be more relevant *in vivo*.

For the interactions between the Grb2-like proteins and VEGFR-1 to be relevant *in vivo*, the effectors need to be naturally coexpressed with the receptor. Grb2 is expressed ubiquitously in many cell types (Suen *et al.*, 1993). By contrast, the expression of Gads has been reported to be specific to haematopoietic cells (Liu and McGlade, 1998; Liu *et al.*, 1999), and VEGFR-1 is primarily expressed in endothelial cells and macrophages (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Wang and Keiser, 1998). By analysing the expression of VEGFR-1 and Gads in PAE cells, Jurkats and monocytes, it was demonstrated that these proteins appear to be coexpressed in monocytes, thereby giving support to the potential relevance of their interaction *in vivo*.

If Gads were to interact with VEGFR-1 in a more physiological setting, it is interesting to speculate on whether this effector would substitute for the role of Grb2 in the Ras–ERK pathway. However, no interaction could be detected between Gads and Sos in Jurkat cell lysates (Liu *et al.*, 1999). Alternatively, it has been proposed that Gads still binds Shc (similar to Grb2), but then initiates a pathway leading to the suppression of apoptosis, rather than the Ras–ERK pathway and cell proliferation (Gotoh *et al.*, 1996). Gads might also couple to effectors that are not bound by Grb2. Indeed, the similar substrate specificity of their SH2 domains, along with the distinct specificity of their SH3 domains, has led to the suggestion that Gads and Grb2 couple common upstream effectors to novel downstream targets.

It is possible that VEGFR-1 couples to both Grb2 and Gads *in vivo*. If this is the case, because the Grb2 SH2 domain seems able to bind a wider variety of phosphopeptide substrates than the Gads SH2 domain, the pool of Grb2 would probably be diluted between many phosphopeptide targets, whereas Gads would be more likely to be concentrated at the VEGFR-1 Y1213 site. Therefore, the expression levels of Grb2 in particular could regulate the relative proportions of VEGFR-1 signalling going through the individual Grb2-like proteins.

Finally, the failure to detect an interaction between Gads and VEGFR-2 is not too surprising given the stringent affinity of the Gads SH2 domain for Y1213. However, given the ability of the Grb2 SH2 domain to bind several VEGFR-1 residues (i.e. Y1213, Y794 and Y1169), it is surprising that the Grb2 SH2 domain did not interact with VEGFR-2.

5.4.2: Interaction between VEGFR-1 and Shc-like proteins

In yeast, VEGFR-1 was shown to bind both Sck and Shc SH2 domains, whereas VEGFR-2 bound only that of Sck. Interactions between Sck and both VEGFRs were subsequently verified in mammalian cells, whereas the SH2 domain of Shc did not precipitate either VEGFR from these cells. The loss of interaction between VEGFR-1 and the Shc SH2 domain upon moving from yeast to mammalian cells was not a consequence of poor functionality of the GST–Shc fusion protein as this protein had been shown to precipitate EGFR from cell lysates (Prigent *et al.*, 1995). Instead, it is more likely to reflect the presence of physiological effectors in mammalian cells that preclude the interaction by binding either Shc or VEGFR-1. These results agree with previous reports showing that Sck bound both VEGFRs in yeast (Igarashi *et al.*, 1998b; Warner *et al.*, 2000), and that a GST fusion protein encoding the SH2 domain of Sck, but not that of Shc, bound VEGFR-2 in VEGFR-2/PAE cells (Warner *et al.*, 2000).

Although the VEGFR-1–Sck interaction was phosphorylation dependent, no clear individual binding site for this effector was revealed by these studies. Instead, mutating any of the residues Y794, Y1169 and Y1213, as well as the Y794/1169F double mutant, decreased binding by ~40–50%. An essential requirement of the Y1175 residue had been reported for the Sck–VEGFR-2 interaction in yeast (Igarashi *et al.*, 1998b; Warner *et al.*, 2000), and a peptide encompassing this residue also inhibited the VEGFR-2–Sck interaction in PAE–VEGFR-2 cells (Warner *et al.*, 2000). However, although the equivalent residue in VEGFR-1 (i.e. Y1169) was significantly involved in the Sck–VEGFR-1 interaction in these studies, it was not essential. Unless the main Sck-binding residue of VEGFR-1 remains to be identified, therefore, converse to the situation with Grb2, it is possible that Sck represents a physiological target of VEGFR-2, but not of VEGFR-1.

The lack of an interaction between the SH2 domain of Shc and the VEGFRs in mammalian cells does not necessarily preclude their interaction; instead this could be mediated by the PTB domain of Shc. However, although VEGFR-1 (not VEGFR-2) has an NPxY site – the consensus target site for the Shc PTB domain (Prigent *et al.*, 1995), no interaction between this domain and VEGFR-1 was detected in yeast (Igarashi *et al.*, 1998b).

If the VEGFRs do indeed interact with Sck *in vivo* [Sck is expressed in endothelial cells (Igarashi *et al.*, 1998b)], it is possible that this effector substitutes for Shc in the Ras–ERK pathway to DNA proliferation. Indeed, a strong activation of ERK was observed in response to VEGF stimulation of SECs despite only a weak phosphorylation of Shc (Seetharam *et al.*, 1995). This hypothesis is strengthened by the observation that Sck can bind both EGFR and Grb2 (Nakamura *et al.*, 1998b). Alternatively, Sck might only be involved in VEGFR signalling in a subset of cells; for

example, not only is Sck highly expressed in nervous tissue, but Schwann cells have also been shown to express VEGFR-2 (Schratzberger *et al.*, 2000).

The downstream responses mediated by Sck, Grb2 and Sck in response to VEGFR activation obviously require further experimentation. However, the results presented here reveal the first interactions between VEGFR-1 and these effectors in mammalian cells and, at least in the case of Gads, define Y1213 as an essential residue for this interaction.

Chapter 6

Chapter 6: Interactions between VEGFR-1 and WW-domain-containing proteins

Chapter 6: Interactions between VEGFR-1 and WW-domain-containing proteins

6.1: Introduction

6.1.1: The role of WW domains in intracellular signalling cascades

Our interest in WW domains was provoked by the isolation of a partial cDNA clone encoding a polypeptide containing WW domains (see below) from a yeast two-hybrid library screen using the intracellular domain of VEGFR-1 as bait (Sally Prigent, unpublished).

WW domains, similar to SH2 and SH3 domains, are protein–protein interaction modules (Bork and Sudol, 1994). However, with a size of only 38 amino acids, WW domains are significantly smaller than their src homology counterparts (Sudol *et al.*, 1995a; 1995b). WW domains are so-called because of the presence of two invariant tryptophan residues, separated by 20–22 primarily aromatic amino acids (reviewed in Bork and Sudol, 1994; Sudol, 1996). In addition, these domains have an invariant proline residue at position +2 with respect to the second tryptophan and a preferential distribution of proline residues towards both termini of the linear sequence (Sudol *et al.*, 1995a; 1995b).

More than 25 distinct WW domain-containing proteins have been characterized, and WW-domain interactions have been reported to effect functions as diverse as protein degradation (e.g. Nedd4; Staub *et al.*, 1996), viral budding (e.g. the M protein of vesicular stomatitis virus; Craven *et al.*, 1999; Harty *et al.*, 1999), RNA splicing (e.g. the PRP40 splicing factor; Bedford *et al.*, 1998), transcriptional coactivation [e.g. RNA polymerase II (RPII) and YAP65; Gavva *et al.*, 1997; Yagi *et al.*, 1999] and mitotic regulation (e.g. the peptidyl-prolyl isomerase Pin 1; Yaffe *et al.*, 1997; Shen *et al.*, 1998).

The recognition motif initially identified for WW domains was a so-called PY motif (PPxY) (Chen and Sudol, 1995). However, as the number of proteins harbouring WW domains increased, it became increasing apt to classify WW domains into four groups on the basis of their target specificity: group I recognize PPxY motifs; group II recognize long stretches of proline residues, often interrupted by leucine residues (PPLP); group III recognize motifs rich in proline and arginine residues (PPR); and group IV recognize motifs in which proline residues are preceded by phosphoserine residues (Bedford *et al.*, 2000).

The preference of WW domains for sequences containing proline residues is reminiscent of the target specificity (PxxP) of SH3 domains (Feng *et al.*, 1994; Yu *et al.*, 1994). However, the SH3 domains of Yes, Fyn, Abl and RasGAP were unable to bind a PY-motif-containing peptide (GTPPPPYTVG) that bound to a WW domain

(Chen and Sudol, 1995), suggesting that these two types of domains do not compete for ligand. However, overlap between the target site specificities of WW and SH3 domains has been detected, although this is generally associated with Group III (PPR) WW domains (Bedford *et al.*, 1997; Lowe *et al.*, 1993). For example, the WW domains of formin-binding proteins [in group III (Bedford *et al.*, 1998)] have been reported to compete with the SH3 domain of Abl for binding to a polyproline peptide in formin (Chan *et al.*, 1996).

6.1.2: WW domains and VEGF signalling cascades

6.1.2 (i) Nedd4

A partial, WW-domain-encoding cDNA clone (encoding residues 467–634) of mNedd4, was previously identified as a VEGFR-1-binding protein in a yeast two-hybrid library screen using VEGFR-1 as bait (Sally Prigent, unpublished). This protein, which was originally isolated from a mouse brain library (Kumar *et al.*, 1992), has a calcium phospholipid binding domain (C2 domain), three WW domains and a HECT (homologous to the E6-AP carboxyl terminus) domain (Fig. 6.1).

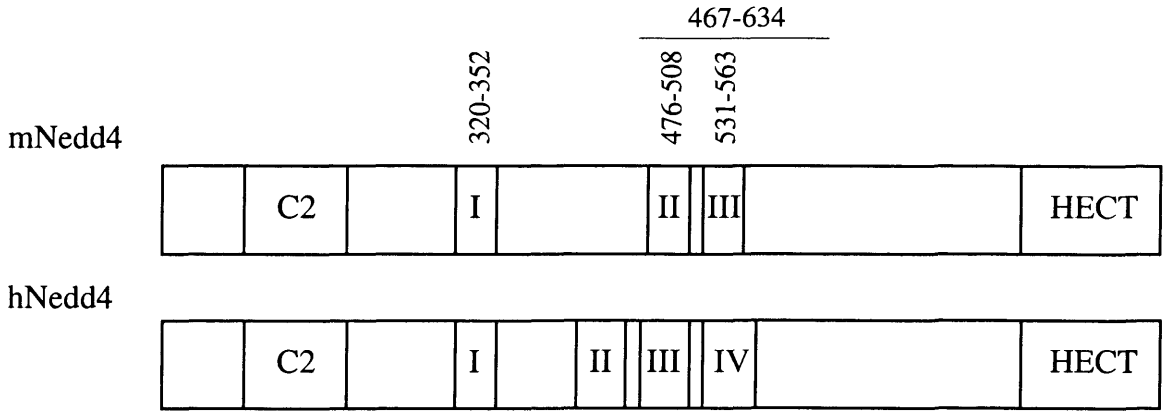


Fig. 6.1: Domain structure of Nedd4 proteins. Nedd4 proteins contain a calcium phospholipid binding domain (C2), WW domains (three in mouse and four in human) and a HECT domain.

HECT domains are generally associated with ubiquitin ligase activity, found in E3 enzymes (see below; Scheffner *et al.*, 1993). Ubiquitination [i.e. the linkage of ubiquitin (a 76 amino acid protein) to the lysine side chain of an acceptor protein] is a method by which cytosolic, misfolded and nuclear proteins are targeted to the 26S proteosome for degradation, and by which membrane proteins are targeted for endocytosis and either degradation in lysosomes or recycling (reviewed in Hicke, 1999). Ubiquination usually involves three enzymes, the third of which (the E3 enzyme

or ubiquitin protein ligase) is responsible for transferring the ubiquitin moiety onto the substrate to be degraded; that is, the E3 enzymes dictate the target specificity. Because Nedd4 contains a HECT domain therefore, it has been suggested that this protein has a role is targeting proteins for ubiquitination. Indeed, both Nedd4 (Staub *et al.*, 1997a) and it's yeast homologue Rsp5 (Huibregtse *et al.*, 1997) have been shown to have ubiquitin ligase activity, mediating the ubiquitination of ENaC (the amiloride-sensitive distal renal epithelial sodium channel) and the large subunit of RPII, respectively. In addition, supporting the role of ubiquitination in protein turnover, the Nedd4-mediated ubiquitination of ENaC leads to degradation of the channel (Staub *et al.*, 2000). Indeed, several ENaC mutations are represented in the human population that, by preventing the channel's interaction with Nedd4, lead to ENaC hyperactivity and the associated condition of Liddle's syndrome (Goulet *et al.*, 1998; Shimkets *et al.*, 1997; Hansson *et al.*, 1995; Tamura *et al.*, 1995). In addition, Nedd4 is thought to initiate degradation of RPII by ubiquitination (Sudol and Hunter, 2000). Nedd4 also binds to the Latent Membrane Protein 2A (LMP2A) of Epstein-Barr Virus, a protein that is known to be degraded by a ubiquitin-dependent mechanism (Ikeda *et al.*, 2000).

In addition to roles in degradation and recycling, ubiquitination has been shown to facilitate correct protein folding and assembly of ribosomes, thereby acting as a chaperone (Finley and Chau, 1991). Furthermore, the ubiquitination of histones has been associated with the formation of transcriptionally active chromatin (Finley and Chau, 1991).

The Nedd4-like proteins might also have functions independent of ubiquitination. For example, both Nedd4 and Rsp5 potentiate hormone-dependent transcriptional activation by human progesterone and glucocorticoid receptors (Imhof and McDonnell, 1996). This function is independent of both ubiquitin ligase activity and WW domains, and is therefore not secondary to the decompaction of chromatin seen upon histone ubiquitination.

Analyses of the interactions between Nedd4 WW domains and ENaC (Schild *et al.*, 1996; Staub *et al.*, 1996), LMP2A (Ikeda *et al.*, 2000), the erythroid-specific transcription factor p45/NF-E2 (Gavva *et al.*, 1997) and WW domain binding protein-1 (WBP-1; Gavva *et al.*, 1997), have shown that the Nedd4 WW domains belong to Group I, recognizing PPxY motifs. However, one group also reported an interaction between the mNedd4 WW domains and phosphoserine-containing (i.e. Group IV) PY motifs (Lu *et al.*, 1999). Interestingly, whereas mNedd contains three WW domains, its human homologue contains four, with the third WW domain of the human protein being novel (Fig. 6.1; Andre and Springael, 1994; Bork and Sudol, 1994; Hofmann and Boucher, 1995). The three common domains are highly conserved, and are therefore expected to have a similar ligand specificity (Kumar *et al.*, 1997).

With the isolation of the partial mNedd4 cDNA clone, encoding WW domains 2 and 3, as a VEGFR-1-binding protein, it was decided to investigate the potential relevance of this protein to VEGF signalling more extensively.

6.1.2 (ii) YAP

Isolation of mNedd4 as a VEGFR-1-binding partner in yeast encouraged us to search for other WW-domain-containing proteins potentially relevant to VEGF signalling. One such protein is YAP65 (Yes-associated protein of 65 kDa) (Andre and Springael, 1994), which was of particular interest because the VEGFRs have previously been shown to bind Yes, albeit rather weakly, *in vitro* (Waltenberger *et al.*, 1994). Moreover, sequence comparisons of various WW domains had revealed that the WW domains of YAP [mYAP has two WW domains whereas human YAP has only one (Sudol *et al*, 1995a)], Nedd4 and Rsp5 are more similar to each other than to the WW domains of other proteins (Sudol *et al.*, 1995a; 1995b), suggesting that these domains might recognize similar targets. Indeed, Nedd4 and YAP have been shown to bind the same PY targets, for example those in p45/NF-E2, RPII and WBP-1 (Gavva *et al.*, 1997). Similar to the WW domains of Nedd4, therefore, those of YAP belong to Group I (for examples see Chen and Sudol, 1995; Yagi *et al.*, 1999, Gavva *et al.*, 1997; Garnier *et al.*, 1996).

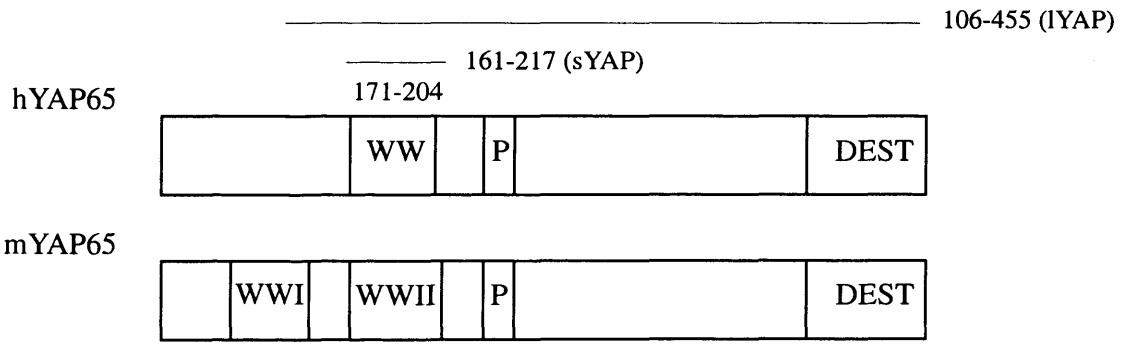


Fig. 6.2: Domain structure of YAP65 proteins. YAP proteins contain WW domains (one in human and two in mouse), a Pro-rich region (P), and a C-terminal region rich in Asp, Glu, Ser and Thr residues (DEST).

The ability of YAP to bind various SH3 domains, including those of Yes, Nck, Crk and Src (Sudol, 1994), has led to the suggestion that this protein might recruit substrates, via its WW domains, to these kinases. In addition, the observation that YAP acts as a potent transcriptional coactivator (Yagi *et al.*, 1999; Gavva *et al.*, 1997) implies that YAP could transmit signals from Src family kinases to the nucleus. This coactivation activity requires the binding of YAP WW domains to PY motifs in

transcription factors, an interaction that presumably recruits YAP to an appropriate promoter, from which it can then activate transcription (Yagi *et al.*, 1999; Kay *et al.*, 2000).

Because mNedd4 had been isolated as a VEGFR-1-binding protein, and because Nedd4 and YAP were thought to have similar substrate specificities, it was decided to analyse the ability of YAP to interact with VEGFR-1.

6.2: Material and methods

An antibody recognizing VP16 (sc-7545) was purchased from Santa Cruz.

6.2.1: Bait construction primers

FL.YAP.F2 (forward):5'–GGTAGCGGCCGCATCAGGCCAGTACTGATGCAGGC–3'
FL.YAP.R (reverse): 5'–GCGGGAATTCCTATAACCATGTAAGAAAGCT–3'

6.2.2: Bait mutagenesis primers

APPDY.F (forward): 5'–ATCGCGTGCTGCTCCGCGCCCCCAGACTACAAC–3'
APPDY.R (reverse): 5'–GTTGTAGTCTGGGGGCGCGGAGCAGCACGCGAT–3'
PAPDY.F (forward): 5'–GCGTGCTGCTCCCCGGCCCCAGACTACAACTCG–3'
PAPDY.R (reverse): 5'–CGAGTTGTAGTCTGGGGCCGGGGAGCAGCACGC–3'
PPADY.F (forward): 5'–TGCTGCTCCCCGCCCGCAGACTACAACTCGGTG–3'
PPADY.R (reverse): 5'–CACCGAGTTGTAGTCTGCGGGCGGGGAGCAGCA–3'
PPPAY.F (forward): 5'–TGCTCCCCGCCCCCAGCCTACAACTCGGTGGTC–3'
PPPAY.R (reverse): 5'–GACCACCGAGTTGTAGGCTGGGGGCGGGGAGCA–3'
PPPAY.F (forward): 5'–TGCTCCCCGCCCCCAGCCTACAACTCGGTGGTC–3'
PPPAY.R (reverse): 5'–GACCACCGAGTTGTAGGCTGGGGGCGGGGAGCA–3'
PPPDF.F (forward): 5´–TCCCCGCCCCCAGACTTCAACTCGGTGGTCCTG–3'
PPPDF.R (reverse): 5'–CAGGACCACCGAGTTGAAGTCTGGGGGCGGGGA–3'
ΔPPPDY.F (forward):

5'–GGAAAATCGCGTGCTGCTCCAACTCGGTGGTCCTGTACTCC–3'

ΔPPPDY.R (reverse):

5'–GGAGTACAGGACCACCGAGTTGGAGCAGCACGCGATTTTCC– 3'

6.2.3: Peptide synthesis

A peptide encompassing the VEGFR-1 PY motif (KIACCSPPPDYNSVVLYSTPPI) was synthesized on an Applied Biosystems model 431A peptide synthesizer using solid-phase Fmoc (fluoren-g-ylmethoxycarbonyl) chemistry [Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, Leicester, UK). The peptide was then dissolved to a concentration of 5 mg/ml in sodium phosphate buffer [0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4 (pH 7.4)], aliquoted and stored at –80°C.

6.3: Results

6.3.1: Interaction of Nedd4 and YAP65 with VEGFRs in yeast

The partial cDNA clone of mNedd4 had previously been isolated as a pVP16 construct from a yeast two-hybrid screen of a mouse embryo cDNA library using VEGFR-1 as bait (Sally Prigent, unpublished). This clone encoded residues 467–634 of mNedd4, thereby completely encompassing the second and third WW domains (Fig. 6.1). The WW domain of human YAP (residues 171–204) was subcloned by PCR from EST image clone 2178810 (accession number AI590105) into pVP16 by Syed Islam and Andrea Warner. These two constructs were individually cotransformed into yeast with either VEGFR-1/pBTM116 or VEGFR-2/pBTM116, and representative colonies were analysed for transactivation of reporter genes by their growth on plates lacking histidine, and their colour changes on X-gal plates and in β-galactosidase filter assays. VEGFR-1 interacted with Nedd4 in all assays (only X-gal results are shown), but failed to interact with YAP [Fig. 6.3 (a)]. VEGFR-2 interacted with neither Nedd4 nor YAP [Fig. 6.3 (b)].

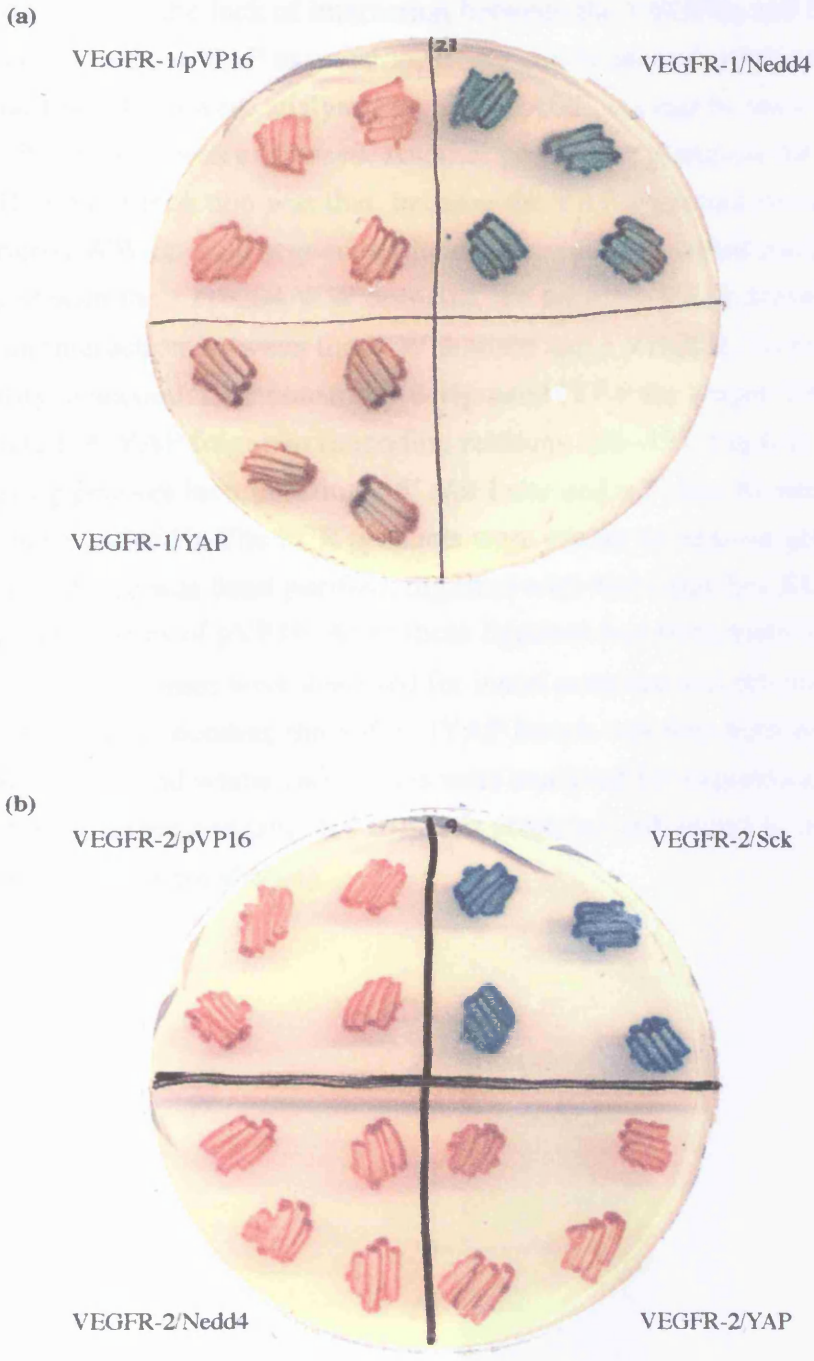


Fig. 6.3: Interaction of VEGFRs with WW domain-containing proteins. (a) VEGFR-1 and (b) VEGFR-2 bait constructs were independently cotransformed into yeast either with pVP16, or with pVP16 constructs encoding WW domains of Nedd4 or YAP. Representative colonies were analysed for transactivation of reporter gene transcription by colour changes on X-gal plates.

To check whether the lack of interaction between the VEGFRs and YAP was a consequence of poor YAP expression, whole cell lysates of colonies cotransformed with YAP and pBTM116 were analysed by SDS–PAGE. As can be seen from Fig. 6.4 (a), this YAP construct was expressed. Another possible explanation for the lack of a VEGFR–YAP interaction was that, because the YAP construct encoded little more than the minimal WW domain sequence (the clone actually encoded residues 161–217; compared with the 171–204 WW domain), the adjacent VP16 domain might sterically hinder an interaction between the WW domain and a VEGFR. To investigate this possibility, a second YAP construct (designated lYAP for longer YAP) was constructed. A YAP fragment (encoding residues 106–455; Fig 6.2) was subcloned by PCR, using primers incorporating a 5′ *Not* I site and a 3′ *Eco* RI site, from the EST image clone 2178810. The PCR products were run on an agarose gel and a fragment of the expected size was band purified, digested with *Not* I and *Eco* RI, and ligated into the corresponding sites of pVP16. After these ligations had been transformed into DH5α, representative colonies were analysed for insert presence and orientation by restriction digest. A plasmid encoding the VP16–lYAP fusion was then cotransformed into yeast with pBTM116, and whole cell lysates were analysed for expression of this protein. Despite being expressed [Fig. 6.4 (b)], this construct still failed to interact with either of the VEGFRs (data not shown).

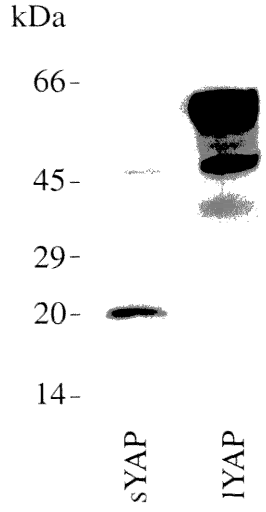


Fig. 6.4: sYAP and lYAP constructs are expressed in yeast. Representative colonies from a transformation of YAP/pVP16 and pBTM116 (sYAP), and lYAP/pVP16 and pBTM116 (lYAP), were analysed for expression of YAP. Whole cell lysates were subjected to SDS–PAGE and probed with an antibody recognizing VP16.

6.3.2: Precipitation of the chimeric VEGFRs from PAE cells with Nedd4 and YAP65 GST fusion proteins

Once an interaction between VEGFR-1 and Nedd4 had been detected in yeast, the next stage was to verify this interaction in mammalian cells. Because a VEGFR-1–Nedd4 interaction had been detected, and as Nedd4 WW domains were thought to recognize similar PY targets as the YAP WW domain, both Nedd4 and YAP GST fusion proteins were analysed for their ability to precipitate VEGFR-1 from mammalian cells. By contrast, this analysis was not performed with VEGFR-2 because this receptor had not interacted with Nedd4 or YAP in yeast.

Both the Nedd4 and YAP (residues 171–204) fragments had previously been subcloned into pGEX3X (by Syed Islam and Andrea Warner). GST fusion proteins of these two constructs were made and conjugated to GST beads. Lysates from untransfected (wild-type) PAE cells and GyrB–VEGFR-1-expressing PAE cells were incubated with the fusion proteins (conditions of stimulation are described in the individual figure legends), and precipitated material was subjected to SDS–PAGE. As can be seen from Fig. 6.5, receptor was precipitated from GyrB–VEGFR-1-expressing, but not from wild-type, PAE cells by both GST–Nedd4 and GST–YAP fusion proteins. (GST alone did not precipitate any receptor.) These interactions were not dependent on receptor phosphorylation.

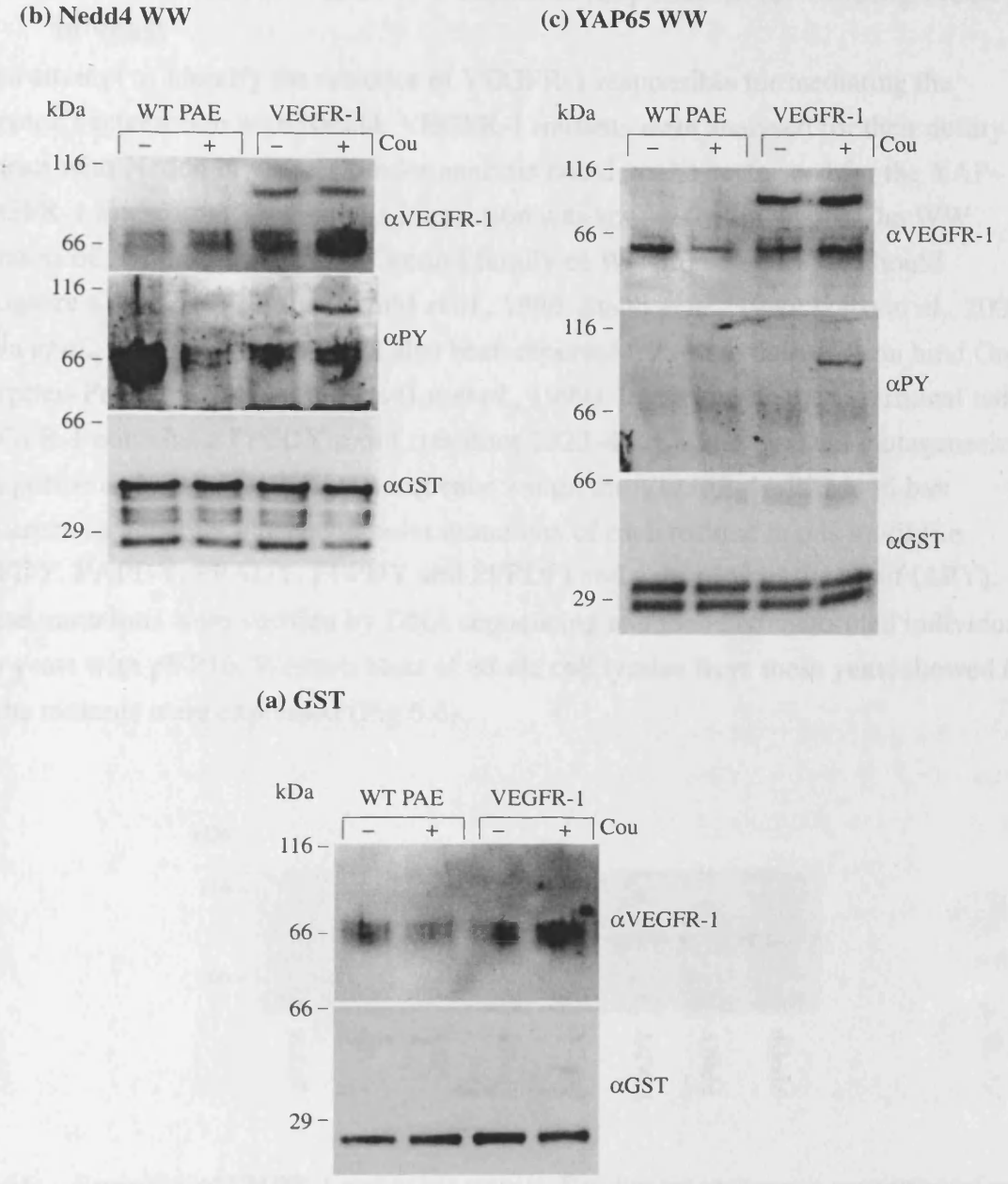


Fig. 6.5: Precipitation of GyrB–VEGFR-1 from PAE cells with GST WW fusion proteins. Wild-type and GyrB–VEGFR-1-expressing PAE cells were either left untreated or treated with 1 μM coumermycin for 15 min. Lysates were then subjected to precipitation with (a) GST, (b) GST–Nedd4 or (c) GST–YAP. Precipitates were analysed for the presence of GyrB–VEGFR-1 (top panels) by immunoblotting with anti-VEGFR-1 antibodies. To control for the amount of GST protein used in the precipitations, immunoblots were stripped and reprobed with an anti-GST antibody (bottom panels). Phosphorylation of VEGFR-1 was detected by reprobing the immunoblots with an antibody against phosphotyrosine (middle panels of b and c).

6.3.3: Identification of VEGFR-1 residues responsible for binding Nedd4 in yeast

In an attempt to identify the residues of VEGFR-1 responsible for mediating the receptor's interaction with Nedd4, VEGFR-1 mutants were analysed for their ability to interact with Nedd4 in yeast. (Similar analysis could not be performed for the YAP–VEGFR-1 interaction because this interaction was not evident in yeast.) The WW domains of Nedd4 belong to the Group I family of WW domains and so should recognize a PPxY consensus (Schild *et al.*, 1996; Staub *et al.*, 1996; Ikeda *et al.*, 2000; Gava *et al.*, 1997), although it has also been reported that these domains can bind Group IV (pSer–Pro or pThr–Pro) targets (Lu *et al.*, 1999). Interestingly, the C-terminal tail of VEGFR-1 contains a PPPDY motif (residues 1322–1326). Site-directed mutagenesis was performed on this VEGFR-1 sequence within the VEGFR-1/pBTM116 bait construct, to generate individual point mutations of each residue in this motif (i.e. APPDY, PAPDY, PPADY, PPPDY and PPPDF) and a deletion of the motif (ΔPY). These mutations were verified by DNA sequencing and then cotransformed individually into yeast with pVP16. Western blots of whole cell lysates from these yeast showed that all the mutants were expressed (Fig 6.6).

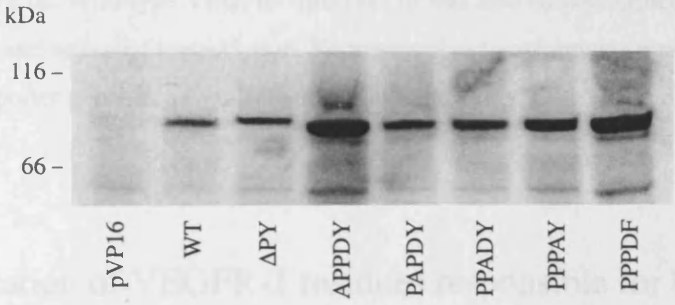


Fig. 6.6: Expression of VEGFR-1 mutant bait proteins. Site-directed mutagenesis was performed on the VEGFR-1/pBTM116 construct to produce five point mutants in the potential PY motif of VEGFR-1 (APPDY, PAPDY, PPADY, PPPDY and PPPDF) and a deletion mutant (ΔPY). Whole cell lysates from yeast transformed with the individual mutants and pVP16 were analysed for expression of the mutant proteins by SDS-PAGE; expression was detected by probing with an anti-LexA antibody.

The wild-type and mutant VEGFR-1/pBTM116 baits were individually cotransformed into yeast with Nedd4/pVP16, and their ability to interact with Nedd4 was assessed in β-galactosidase solution assays. A kinase inactive (K861A) VEGFR-1/pBTM116 mutant was also analysed in this respect [the synthesis and expression of this mutant are described in 3.3.4 (i)]. As can be seen from Fig. 6.7, the Nedd4–VEGFR-1 interaction

was ablated by deleting the entire PPPDY motif. By contrast, none of the single point mutations, including the kinase inactive mutant, had any significant effect on the Nedd4–VEGFR-1 interaction.

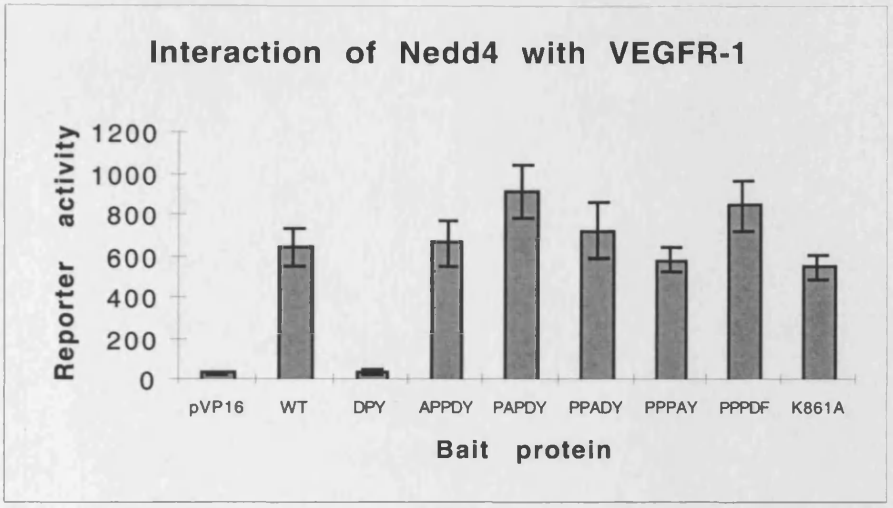


Fig. 6.7: Identification of receptor residues responsible for the interaction between Nedd4 and VEGFR-1. Wild-type and mutant VEGFR-1/pBTM116 constructs were individually cotransformed into yeast with Nedd4/pVP16. Wild-type VEGFR-1/pBTM116 was also cotransformed with pVP16 to enable comparison of basal and activated transcription. Representative transformants were analysed for transactivation of reporter genes in a β-galactosidase solution assay. Data are ±SE, and are representative of five experiments.

6.3.4: Identification of VEGFR-1 residues responsible for binding Nedd4 and YAP65 GST fusion proteins

To verify the importance of the PPPDY motif in the Nedd4–VEGFR-1 interaction in mammalian cells, and to assess the importance of this motif in the VEGFR-1–YAP interaction, various concentrations (0–100 μg) of a PPPDY-containing peptide (KIACCSPPPDYNSVVLYSTPPI) were included in the incubation mixture during GST precipitations to see whether it could compete with GyrB–VEGFR-1 for binding to Nedd4 and/or YAP.

As can be seen from Fig. 6.8, increasing concentrations of PPPDY-containing peptide were correlated with a reduced precipitation of GyrB–VEGFR-1 with both GST–Nedd4 and GST–YAP. The precipitation of GyrB–VEGFR-1 with GST–Gads was not affected by inclusion of the PPPDY-containing peptide, suggesting that this peptide was specifically interacting with the Nedd4 and YAP proteins. In addition, equivalent concentrations of a control peptide

(RQIKIWFQNRRMKWKKQDGKDYIVLPIS, encompassing the Y1175 site of VEGFR-2) did not affect the ability of the Nedd4 and YAP fusion proteins to precipitate VEGFR-1, thereby suggesting that these proteins specifically bind the PPPDY motif.

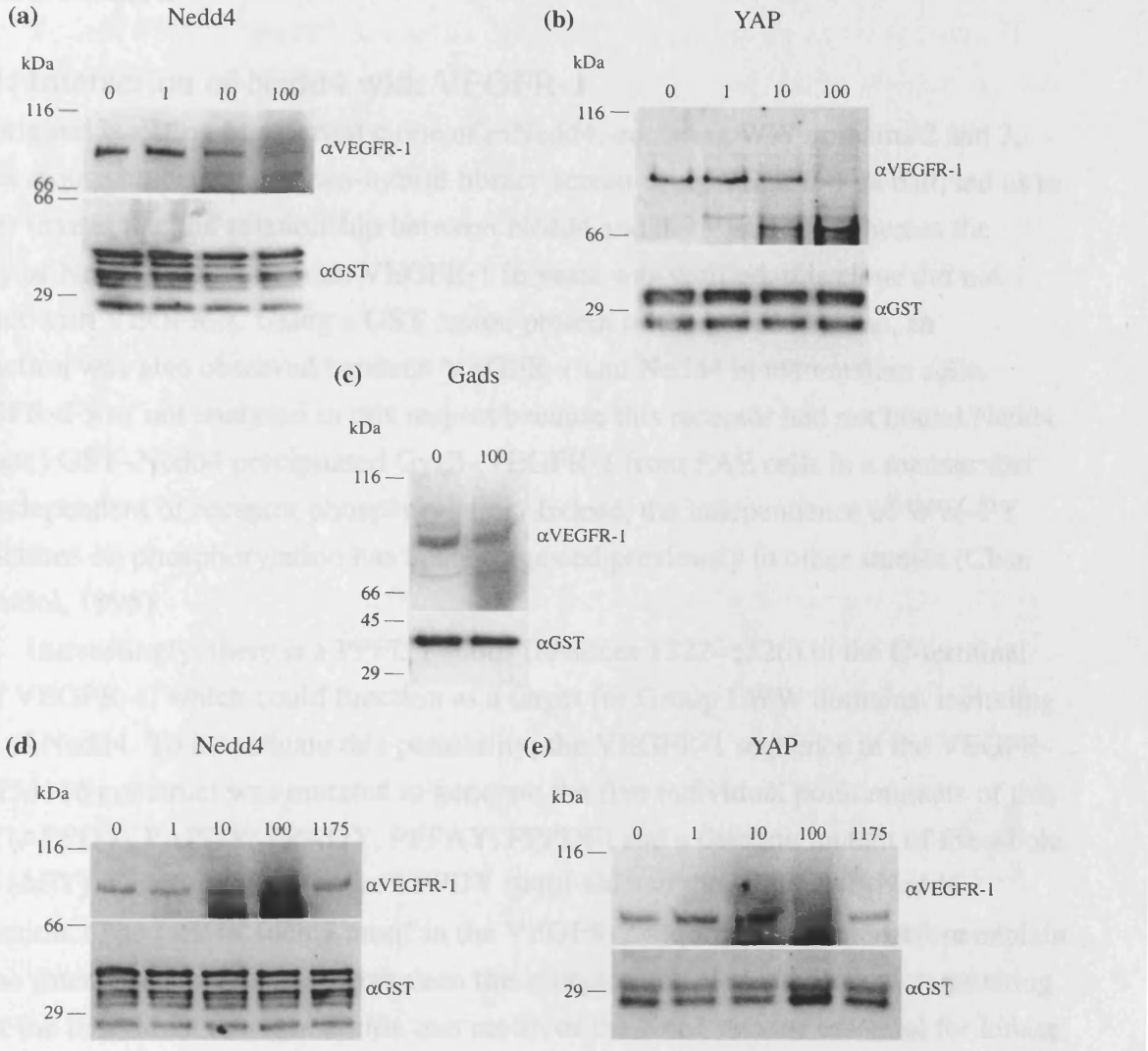


Fig. 6.8: A PPPDY-containing peptide competes with VEGFR-1 for binding Nedd4. Lysates from PAE cells expressing GyrB–VEGFR-1 were subject to precipitation with (a) GST–Nedd4 or (b) GST–YAP, in the presence of increasing concentrations (1, 10 or 100 μg) of PPPDY-containing peptide. Precipitates were analysed for the presence of GyrB–VEGFR-1 by immunoblotting with antibodies to the intracellular domain of VEGFR-1 (top panels). To control for the amount of GST protein used in the precipitations, immunoblots were reprobed with an anti-GST antibody (bottom panels). (c) Lysates from PAE cells expressing GyrB–VEGFR-1 were subject to precipitation with GST–Gads either in the absence of peptide, or in the presence of 100 μg PPPDY peptide. Precipitates were analysed for the presence of GyrB–VEGFR-1 and controlled for variations in GST protein levels as in (a) and (b). (d) Lysates from PAE cells expressing GyrB–VEGFR-1 were subject to precipitation with GST–Nedd4 either in the absence of peptide, or in the presence of 100 μg control Y1175 peptide. Precipitates were analysed for the presence of GyrB–VEGFR-1 and controlled for variations in GST protein levels as in (a) and (b). (e) Lysates from PAE cells expressing GyrB–VEGFR-1 were subject to precipitation with GST–YAP either in the absence of peptide, or in the presence of 100 μg control Y1175 peptide. Precipitates were analysed for the presence of GyrB–VEGFR-1 and controlled for variations in GST protein levels as in (a) and (b).

6.4: Discussion

6.4.1: Interaction of Nedd4 with VEGFR-1

Our original isolation of a partial clone of mNedd4, encoding WW domains 2 and 3, from a mouse embryo yeast two-hybrid library screen using VEGFR-1 as bait, led us to further investigate the relationship between Nedd4 and the VEGFRs. Whereas the ability of Nedd4 to interact with VEGFR-1 in yeast was verified, this clone did not interact with VEGFR-2. Using a GST fusion protein of the mNedd4 clone, an interaction was also observed between VEGFR-1 and Nedd4 in mammalian cells. (VEGFR-2 was not analysed in this respect because this receptor had not bound Nedd4 in yeast.) GST–Nedd4 precipitated GyrB–VEGFR-1 from PAE cells in a manner that was independent of receptor phosphorylation. Indeed, the independence of WW–PY interactions on phosphorylation has been suggested previously in other studies (Chen and Sudol, 1995).

Interestingly, there is a PPPDY motif (residues 1322–1326) in the C-terminal tail of VEGFR-1, which could function as a target for Group I WW domains, including those of Nedd4. To investigate this possibility, the VEGFR-1 sequence in the VEGFR-1/pBTM116 construct was mutated to generate the five individual point mutants of this motif (APPDY, PAPDY, PPADY, PPPAY, PPPDF) and a deletion mutant of the whole motif (ΔPY). Deletion of the entire PPPDY motif ablated the VEGFR-1–Nedd4 interaction. (The lack of such a motif in the VEGFR-2 sequence might therefore explain why no interaction was detected between this receptor and Nedd4.) However, mutating any of the individual residues within this motif, or the K861 residue essential for kinase activity, had no significant effect on the VEGFR-1–Nedd4 interaction. Considering results in the literature about WW–PY interactions, this result was surprising. For example, the Y and the second P of PPxY motifs have been shown to be essential for interactions between Nedd4 WW domains and the subunits of ENaC (Farr *et al.*, 2000; Staub, *et al.*, 1996) and p45 (Gavva *et al.*, 1997). Indeed, natural mutations of these residues are also associated with Liddle's syndrome (Hansson *et al.*, 1995; Tamura *et al.*, 1995). In addition, both P residues of the PPxY motif in LMP2A were needed for the interaction of this protein with Nedd4 (Ikeda *et al.*, 2000). The corresponding mutations in the VEGFR-1 sequence (PAPDY, PPADY and PPPDF) did not significantly disrupt the VEGFR-1–Nedd4 interaction. Hence, it is possible that disruption of this interaction requires a double mutation in this motif. Alternatively, the ΔPY mutation might preclude Nedd4 binding by disrupting the structure of the C-terminal domain and thereby an alternative Nedd4-binding site. Indeed, PPxY motifs might not be the only substrates for Nedd4 WW domains; these domains have also been reported to bind pSer–Pro or pThr–Pro motifs (Lu *et al.*, 1999), and the C-terminal domain of VEGFR-1 does contain such a motif: STPP (residues 1333–1336). The

Nedd4 WW construct might, therefore, interact with VEGFR-1 via this STPP motif rather than the PPPDY sequence. It is also possible that the other domains of Nedd4 proteins that are not encoded by this construct (i.e. mWW1 and hWW3) are responsible for the more physiological binding of VEGFR-1, and would be more affected by single mutations in the PPPDY sequence. Indeed, although all Nedd4 WW domains recognize PPxY motifs (Staub *et al.*, 1996; Ikeda *et al.*, 2000; Gava *et al.*, 1997), they do so with varying affinities (Harvey *et al.*, 1999; Staub *et al.*, 1996; Mosser *et al.*, 1998). In addition, the strength of binding can be regulated by the number of WW domains present (Staub *et al.*, 1996). It will therefore be important to isolate the other WW domains of Nedd4 and analyse their individual and combined abilities to interact with both wild-type VEGFR-1 and the PY mutants.

Support for a role of the PPPDY motif in binding Nedd4 was provided by peptide competition experiments: a peptide (KIACCSPPPDYNSVVLYSTPPI) containing this motif efficiently competed with the receptor for binding to the GST–Nedd4 WW construct. This inhibition was specific as addition of an unrelated peptide had no effect on the VEGFR-1–Nedd4 interaction and the PY peptide did not compete with VEGFR-1 for binding to a control GST–Gads SH2 fusion protein. However, this peptide also contains the STPP sequence that might have a role in WW-domain binding. It is therefore necessary to assess the interaction of Nedd4 with a VEGFR-1 mutant lacking this STPP sequence.

6.4.2: Physiological relevance of the VEGFR-1–Nedd4 interaction

For the VEGFR-1–Nedd4 interaction to be of potential physiological relevance, VEGFR-1 and Nedd4 must be naturally coexpressed. Although our experiments did not analyse expression, Nedd4 has been shown to be widely expressed, both in adult and embryonic tissues (Staub, *et al.*, 1996; 1997b; Kumar *et al.*, 1992; 1997; Hatakeyama *et al.*, 1997; Farr *et al.*, 2000). In addition, we have previously detected coexpression of Nedd4 and VEGFR-1 in HUVECs (Alex Bell, data not shown).

By analogy to the ENaC studies, in which Nedd4 binding induces ubiquitination and subsequent degradation of the channel, it is not unlikely that an interaction between Nedd4 and VEGFR-1 serves to mark the receptor for ubiquitination. This theory is supported by the observations of several groups demonstrating ligand-induced ubiquitination of RTKs. Interestingly, Mori *et al.* demonstrated such modification of the monomeric RTKs – EGFR, PDGFβR, PDGFαR, CSF-1R and FGFR – but not of the tetrameric insulin receptor (Mori *et al.*, 1995). This group also showed that the ligand-induced ubiquitination was most prominent in the Class III receptors; that is, the class to which the VEGFRs belong. Ligand-induced ubiquitination has been suggested to lead to degradation and/or internalization of growth hormone receptor (Strous *et al.*, 1996), PDGFβR (Mori *et al.*, 1993) and EGFR (Galcheva-Gargova *et al.*, 1995).

If Nedd4 does couple VEGFR-1 to the ubiquitin conjugation machinery, it is of note that receptor phosphorylation does not appear to be necessary for the binding of Nedd4. However, in the better-characterized receptor systems, ubiquitination was ligand-dependent. Therefore, it is possible that Nedd4 can bind receptors irrespective of their phosphorylation status, but does not transfer ubiquitin onto the receptor until it has been activated by ligand.

Nedd4-mediated ubiquitination might, therefore, be a mechanism by which to regulate VEGFR-1 activity, whereas VEGFR-2 is unable to bind Nedd4 and so would not be regulated by these mechanisms. Interestingly, a WW-like domain that is able to bind PY motifs has been found in the cytoplasmic juxtamembrane region of the Class III RTKs (Irusta *et al.*, 1998). Mutation of a single amino acid in this motif generated constitutively active receptors, thereby leading to the suggestion that the wild-type WW-like domain might interact with a negative regulator such as a phosphatase (Fig. 6.9). Alternatively, this domain could recruit the ubiquitin conjugation machinery, leading to receptor downregulation. These hypotheses could be extended to an explanation of the differing abilities of VEGFR-1 and VEGFR-2 to autophosphorylate upon ligand stimulation. Although both receptors have a WW-like domain in their juxtamembrane regions, it is possible that negative regulators bind the region in VEGFR-1 but not VEGFR-2, thereby only precluding ligand-induced activation of VEGFR-1. As Nedd4 has been shown to selectively interact with VEGFR-1 but not VEGFR-2, it is tempting to speculate that the mechanism behind the ligand-induced autophosphoryation discrepancies involves the binding of Nedd4 to the C terminus of VEGFR-1 and the recruitment of some other component of the ubiquitin conjugation machinery to the WW-like domain in the juxtamembrane region of the receptor. Three residues (SSS) located just upstream of the putative WW-like domain in VEGFR-1 have been shown to constitutively inhibit the receptor activity (Gille *et al.*, 2000). These residues might be essential for the interaction between a negative regulator and the WW-domain in VEGFR-1. Finally, an intriguing hypothesis is that the C-terminal PY motif of VEGFR-1 could interact with the WW-like region in the juxtamembrane domain. This would 'loop' the entire cytoplasmic domain of the receptor, possibly into a conformation that is resistant to ligand-induced activation (Fig. 6.9).

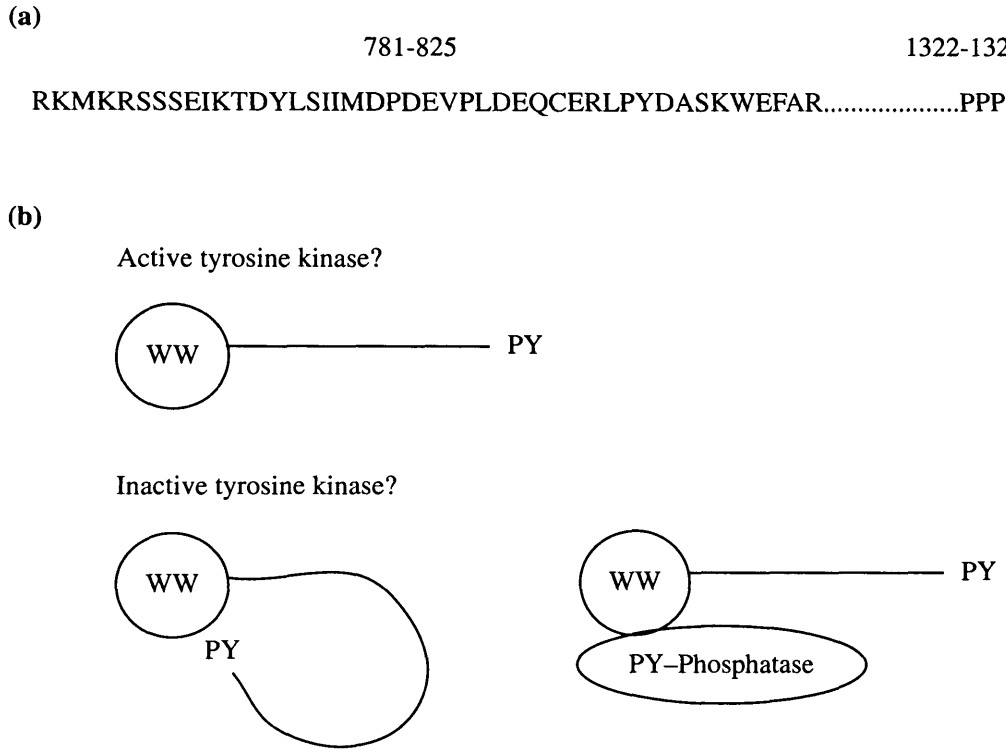


Fig. 6.9: VEGFRs contain a WW-like domain in their juxtamembrane domains. (a) Residues 781–825 of VEGFR-1 represent a WW-like domain. The putative PY motif of this receptor is located at residues 1322–1326, with the C terminus of the receptor being at residue 1337. (b) In our model, the ligand-induced tyrosine kinase activity of VEGFR-1 is thought to be inhibited either by steric hindrance, caused by looping of the entire cytoplasmic domain of the receptor when the C-terminal PY motif interacts with the WW-like domain, or by binding of a negative regulator such as a phosphatase to the WW-like domain.

6.4.3: Relevance of YAP65 to VEGFR signalling

When looking for other WW-domain containing proteins that might be of relevance to VEGF signalling, YAP was chosen for analysis because this factor had been cloned as a protein that interacted with c-yes, a protooncogene that had been shown to bind both VEGFRs 1 and 2 in response to VEGF (Waltenberger *et al.*, 1994). Moreover, sequence comparisons of various WW domains had revealed that the WW domains of YAP and Nedd4 were highly homologous and could therefore be envisaged to bind similar PY targets (Sudol *et al.*, 1995a; 1995b).

Despite being expressed, LexA–YAP did not interact with either of the VEGFRs in yeast. The failure to observe an interaction between YAP and VEGFR-1 could have been a consequence of steric hindrance, caused by the VP16 activation domain being too close to the WW domain sequence of YAP as only the minimal WW domain

sequence was encoded by this construct. However, a second construct, lYAP, encoding extra sequence flanking the WW domain, also failed to interact with VEGFR-1 in yeast. By contrast, when GST fusion proteins of the YAP sequence (encoding just the minimal WW domain) were mixed with mammalian cell lysates, this protein did precipitate VEGFR-1. (GyrB–VEGFR-2 was not analysed here because this receptor did not interact with Nedd4 in yeast.) The apparent discrepancy between the yeast and mammalian results could reflect the lower sensitivity of the yeast two-hybrid assay relative to the *in vitro* binding assay.

Because of the lack of interaction in yeast, the ability of VEGFR-1 PY mutants to interact with YAP could not be analysed in this system. However, the results from peptide competition experiments were similar to those for Nedd4; that is, increasing concentrations of a PY peptide, but not of an unrelated control peptide, significantly inhibited the VEGFR-1–YAP interaction. The YAP WW domain is therefore likely to bind VEGFR-1 via either the PPPDY or the STPP sequence. Similar to the VEGFR-1–Nedd4 interaction, that of VEGFR-1 and YAP was not phosphorylation dependent. Mutagenesis studies of other groups have shown that a minimal PPxY motif is essential for an interaction of YAP with WBP-1, WBP-2 and p45 (Gava *et al.*, 1997). In addition, with respect to the WBP proteins, even the APPPY and PPPAY mutants decreased binding to YAP by twofold (Gava *et al.*, 1997). To analyse the residues responsible for the YAP–VEGFR-1 interaction further, the GST–YAP WW fusion protein could be tested for its ability to precipitate the VEGFR-1 PY mutants from yeast.

YAP has been shown to function as a potent transcriptional co-activator (Yagi, *et al.*, 1999; Gavva *et al.*, 1997). However, it is difficult to envisage such a role for YAP when bound to VEGFR-1 unless, as demonstrated for VEGFR-2 (Feng *et al.*, 1999b), this receptor relocates to the cell nucleus. Alternatively, YAP might serve to recruit c-yes (and/or other src-like kinases) to VEGFR-1, where they are then optimally positioned to phosphorylate downstream substrates.

As yet, no interactions between the VEGFRs and WW-domains have been reported in the literature. It is therefore extremely important to further define the interactions observed during this work. Investigation of the YAP–VEGFR-1 interaction could define novel signalling pathways involving the src-like kinases. In addition, it is essential to determine whether VEGFR-1 is regulated by ubiquitin-mediated degradation as this could have significant implications on the relative activation kinetics of the two VEGF receptors.

Chapter 7

Discussion

Chapter 7: Discussion

VEGF signalling cascades are important for the endothelial cell activities that lead to the formation of blood vessels (i.e. vasculogenesis and angiogenesis). Current research into these pathways is intense, not least because of the association of angiogenesis with the progression of solid tumours to a state of malignancy. Misregulated angiogenesis is also a factor in the cartilage destruction that gives rise to rheumatoid arthritis, and in the blindness associated with diabetic retinopathy. An understanding of the angiogenic signalling cascades could therefore lead to development of anti-angiogenic agents that would specifically alleviate these pathologies. In addition, such research could identify suitable proangiogenic factors for the treatment of ischaemic heart disease, peripheral vascular disease, chronic wounds and gastric ulcers – pathologies associated with inadequate vascularization.

Most molecular signalling studies of the VEGF pathways have focussed on VEGFR-1 and VEGFR-2 because these two receptors are essential for blood vessel development and their expression is virtually restricted to endothelial cells. So far, the vast majority of VEGF-induced signalling has been attributed to VEGFR-2; however, this might be a misguided conclusion caused by the relative difficulty researchers have experienced in trying to promote ligand-induced phosphorylation of VEGFR-1. To add to the confusion over VEGF-induced signalling cascades, the VEGF receptors might couple to downstream effectors distinct from those used by other growth factor receptors. For example, although phosphorylated Shc is a common requirement for ERK1/2 activation downstream of most RTKs, VEGF induced significant ERK1/2 activation in the absence of Shc phosphorylation (other examples are given in section 3.1.1).

We have generated chimeras of the VEGFRs in which the cytoplasmic domains of the receptors are fused downstream of the N-terminal dimerization domain of the GyrB subunit of bacterial DNA gyrase. Addition of coumermycin to the medium of cells expressing these receptors induced the tyrosine phosphorylation of the cytoplasmic domains of both GyrB–VEGFR-1 and GyrB–VEGFR2. This ligand-induced phosphorylation required the kinase activities of the receptors and was also mediated by ligand-induced dimerization (as shown by using a monomeric counterpart of coumermycin), thereby suggesting that signalling downstream of these receptors could be analagous to that occurring downstream of the full-length receptors. These results also show that the kinase domain of VEGFR-1 is able to catalyse receptor autophosphorylation; an observation that has been difficult to detect using other ligand-induced systems. Using these chimeras, it has therefore been possible to study VEGF-induced association of effector proteins with both VEGF receptors.

With respect to VEGF-'specific' effectors, we have shown that both chimeric VEGFRs 1 and 2 bind to the SH2 domain of the Shc-like protein Sck in preference to that of Shc itself. In addition, VEGFR-1, but not VEGFR-2, bound the SH2 domains of Grb2 and Gads. No extensive studies comparing the signalling competencies of Shc and Sck have been reported, so it can only be speculated that Sck might couple the VEGFRs to effectors distinct to those of Shc. By contrast, although the SH2 domains of Grb2 and Gads are known to have similar target specificities, that of their SH3 domains differs. These two proteins could therefore couple common upstream factors to distinct downstream effectors. Gads is not necessarily an alternative to Grb2 for coupling RTKs to the Ras–ERK pathway. Indeed, Gads has been suggested to couple to suppression of apoptosis rather than to cell proliferation (Gotoh *et al.*, 1996). The results of the experiments described in this thesis also suggest that VEGFR-1 might couple to pathways that are distinct to those downstream of VEGFR-2 as VEGFR-1 can bind Gads, Grb2 and Sck, whereas VEGFR-2 binds only Sck (Fig. 7.1). Interestingly, although we found no interaction between VEGFR-2 and Gads, Grb2 or Shc, this receptor still mediated ERK1/2 activation, thereby suggesting that these three factors are not required for this coumermycin-induced pathway (Fig. 7.2). In addition, although VEGFR-2 bound Sck, mutating a residue shown to be essential for the binding of the receptor to this factor (Y1175) had no effect on the activation of ERK1/2. Therefore, unless Sck binds to GyrB–VEGFR-2 via alternative residues, or couples indirectly, this effector cannot be essential for the activation of ERK1/2 seen downstream of this chimera.

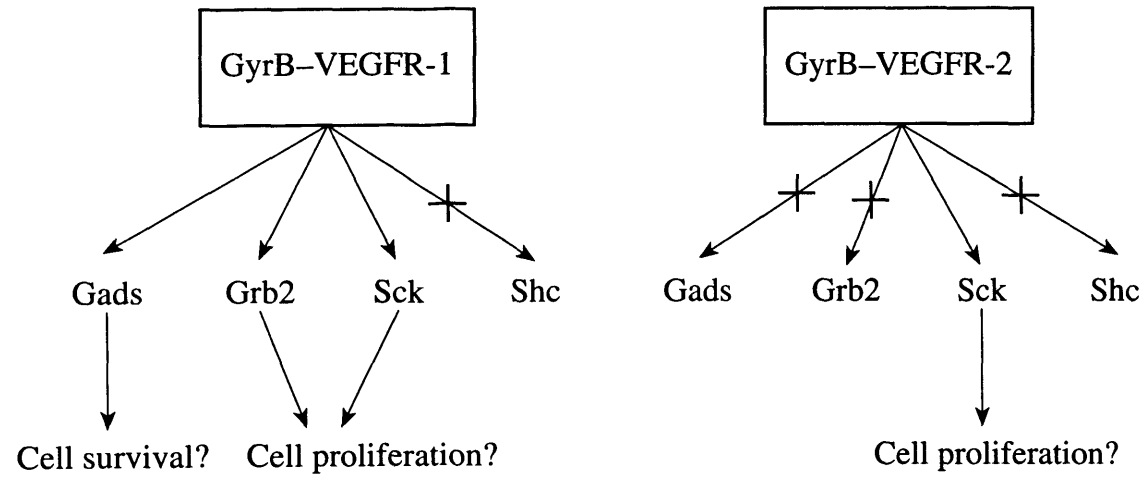


Fig 7.1: Distinct signalling pathways mediated by GyrB–VEGFR-1 and GyrB–VEGFR-2. Neither receptor bound the SH2 domain of Shc. By contrast, the VEGFR-1 chimera bound the SH2 domains of Gads, Grb2 and Sck, whereas GyrB–VEGFR-2 bound only that of Sck. These discrepancies could reflect a difference in the cellular responses mediated by the two receptors.

One protein whose relevance in VEGF signalling cascades has been extensively studied is PLCγ. We observed an interaction between PLCγ and VEGFR-1 in yeast, and also demonstrated that both chimeric VEGFRs can induce the phosphorylation of PLCγ in mammalian cells, although that mediated by GyrB–VEGFR-2 appeared slightly more efficient than that of GyrB–VEGFR-1. In the case of GyrB–VEGFR-2, this phosphorylation required the Y1175 residue, thereby implicating this residue as a major binding site for PLCγ. Interestingly, ERK1/2 activation mediated by GyrB–VEGFR-2 was not affected by a Y1175F mutation, thereby suggesting that PLCγ is not needed for ERK1/2 activation in this system. As noted above, the involvement of Gads, Grb2, Shc and Sck in this pathway is also unlikely. Roles for PI3K and NO in linking VEGFRs to ERK1/2 activation have also been suggested previously (Thakker *et al.*, 1999; Parenti *et al.*, 1998; Kroll and Waltenberger, 1997). Although we have not studied PI3K directly, coumermycin-induced Akt activation was maintained downstream of GyrB-VEGFR-2 despite a Y1175F mutation. Because PI3K is probably responsible for the activation of this Ser/Thr protein kinase, it is therefore possible that PI3K could mediate the GyrB–VEGFR-mediated activation of ERK1/2, as could NO (Fig. 7.2).

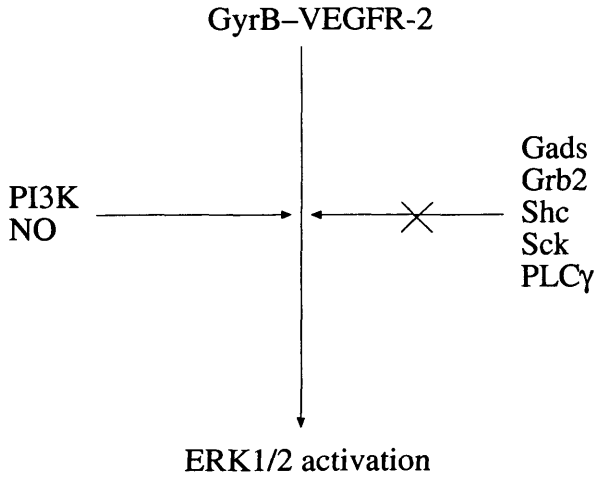


Fig. 7.2: Putative effectors that could mediate ERK1/2 activation downstream of GyrB–VEGFR-2.

One point of note highlighted by the studies described in this thesis is the possibility that VEGFRs 1 and 2 might be regulated by distinct mechanisms. Indeed, the kinetics of coumermycin-induced phosphorylation differed markedly for the two receptors (although this could just be an artefact of the chimeric system). Whereas the phosphorylation of VEGFR-2 peaked 5–15 minutes after stimulation, that of VEGFR-1 was still sustained after 90 minutes. It is therefore possible that the two receptors could activate different effectors depending on the length of time for which they are activated.

The relatively fast turnover of GyrB–VEGFR-2 phosphorylation suggests that this is regulated by phosphastases. Indeed, phosphatases that regulate VEGFR-2 but not VEGFR-1 have been reported. Interestingly, one of the proteins investigated through our work is a ubiquitin ligase – a class of enzyme thought to select targets for ubiquitination, and thereby either recycling or degradation. VEGFR-1, but not VEGFR-2, was able to bind this protein, Nedd4, in yeast, and also in precipitates from mammalian cells. In addition, ubiquitination of other RTKs has previously been shown to lead to their degradation. These observations have led to our hypothesis that VEGFR-2 is regulated primarily by phosphatases, whereas the activity of VEGFR-1 is more sensitive to ubiquitin-induced degradation and/or recycling (Fig 7.3).

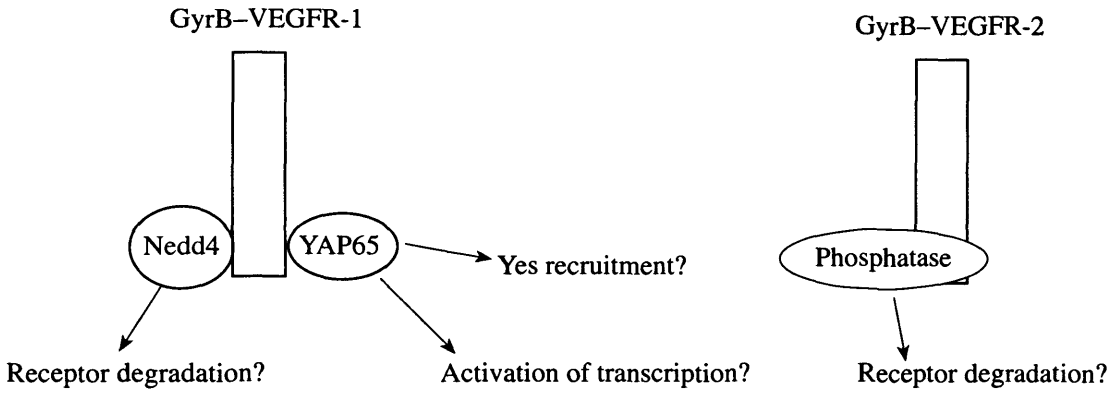


Fig. 7.3: Possible roles of Nedd4 and YAP65 with VEGFRs. Nedd4 and YAP65 both bind VEGFR-1. With repect to Nedd4, this could have important implications for the mechanisms of receptor regulation, with VEGFR-2 being regulated by phosphatases whereas VEGFR-1 could be degraded by Nedd4-mediated ubiquitination.

Intriguingly, WW-like domains capable of binding PY motifs have been identified in members of the Class III subfamily of RTKs, including VEGFRs 1 and 2. A single amino acid mutation in the WW domain of PDGFβR (and in all other members tested) resulted in constitutive activation of the receptor's intrinsic tyrosine kinase activity. It is therefore possible that this mutation prevents association of the receptor with a PY-containing negative regulator such as a phosphatase or a component of the endocytic machinery (Fig. 6.9). In the case of VEGFR-1, three residues (SSS) were shown to inhibit responses mediated by this receptor; deletion of just these three residues restored receptor activity. Because this SSS motif is located less than ten residues N-terminal to the WW-like domain, it might be involved in the interaction of this domain with a negative regulator. Taken further still, the C-terminal tail of

VEGFR-1, but not that of VEGFR-2, has a putative PY motif. Therefore, it is intriguing to speculate that this PY motif could interact with the WW-like domain, looping the entire cytoplasmic domain of the receptor and thereby preventing receptor activation and downstream signalling. Such an interaction could explain the discrepancies observed in relation to the ligand-induced activation of the two VEGFRs. However, both the WW-like domain and the putative PY motif are present in the GyrB–VEGFR-1 construct, and this receptor is significantly activated in response to coumermycin. Although this threatens our hypothesis about the WW–PY intramolecular interaction in VEGFR-1 being responsible for the 'resistance' of the receptor to ligand activation, it is possible that fusion of the VEGFR-1 and GyrB sequences alters the confomation of the juxtamembrane WW-like domain, thereby preventing an interaction with the C-terminal PY motif, and permitting receptor activation.

As with all molecular studies, it is important to remember that the effects seen in cell culture experiments might not faithfully reflect the situation *in vivo* where, instead of being in a relatively homogeneous monolayer, cells are surrounded by diverse tissues and factors. In addition, the receptor residues phosphorylated upon coumermycin stimulation might not truthfully reflect those phosphorylated upon ligand stimulation *in vivo*, and the kinetics of phosphorylation and the mechanisms regulating activation might also differ between the chimeric and the full-length receptors. Nevertheless, use of the system has shown that the VEGFR-1 cytoplasmic domain can be activated and is capable of mediating downstream signalling cascades. Attribution of the majority of VEGF-induced responses to VEGF-2 is likely to have been influenced by the inability to demonstrate ligand-induced VEGFR-1 activation. Using the chimeric system, we have shown that once sufficiently activated, VEGFR-1 can indeed mediate the activation of downstream effectors, suggesting that the relative signalling capabilities attributed to the individual full-length receptors need to be reanalysed.

Appendices

Appendix

Publications

Full papers

Data from Chapter 4, regarding the GyrB–VEGFR chimeras, has been published in:

Knight, E.L., Warner, A.J., Maxwell, A. and Prigent, S.A. (2000) Chimeric VEGFRs are activated by a small-molecule dimerizer and mediate downstream signalling cascades in endothelial cells. *Oncogene* 19, 5398–5405

Some of the data from Chapter 5, regarding the interaction of VEGFRs with Shc-like proteins in yeast, has been published, along with other data, in:

Warner, A.J., Lopez-Dee, J., Knight, E.L., Feramisco, J.R. and Prigent, S.A. (2000) The Shc-related adaptor protein, Sck, forms a complex with the vascular endothelial growth factor receptor KDR in transfected cells. *Biochem. J.* 347, 501–509

Data from Chapter 6, regarding the interactions of the VEGFRs with Nedd4 and YAP65 constructs, are presently being prepared for publication.

Abstracts

Prigent, S.A., Knight, E., Warner, A., Bell, A., Lopez-Dee, J. and Feramisco, J. (1999) Identification of novel effectors for vascular endothelial growth factor receptors. Keystone Symposium: Oncogene Networks in Signal Transduction, p148

Knight, E.L. and Prigent, S.A. (2000) Signalling through chimeric VEGF receptors. Keystone Symposium: Experimental and Clinical Regulation of Angiogenesis, p. 70

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