Investigation into Glucagon Like Peptide-1 Signalling in Pancreatic β-cells

Thesis submitted for the degree of Doctor of Philosophy

August 2008

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Abstract

Glucagon like peptide-1 (GLP-1) is a G_S-coupled receptor agonist that exerts multiple effects on pancreatic β-cells, including the stimulation of insulin gene expression and secretion, growth and survival. A number of kinases are activated in response to GLP-1R activation, including extracellular regulated kinases (Erk1/2), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB) and mammalian target of rapamycin mTOR, all of which contribute in regulating various aspects of β-cell function. However, the mechanism by which GLP-1 activates these signalling pathways in pancreatic β-cells is not fully understood. Therefore, the objectives of this thesis were to investigate how GLP-1 signals to Erk1/2, PI3K/PKB and mTOR. It has previously been reported that GLP-1 stimulated Erk1/2 activation is dependent on the influx of Ca^{2+} specifically through L-Type VGCC. In this thesis I provide evidence that this increase in Ca^{2+} activates the Ca^{2+} dependent phosphatase, calcineurin which in turn activates IKK leading to the activation of the MEK kinase, Tpl2. Ca^{2+} entry through L-Type VGCC also plays a key role in stimulating insulin secretion which I show is responsible for glucose stimulated PI3K activation and PKB phosphorylation. In contrast, GLP-1 can activate PI3K independent of insulin secretion which is unable to couple to PKB. Interestingly, GLP-1 is able to potentiate glucose stimulated mTOR activation via a PI3K leading to the phosphorylation of rpS6 on Ser240/244. Moreover, GLP-1 can stimulate the phosphorylation of rpS6 on Ser235/236 which is not dependent on mTOR activation or the two currently known S6Ks, S6K1/2 or p90RSK. In summary, this thesis has contributed to increase our understanding of GLP-1 signalling in pancreatic β-cells.

Publications

Greenman IC, Gomez E, **Moore CE**, Herbert TP. *The selective recruitment of mRNA to the ER and an increase in initiation are important for glucose-stimulated proinsulin synthesis in pancreatic beta-cells.* Biochemical Journal 2005 Oct 15;**391**(Pt 2):291-300

Greenman IC, Gomez E, **Moore CE**, Herbert TP *Distinct glucose-dependent stress responses revealed by translational profiling in pancreatic beta-cells.* J Endocrinol. 2007 Jan;**192**(1):179-87.

Moore C E, Xie J, Gomez E and Herbert TP, *Identification of cAMP dependent kinase as a third in vivo ribosomal protein S6 kinase in pancreatic beta-cells.* Manuscript in preparation.

Abstracts

C E Moore *cAMP Dependent phosphorylation of ribosomal protein S6 by glucagon like peptide 1 in pancreatic β-cells.* Translation UK, University of Nottingham, Nottingham, 5-6th July 2007.Oral

C E Moore, E Gomez, R Rigatti and T P Herbert. *GLP-1 Signalling via L-Type voltage gated calcium channels in pancreatic beta cells.* The European Association for the Study of Diabetes, 41st Annual Meeting 12-15 September 2005 Athens, Greece. Poster

C E Moore, E Gomez, R Rigatti and T P Herbert. *GLP-1 Signalling via L-Type voltage gated calcium channels in pancreatic beta cells* .Islet Study Group Post-EASD Symposium Alicante November 11-12th 2005. Poster

C E Moore and Herbert, T P. *Regulation of S6 phosphorylation by incretins and nutrients in pancreatic β-cells.* Translation UK 2006 Newcastle July 3-5th July 2006. Poster

C E Moore and Herbert, T P. *Regulation of S6 phosphorylation by incretins and nutrients in pancreatic β-cells.* Islet Study Group Post-EASD Symposium Helsingør, Denmark September 17.-19. 2006. Poster

C E Moore and Herbert, T P. *cAMP Dependent phosphorylation of ribosomal protein S6 by glucagon like peptide 1 in pancreatic β-cells.* Islet Study Group Post-EASD Symposium Brussels, September 21-23rd. 2007. Poster

Acknowledgments

First and foremost I'd like to thank my supervisor, Terry Herbert for expert scientific direction, supervision and support over the last three years. I would also like to say a special thanks to Edith for all technical and teaching support throughout the three years.

Thanks also to my committee members, Gary Willars and Catrin Pritchard for advice and discussion regarding the work in this thesis.

A special thanks to two of my colleagues who were with me for almost the entire journey, Jo and Mike. Jo and Mike have been great lab mates, always willing to go the extra mile to help. I would also like to thank all the lab members past and present which include Isabel, Kate, Tola, Adam and Jianling.

I would also like to thank the good friends (too many to list) I have made within the department for their support and encouragement.

A special thanks to my family and friends for their support and encouragement over the last three years. At the heart of my support system were my parents Mike and Lily who lend me unending support in everything I do.

Last but not least I would like to thank James for the continued support in everything I do.

This thesis is dedicated to the loving memory of my brother Simon.

Abbreviations

aa	Amino acids
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATF4	Activating transcription factor 4
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP
CaM	Calmodulin
CaMK	Calcium and calmodulin-dependent protein kinase
CREB	cAMP response element-binding
CHO	Chinese hamster ovary cells
CHOP	C/EBP homologous protein
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide triphosphate
dsRNA	Double stranded RNA
DTT	Dithiotreitol
4E-BP	eIF4E binding protein
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
eIFs	Eukaryotic initiation factor
ER	Endoplasmic reticulum
Epac	Exchange protein activated by cAMP
FCS	Foetal calf serum
FOXO	Forkhead transcription factor
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanosine-5'-Diphosphate
GLP-1	Glucagon Like peptide-1
Grb2	Growth factor receptor-bound protein 2.
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-Triphosphate
HA	Hemagglutinin
HBS	HEPES buffered saline
HEK-293	Human embryonic kidney 293 cells
IBMX	Isobutylmethylxanthine
INS-1	Rat insulinoma cell line
IP	Immunoprecipitation
IPTG	Isopropyl-beta-D-thiogalactopyranoside

JNK	c-jun N-terminal kinase
kb	Kilobases
kDa	Kilodaltons
LPS	Lipopolysaccharide
KRB	Krebs-Ringer bicarbonate buffer
MAPK	Mitogen activated protein kinase
MEK	Mitogen and extracellular signal regulated protein kinase
MIN6	Mouse insulinoma 6 cells
Mnk	MAP kinase signal interacting kinase
mRNA	Messenger ribonucleic acid
mSOS	Son of Sevenless
MSK1/2	Mitogen- and Stress-activated protein Kinases 1 and 2
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PC2	Prohormone convertase 2
PC3	Prohormone convertase 3
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PDK-1	Phosphoinositide-dependent protein kinase-1
PKA	Protein Kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phosholipase C
PTB	Phosphotyrosine binding domain
PMA	Phorbol 12-Myristate 13-Acetate
PYK2	Proline-rich tyrosine kinase 2
Raptor	Regulatory associated protein of mTOR
Rheb	Ras homologue enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
rpS6	Ribosomal Protein S6
RSK	p90 Ribosomal S6 kinase
Shc	Src homology
S6K	p70 S6 kinase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tpl2	Tumor progression locus 2
TSC1	Tuberous sclerosis 1 (Tuberin)
TSC2	Tuberous sclerosis 2 (Hamartin)
UV	Ultraviolet
VGCC	Voltage gated calcium channel
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

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Chapter 1: Introduction

1.1 Background

The incretin effect refers to the augmented insulin secretory response observed during oral glucose administration compared to that seen during intravenous infusion of glucose (Elrick, Stimmler et al. 1964). The two most important incretin hormones responsible for this effect are Glucose-dependent insulinotropic polypeptide (GIP) and Glucagon-like peptide-1 (GLP-1) which account for about 20% and 80% of the intestinal incretin effect respectively (Holst 1994). In response to nutrient ingestion, GIP is released from enteroendocrine K cells in the duodenum (Yip and Wolfe 2000), whereas GLP-1 is released from intestinal L-cells, which are distributed throughout the intestine (Eissele, Goke et al. 1992). The actions of both of these hormones are mediated via their interactions with specific G-protein coupled receptors located on the β-cells of the islets of langerhans, and receptor activation results in potentiation of glucose stimulated insulin secretion (Drucker 2006). Besides potentiating insulin secretion, the incretin hormones also exert additional effects. For example, GLP-1 enhances β-cell growth and survival, via increasing cell proliferation, reducing apoptosis and promoting neogenesis from precursor cells (Holst 2007). These multiple actions of GLP-1 on the β-cell make GLP-1 an excellent candidate for the treatment of Type 2 diabetes. Indeed, in subjects with Type 2 diabetes, GLP-1 receptor agonist administration stimulates insulin secretion in a glucose dependent manner which then reduces the levels of glucose in both the fed and fasting state (Zander, Madsbad et al. 2002).

1.2 GLP-1 Synthesis and Secretion

GLP-1 is a 30 amino acid derivative of the prohormone proglucagon (Fehmann, Goke et al. 1995). In the pancreas, post translational processing of proglucagon occurs in the α-cells and gives rise to glucagon, glicentin-related polypeptide (GRPP) and major proglucagon fragment (MPGF) generated via the action of prohormone convertase 2 (PC2) (Fehmann, Goke et al. 1995). This processing via PC2 also gives rise to small amounts of biologically inactive GLP-1 (1-36). In the intestinal L-cells, proglucagon is processed to glicentin, oxyntomodulin, GLP-1(1-37) and GLP-2 via the actions of prohormone convertase 1 and 3 (Mojsov, Heinrich et al. 1986; Rouille, Westermark et al. 1994). GLP-1(1-37) is then

cleaved to form the bioactive GLP-1(7-37) which is further cleaved at the C-terminal end and subsequently amidated to give rise to the 30 amino acid biologically active GLP-1(7-36) amide gut hormone (Fehmann, Goke et al. 1995) (**Figure 1.1**).

The proglucagon gene is expressed in the central nervous system, mainly in the hypothalamus and the brain stem, as well as in the α-cells of the islets of langerhans (Drucker and Asa 1988; Holst 1997). However, the intestinal L-cells are the main site of GLP-1 expression. L-cells are distributed throughout the intestine but are preferentially located in the jejunum, ileum, and colon (Eissele, Goke et al. 1992). L-cells are open-type endocrine cells with slender apical processes which are covered in microvilli that emanate towards the lumen of the gut (Eissele, Goke et al. 1992). The microvilli covering these cells are thought to assist in nutrient sensing following the ingestion of a meal (Holst 2007). The base of the cell which rests on the basal lamina contains secretory granules which are packed with fully processed GLP-1 and are predominant in the distal ileum. The main stimuli for GLP-1 release from intestinal L-cells are nutrients such as carbohydrate and fat (Holst 1994). While GLP-1 is released from the lower small intestine where the density of L-cells is highest, GIP is secreted from K cells, located predominately in the upper small intestine. Therefore, the ingestion of smaller nutrient loads would stimulate GIP release whilst larger nutrient loads, such as after a large meal which requires extensive processing, will stimulate GLP-1 secretion (Qualmann, Nauck et al. 1995). The mechanism by which nutrients stimulate GLP-1 secretion is not clearly understood. GLP-1 secretion after a meal can be divided into two phases: an early phase that begins within minutes and lasts for 30–60min and an overlapping second phase that causes prolonged secretion for 1–3h (Elliott, Morgan et al. 1993). It had previously been demonstrated that inhibition of the sodium/glucose co-transporter (SGLT-1) can inhibit GLP-1 secretion in canine ileum (Sugiyama, Manaka et al. 1994). It was later shown in GLUT-ag cells (pro-glucagon expressing cell line, which is a model for L-cells) that GLP-1 is secreted in response to glucose, this effect is impaired by the sodium-glucose co-transporter (SGLT) inhibitor, phloridzin (Gribble, Williams et al. 2003). SGLT substrates generate small inward currents as a result of the electrogenic action of the transporter. This transporter-associated current can trigger electrical activity and secretion when the concentration of substrate is high or when outward currents are reduced by metabolic closure of the K_{ATP} channels (Gribble, Williams et al. 2003). In addition, glucose triggers GLP-1 release through closure of K_{ATP} channels and action potential generation (Reimann and Gribble 2002).

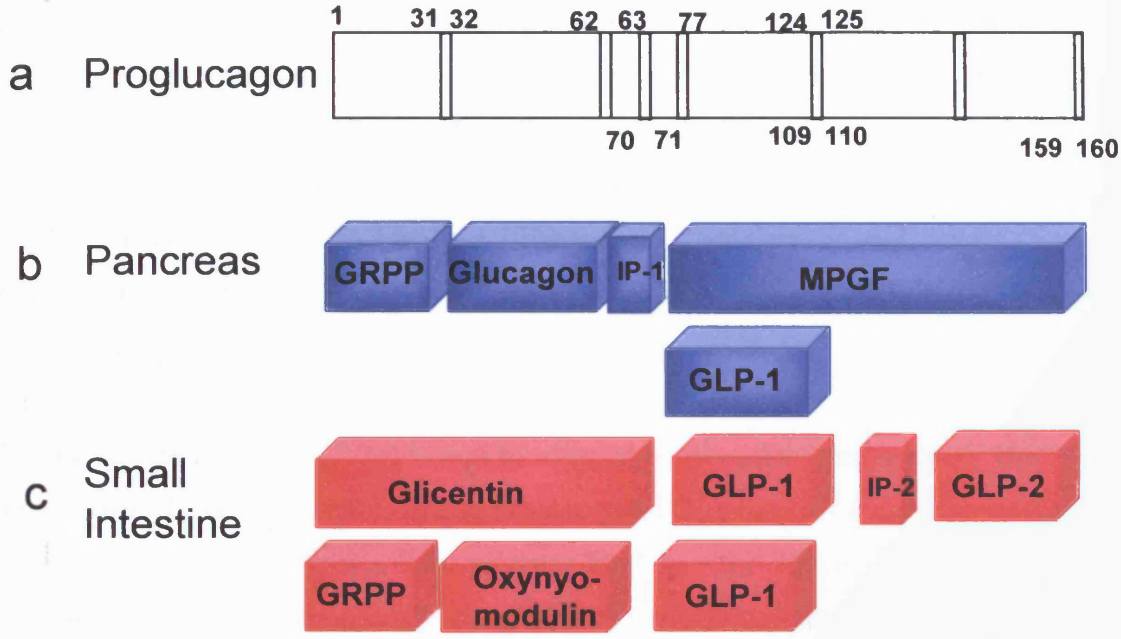


Figure 1.1: Differential Processing of Proglucagon in the Pancreas and Gut

a) Representation of the proglucagon gene, b) Post-translational processing of the proglucagon gene in the pancreas and c) small intestine. In the pancreas, the prohormone proglucagon is cleaved to produce glucagon, glicentin-related pancreatic polypeptide and a large peptide, major proglucagon fragment. During this processing, GLP-1 is also produced but it remains inactive. In the small intestine, the L-cells produces glicentin, oxyntomodulin and GLP-1/2 (Adapted from Holst J 2007).

GLP-1 secretion from the intestine is also regulated by hormones and neurotransmitters. For example, GLP-1 secretion can be stimulated by GIP which activates the L-cell indirectly through vagal afferent pathways (Rocca and Brubaker 1999). Additionally, it has been reported that stimulation with acetylcholine or somatostatin increases GLP-1 secretion (Dumoulin, Dakka et al. 1995) (Hansen, Hartmann et al. 2000). Other strong stimuli for GLP-1 secretion are lipids. The GPCR receptor GPR120, which is activated by long chain fatty acids (FFAs), promotes the secretion of GLP-1 *in vitro* and *in vivo* (Hirasawa, Tsumaya et al. 2005). Additionally, in GLUT-ag cells, GPR120 and PKCζ, which have been shown to be involved in fatty acid signalling in many cells (Yaney, Korchak et al. 2000), were found to be required for oleic acid-induced GLP-1 secretion (Iakoubov, Izzo et al. 2007).

Following secretion, GLP-1 (7-36) is rapidly cleaved by dipeptidyl peptidase IV (DPP-IV) which removes the two N-terminal amino acids to yield GLP-1(9-36) amide. Given that the N-terminus of GLP-1 is important for receptor binding, GLP-1(9-36) amide is rendered biologically inactive (Mentlein, Gallwitz et al. 1993; Deacon, Johnsen et al. 1995). As a result of this, the plasma half life of GLP-1 is around 5min and the clearance rate is approximately 12min (Holst 1994). It has also been reported that GLP-1 is also degraded *in vivo* by the actions of a neutral endopeptidase, a zinc metallopeptidase which is membrane bound and found predominately in the kidney (Plamboeck, Holst et al. 2005).

1.3 Molecular Mechanisms of GLP-1 Actions

1.3.1 The GLP-1 Receptor

The GLP-1 receptor (GLP-1R) is a G protein coupled receptor that was first cloned from a rat pancreatic islet cDNA library (Thorens 1992). The GLP-1R is a member of the class B family, which is the same family as the GIP and glucagon receptors (Mayo, Miller et al. 2003). The receptor is expressed in pancreatic β-cells, brain, heart, kidney and gastrointestinal (GI) tract (Bullock, Heller et al. 1996; Dunphy, Taylor et al. 1998). Site directed mutagenesis of selected residues within the GLP-1R revealed which residues are important for GLP-1 binding including K197, D198, K202, D215, R227 (for a detailed review see Doyle and Egan 2007) (**Figure 1.2a**). Following receptor stimulation, the GLP-1R is cycled between the plasma membrane and endosomal compartments via a clathrin coated pit dependent mechanism (Widmann, Dolci et al. 1995). The GLP-1R also

undergoes caveolin-1 dependent trafficking. GLP-1R localizes in lipid rafts and interacts with caveolin-1 and upon agonist stimulation, the GLP-1R undergoes rapid and extensive endocytosis in association with caveolin-1 (Syme, Zhang et al. 2006). Six serine residues (441/442, 444/445 and 451/452) located within the C-terminus have been associated with GLP-1R internalization as deletion of these sites prevents receptor internalization in CHO cells over-expressing the GLP-1R (Widmann, Dolci et al. 1997). Furthermore, this study demonstrated that the phosphorylation of these sites by PKC also plays a role in receptor desensitization as assessed by a decrease in the levels of cAMP produced following an initial exposure of cells to GLP-1 (Widmann, Dolci et al. 1997) (Widmann, Dolci et al. 1996). In the pancreatic β-cell line INS-1, treatment with the GLP-1R agonist exendin-4 or the phorbol ester PMA for various time intervals followed by a rest period and then re-stimulated with exendin-4 results in a decrease in cAMP levels as a consequence of GLP-1R down-regulation (Baggio, Kim et al. 2004) (**Figure 1.2a**).

1.3.2 GLP-1 Signalling in β-cells

It was first demonstrated in rat islet cell line RIN 1046-38 that the GLP-1R is coupled to the Gαs subunit and agonist engagement with the receptor leads to adenylyl cyclase (AC) activation and the formation of cAMP (Drucker, Philippe et al. 1987). Rat islets express multiple isoforms of AC including isoforms 2 to 7 (Leech, Castonguay et al. 1999). However, it has been proposed that in RINm5F β-cell line that the GLP-1R couples to the AC8 isoform which is synergistically activated by both the Gαs and calcium/calmodulin (Delmeire, Flamez et al. 2003). The production of cAMP by adenylyl cyclase is tightly regulated via a balance between production by AC and degradation by cyclic nucleotide phosphodiesterases (PDEs). In pancreatic β-cells, several studies have shown that β-cells express several PDE isoforms, including PDE1C, PDE3B and PDE4 (Pyne and Furman 2003). PDE3B has been shown to play an important role in regulating insulin secretion as inhibition of PDE3 enhances GLP-1 stimulated insulin secretion and, more recently, PDE3B has been shown to be localized within insulin granules (Pyne and Furman 2003; Walz, Wierup et al. 2007). The rise in cAMP as a consequence of GLP-1R activation leads to the activation of protein kinase A (PKA) and cAMP regulated exchange factor (Epac) and to the subsequent activation of downstream signalling pathways including the MAPK and PI3K/PKB pathway which are discussed in detail in chapter 3 and 5 and PKC (**Figure 1.2b**).

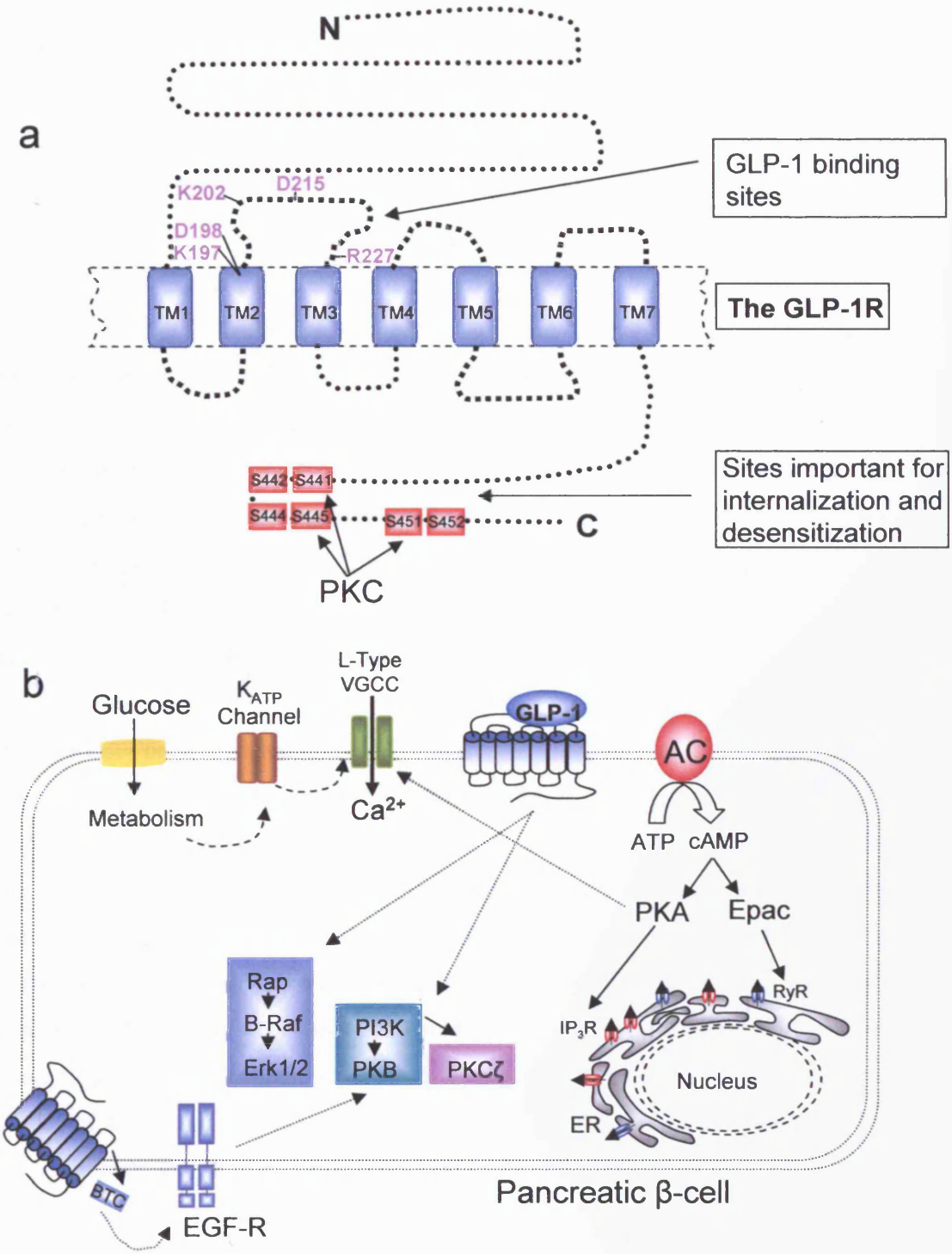


Figure 1.2: The GLP-1 Receptor and Associated Signal Transduction Pathways in the Pancreatic β-cell

a) Schematic representation of the GLP-1 receptor.

b) Binding of GLP-1 to the receptor is coupled to adenylyl cyclase activation. Intracellular cAMP levels are then increased leading to PKA and Epac activation. Following GLP-1R activation, signalling mechanisms leading to β-cell proliferation and survival are activated.

GLP-1 potentiates glucose stimulated Erk1/2 activation in several insulinoma cell lines, including MIN6 cells and INS-1 cells, and human islets (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003; Trumper, Ross et al. 2005). The activation of Erk1/2 by GLP-1, like glucose, has been shown to be dependent on a rise in intracellular free Ca^{2+} levels via Ca^{2+} influx through L-type voltage-gated calcium channels (L-type VGCC) (Gomez, Pritchard et al. 2002) and the release of intracellular Ca^{2+} (Arnette, Gibson et al. 2003; Gibson, Lawrence et al. 2006). Reports in the literature disagree as to whether Ras and Raf are required for glucose and GLP-1 stimulated Erk1/2 activation in pancreatic β-cells. However, a recent study in human islets showed that glucose and GLP-1 promoted the association of Rap with B-Raf (Trumper, Ross et al. 2005). In pancreatic β-cells, the MAPK cascade regulates cell proliferation, differentiation and gene transcription. For example, glucose stimulated Erk1/2 activation up-regulates insulin gene transcription in MIN6 cells (Lawrence, McGlynn et al. 2005) and β-cell proliferation in response to glucose and GLP-1 in INS-1 cells (Briaud, Lingohr et al. 2003).

It has previously been reported that GLP-1 activates PI3K and protein kinase-B (PKB) via the transactivation of the epidermal growth factor receptor (EGFR) in INS(832/13) cells (Buteau, Foisy et al. 2003). The ability of GLP-1 to activate PI3K via the transactivation of the EGFR is dependent on GLP-1R mediated activation of c-src and subsequent activation of a metalloprotease which cleaves membrane bound betacellulin (BTC) (endogenous EGF-like ligands) allowing transactivation of the EGFR (Buteau, Foisy et al. 2003). An increase in PI3K activity was associated with Rap1 immunoprecipitates in human islets stimulated by GLP-1 (Trumper, Ross et al. 2005). A study in which mice are carrying a knock-out of the Class 1_B PI3Kγ revealed that this isoform played an important role in regulating insulin secretion in pancreatic β-cells (Li, MacDonald et al. 2006). The loss of PI3Kγ and the resulting reduction in the glucose secretory response could be restored by the administration of exendin, a GLP-1R agonist (Li, MacDonald et al. 2006).

Following PI3K activation, the production of PtdIns(3,4,5)P_3 recruits PDK-1 as well as PKB to the plasma membrane, which enables the phosphorylation and activation of PKB. In pancreatic β-cells, all 3 isoforms of PKB have been shown to be expressed (Muller, Huang et al. 2006). Furthermore, PKB is phosphorylated in response to GLP-1 in INS-1

cells and this has been shown to play an important role in promoting β-cell proliferation and survival (Trumper, Trumper et al. 2000).

GLP-1 signalling has also been demonstrated to activate an atypical isoform of PKC, PKCζ, resulting in its translocation from the cytoplasm to the nucleus in INS-1 cells (Buteau, Foisy et al. 2001). The over-expression of a dominant negative form of PKCζ resulted in a significant inhibition of β-cell proliferation, indicating that GLP-1 stimulated PKC activation plays an important role in β-cell proliferation (Buteau, Foisy et al. 2001). In the clonal β-cell line, INS-1 cells, GLP-1 activation also results in the translocation of conventional PKCα and novel PKCε from the cytoplasm to the nucleus through Ca^{2+}-dependent PLC-mediated activation (Suzuki, Zhang et al. 2006). The authors hypothesised that GLP-1R activation and subsequent PKA activation results in the activation of IP_3 sensitive Ca^{2+} channels, which triggers Ca^{2+} release. Indeed, they show that inhibition of the IP_3 receptor with 2-APB inhibited GLP-1 induced Ca^{2+} release. The elevations in intracellular Ca^{2+} in turn leads to the activation of PLC which is able to hydrolyse PIP2 to generate IP_3 and DAG which then activates PKC (Suzuki, Zhang et al. 2006).

1.4 Functions of GLP-1

1.4.1 GLP-1 Actions on Insulin Secretion

Nutrients such as glucose are potent stimulators of insulin secretion, an effect that can be augmented by hormones such as GLP-1 (Doyle and Egan 2007; Holst 2007). In pancreatic β-cells, glucose enters the β-cell via the GLUT1 and 2 transporters. Upon entry, glucose is rapidly phosphorylated by glucokinase to glucose 6-phosphate. Its subsequent metabolism leads to an increase in the ATP/ADP ratio within the cell. This increase in ATP promotes the closure of ATP-sensitive K^+ channels, resulting in the depolarization of the cell membrane, and the opening of L-type VGCC. The subsequent influx of extracellular calcium into the cell, triggers the fusion of insulin containing secretory granules to the plasma membrane, which is followed by the exocytosis of insulin (Straub and Sharp 2002) (**Figure 1.3**). Insulin secretion occurs in two phases; the first is the rapid increase in intracellular calcium following membrane depolarization whilst the second involves augmentation of calcium induced insulin secretory vesicle exocytosis (Gromada, Brock et al. 2004). Both of these phases are enhanced by GLP-1. A main signalling mechanism

initiated by GLP-1 is the activation of adenylyl cyclase and formation of cAMP. Elevations in cAMP lead to the subsequent activation of PKA and cAMP regulated exchange factor II (Epac) which regulate a multitude of events, which include the altered ion channel activity, increases in intracellular calcium, and enhanced secretion of insulin containing granules (Drucker 2006). PKA activation is a key component in the regulation of insulin secretion as inhibition of PKA blocks GLP-1 and glucose stimulated insulin secretion (Wang, Zhou et al. 2001). The mechanisms by which PKA regulates insulin secretion include the modulation of ion channels, namely L-type VGCC and K_{ATP} channels. For example, PKA has been shown to phosphorylate the α1.2 subunit of L-type VGCC in pancreatic β-cells (Leiser and Fleischer 1996). Phosphorylation of Ser1928 of the α1.2 subunit and both Ser478/479 of the β_{2a}-subunit by PKA increase the activity of VGCC, thus increasing Ca^{2+} influx and insulin exocytosis (Bunemann, Gerhardstein et al. 1999) (Gao, Yatani et al. 1997). Closure of K_{ATP} channels, as a result of an increase in intracellular ATP may also be affected by PKA. It has been demonstrated that a point mutation in the ADP-sensing region of sulfonylurea receptor (SUR1) which forms part of the K_{ATP} channel leads to K_{ATP} channel closure via an ADP-dependent mechanism at position Ser1448Ala removed the modulatory effects of PKA (Light, Manning Fox et al. 2002). PKA-mediated phosphorylation at Ser1448 by PKA not only regulates insulin secretion via increasing Ca^{2+} influx but it also regulates the insulin secretion by release of intracellular Ca^{2+} from ER stores. It has been reported that PKA is able to promote the release of Ca^{2+} via the IP_3R in rodent islets and MIN6 cells to amplify insulin release (Tsuboi, da Silva Xavier et al. 2003; Dyachok and Gylfe 2004). Furthermore, this increase in intracellular Ca^{2+} was proposed to activate mitochondrial dehydrogenases which increase ATP synthesis and which may lead to the closure of K_{ATP} channels, enhancing the influx of extracellular Ca^{2+} within cells (Tsuboi, da Silva Xavier et al. 2003).

In islets, GLP-1 stimulated insulin secretion is not solely mediated via a PKA dependent mechanism, as PKA inhibitors do not completely abolish GLP-1 stimulated insulin secretion (Renstrom, Eliasson et al. 1997). These PKA independent effects of GLP-1 are attributed to guanine nucleotide exchange factors (GEFs) Epac1 and 2, in particularly Epac2, as reduction of Epac2 expression attenuates the effects of GLP-1 on insulin secretion (Kashima, Miki et al. 2001). In pancreatic β-cells, Epac2 is the predominate isoform (Ueno, Shibasaki et al. 2001) and is activated by the binding of cAMP to two cAMP binding domains. Once active, Epac functions as a guanine nucleotide exchange factor (GEF) for the small G-protein Rap. Epac also interacts with Ras, microtubule

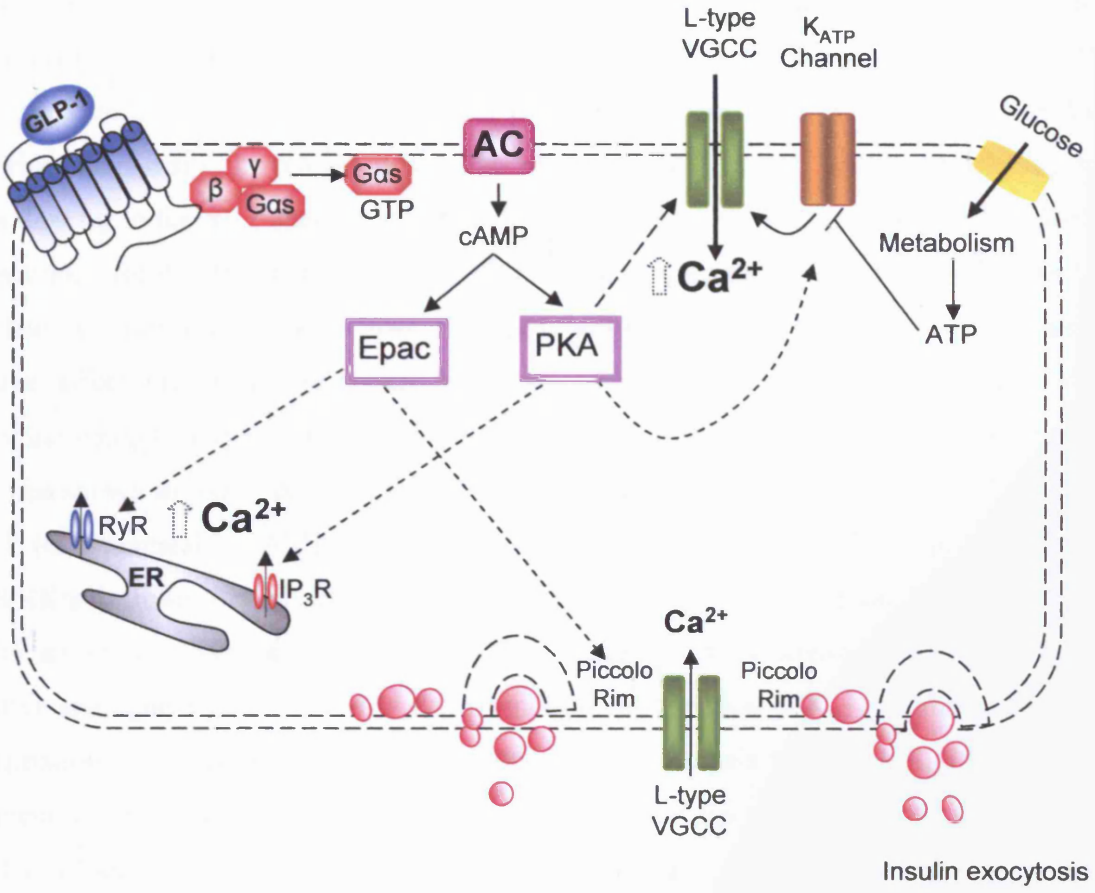


Figure 1.3: GLP-1 Actions Stimulate Insulin Secretion from the Pancreatic β-cell

Binding of GLP-1 to its receptor couple to adenylyl cyclase activation which cause intracellular cAMP levels to increase, leading to PKA and Epac activation. Following GLP-1R activation, insulin is released by stimulation of exocytotic pathways.

associated proteins, secretory granule associated proteins, such as Rim2 and Piccolo, and the SUR1 subunit of the K_{ATP} channel. A recent study revealed that GLP-1 or agents which increase cAMP within the β-cell increase the levels of Rap.GTP and that siRNA knock-down of Rap1 inhibits cAMP-dependent insulin secretion (Shibasaki, Takahashi et al. 2007). Epac2 can also interact with Rim2, a Rab3A interacting protein (Ozaki, Shibasaki et al. 2000) which is believed to be involved in the priming of secretory granules, thereby rendering them ready for release. Epac also interacts with the Ca^{2+} sensor piccolo which binds directly to the α1.2 subunit of L-type VGCC (Shibasaki, Sunaga et al. 2004). These interactions promote the interaction of cAMP and Ca^{2+} signals to specialized domains to facilitate exocytosis. Like PKA, Epac also increase intracellular Ca^{2+} levels and this has a positive effect on insulin secretion. Epac interacts with a ER resident channel, the ryanodine receptor (RyR) (Holz, Kang et al. 2006). Confirmation that Epac acts via the RyR was shown in INS-1 cells incubated with ryanodine which blocked 8-pCPT-2'-*O*-Me-cAMP (cell permeable cAMP analogue which can activate Epac but not PKA) induced CICR (Kang, Joseph et al. 2003). An additional mechanism by which Epac regulates insulin secretion is via the modulation of K_{ATP} channels. In pancreatic β-cells, the Epac selective analogue 8-pCPT-2'-*O*-Me-cAMP inhibits the function of K_{ATP} channels. Further confirmation that Epac leads to the inhibition of K_{ATP} channels is that cAMP itself, when administered intracellularly, leads to the inhibition of K_{ATP} channels whereas no such effect is observed in the presence of a cAMP analogue (N6-Bnz-cAMP) that activates protein kinase A (PKA) but not Epac (Kang, Chepurny et al. 2006). Additionally, a cyclic nucleotide phosphodiesterase-resistant Epac selective agonist (Sp-8-pCPT-2'-O-Me-cAMPS) inhibits K_{ATP} channel activity (Kang, Leech et al. 2008). This demonstrates that the inhibitory actions of Epac are unlikely to arise as a consequence of their hydrolysis to bioactive derivatives of adenosine.

cAMP generation also regulates exocytotic processes. Insulin secretory granules exist in distinct pools. One such pool which are docked and ready for release are referred to as the readily releasable pool (RRP). Another is a subset of granules located in close proximity to the Ca^{2+} channels and are capable of undergoing exocytosis following Ca^{2+} entry and are termed immediate releasable pool (IRP) (Straub, Shanmugam et al. 2004). The remaining pool is a large reserve pool that requires a series of priming reactions in order to achieve release competence. cAMP acting via PKA is able to accelerate granule mobilization whilst Epac functions to increase the size of the RRP (Gromada, Brock et al. 2004).

1.4.2 GLP-1 and Gene Expression

GLP-1 has been shown to increase insulin mRNA expression in pancreatic β-cells (Drucker, Philippe et al. 1987). It was later demonstrated that GLP-1 not only induces insulin mRNA levels but also the levels of the glucose transporter (GLUT-1) and hexokinase (Wang, Egan et al. 1995). The action of GLP-1 on insulin mRNA levels is a combination of increased stabilization and induction of insulin gene transcription. The mechanism of GLP-1 modulation of β-cell specific genes involves the pancreatic and duodenal homeobox 1 transcription factor (PDX-1). PDX-1 is implicated in regulating the expression of insulin in β-cells (Wang, Cahill et al. 1999). In pancreatic β-cells, GLP-1 has been shown to be involved in the regulation of PDX-1 by increasing its protein level, its translocation to the nucleus and its binding to the A1 element of the insulin promoter, resulting in a increase in the activity of the insulin gene promoter in a PKA dependent manner (Wang, Zhou et al. 2001). The increased stability of the insulin gene transcript in response to GLP-1 is dependent on the polypyrimidine tract binding protein (PTB) (Knoch, Bergert et al. 2004). PTB binds and stabilizes the mRNA encoding insulin. In INS-1 cells, PTB is translocated from the nucleus to the cytoplasm following glucose and GLP-1 stimulation, where PTB increases insulin expression (Knoch, Meisterfeld et al. 2006). Furthermore, PKA activation following GLP-1R activation increases PTB translocation by phosphorylating Ser16, as demonstrated by PKA inhibitors or siRNA of both the regulatory and catalytic subunits of PKA (Knoch, Meisterfeld et al. 2006). In pancreatic β-cells, the transcription factor FOXO1 (forkhead transcription factor 1) has been shown to be the predominate mediator of growth factor signalling which regulates β-cell mass through PDX-1 (Kitamura, Nakae et al. 2002). Subsequent studies have demonstrated that GLP-1 is able to induce the nuclear localization of PDX-1 via the phosphorylation of FOXO1. Phosphorylation of FOXO1 causes nuclear exclusion and GLP-1 is thought to promote the phosphorylation of FOXO1 in a PI3K dependent manner via the transactivation of the EGF-R (Buteau, Spatz et al. 2006). FOXO1 inactivation by phosphorylation leads to increased PDX-1 expression and β-cell proliferation (Kitamura, Nakae et al. 2002).

The transcription factor CREB (cAMP response element binding protein) has been shown to play an important role in β-cell survival in response to GLP-1 (Jhala, Canettieri et al. 2003). GLP-1R activation and subsequent elevations in cAMP activate PKA which phosphorylates CREB on Ser133 and promotes the induction of IRS-2, a downstream

target of the insulin signalling pathway which is important for the activation of PI3K/PKB mediation of β-cell survival. Furthermore, the over-expression of dominant-negative CREB in transgenic mice results in apoptosis due to the reduced expression of IRS-2 (Jhala, Canettieri et al. 2003). In addition, a family of CREB coactivators called transducers of CREB activity (TORCs), in particular TORC2, have also been shown to be regulated by the GLP-1R agonist exendin-4. In basal conditions, TORC2 is sequestered in the cytoplasm by the binding to 14-3-3 proteins. An elevation in Ca^{2+} and cAMP following GLP-1R activation promotes the release of TORC2 from 14-3-3 by activating a TORC2 specific phosphatase and inhibiting a TORC2 kinase via PKA dependent phosphorylation. Dephosphorylated TORC2 migrates into the nucleus and is recruited to the promoter via an interaction with the bZIP domain of CREB (Screaton, Conkright et al. 2004).

1.4.3 GLP-1 Actions on β-cell Proliferation

GLP-1 receptor activation leads to an increase in β-cell mass by regulating proliferation, growth, apoptosis, differentiation and neogenesis (**Figure 1.4**). The activation of the GLP-1 receptor couples to multiple signal transduction pathways important for control of β-cell proliferation. In INS-1 cells, GLP-1 has been shown to increase β-cell proliferation as measured by thymidine incorporation into DNA (Buteau, Foisy et al. 2003). The increase in β-cell proliferation was paralleled by an increase in PI3K activity (Buteau, Foisy et al. 2003). It was subsequently demonstrated that the PI3K effector PKCζ, which is an atypical PKC isoform is translocated to the nucleus following GLP-1 stimulation and also that inhibition of PKCζ suppresses the proliferative action of GLP-1 (Buteau, Foisy et al. 2001). Furthermore, inhibition of p38 MAPK with SB203580 also suppressed GLP-1-induced β-cell proliferation (Buteau, Foisy et al. 2001). It has been reported that GLP-1 induces β-cell proliferation via the transactivation of the epidermal growth factor receptor (EGFR) and subsequent activation of PI3K (Buteau, Foisy et al. 2003). In addition, GLP-1 has been shown to increase cell proliferation and survival via EGFR transactivation resulting in PKB dependent phosphorylation of FOXO1 which then promotes its nuclear exclusion (Buteau, Spatz et al. 2006). PKB dependent phosphorylation of FOXO1 inhibits its activity and promotes GLP-1 induced β-cell proliferation. PKB also phosphorylates a negative regulator of the mTOR pathway, TSC2 and phosphorylation of TSC2 inhibits the GAP activity towards Rheb (Inoki, Li et al. 2002). Previously, it has been shown that nutrients and hormones are able to activate mTOR via a cAMP dependent mechanism (Kwon, Marshall et al. 2004). mTOR is a multidomain protein which plays a key role in

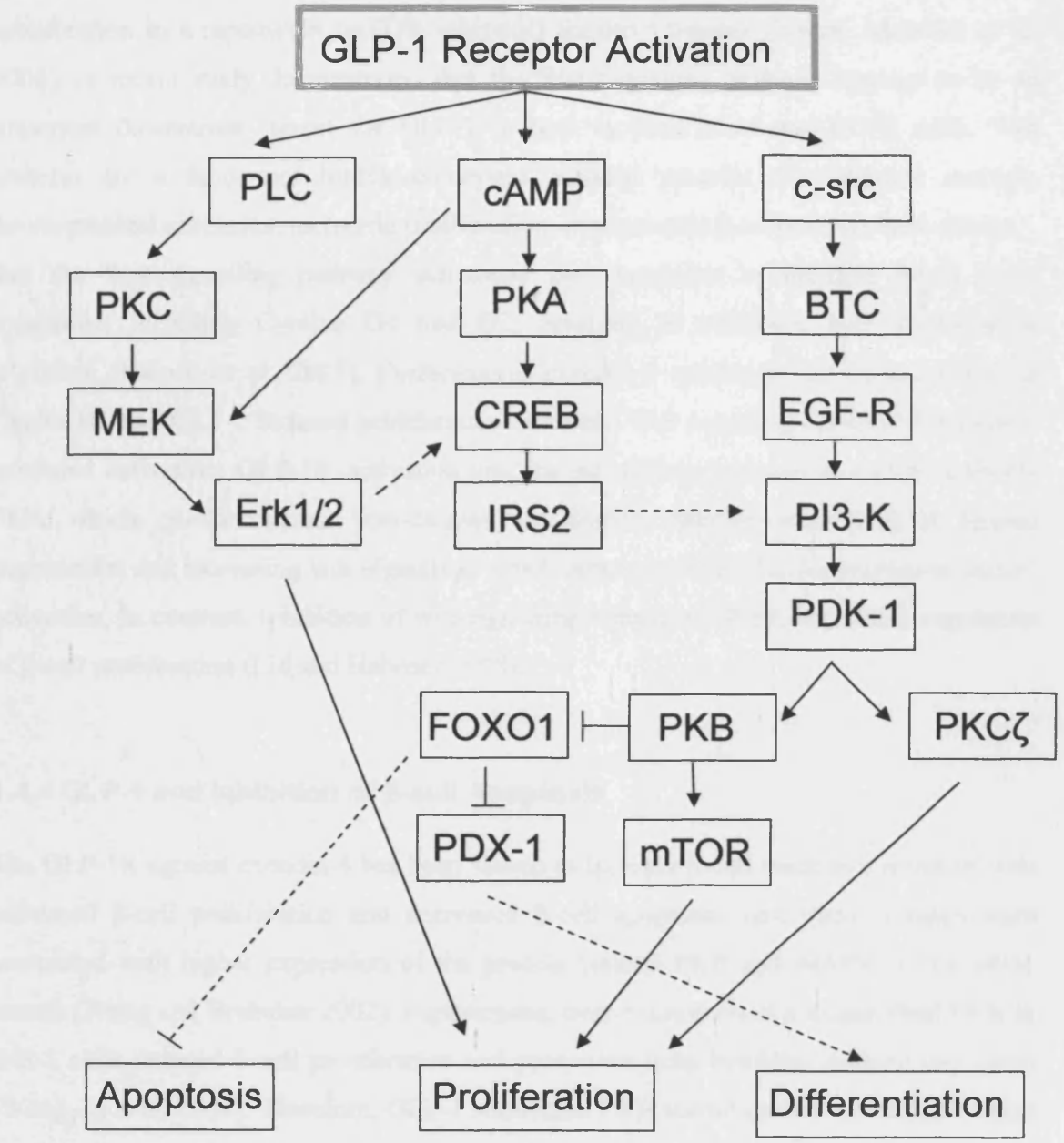


Figure 1.4: GLP-1 Receptor Signal Transduction Pathways in the Pancreatic β-cell Reported to be Involved in Proliferation, Apoptosis and Differentiation.

regulating many aspects of cell growth and proliferation via the regulation of protein synthesis. In rat islets, the GLP-1R agonist exendin-4 has been shown to increase β-cell proliferation in a rapamycin (mTOR inhibitor) sensitive manner (Kwon, Marshall et al. 2004). A recent study demonstrated that the Wnt signalling pathway appears to be an important downstream target for GLP-1 in both isolated islets and INS-1 cells. Wnt proteins are a family of highly conserved secreted proteins that regulate multiple developmental processes, including proliferation. In pancreatic β-cells, it has been shown that the Wnt signalling pathway stimulated the expression of multiple β-cell cycle regulators, including Cyclins D1 and D2, resulting in enhanced islet proliferation (Rulifson, Karnik et al. 2007). Furthermore, exendin-4 results in the up-regulation of Cyclin D1 and GLP-1 induced proliferation enhances Wnt signaling via GLP-1 receptor-mediated activation. GLP-1R activation and the subsequent increase in cAMP activates PKA which phosphorylates beta-catenin on Ser675, thereby stabilizing it against degradation and increasing wnt signalling which enhances TCF7-L2 (transcription factor) activation. In contrast, inhibition of wnt signalling reduces GLP-1R dependent regulation of β-cell proliferation (Liu and Habener 2008).

1.4.4 GLP-1 and Inhibition of β-cell Apoptosis

The GLP-1R agonist exendin-4 has been shown to increase β-cell mass as a result of both increased β-cell proliferation and decreased β-cell apoptosis, and these changes were associated with higher expression of the protein kinases PKB and MAPK in the *db/db* mouse (Wang and Brubaker 2002). Furthermore, over-expression of a kinase dead PKB in INS-1 cells reduced β-cell proliferation and protection from cytokine induced cell death (Wang, Li et al. 2004). Therefore, GLP-1 stimulated PKB activation has a protective effect on the β-cells. The anti-apoptotic properties of GLP-1 agonists have also been demonstrated in Zucker diabetic rats (Farilla, Hui et al. 2002). GLP-1 treatment of Zucker diabetic rats increased islet size and β-cell mass. Additionally, GLP-1 treatment reduced the numbers of apoptotic cells in the exocrine pancreas (Farilla, Hui et al. 2002). Activation of the GLP-1 receptor has also been shown to inhibit hydrogen peroxide-induced apoptosis in MIN6 cells, an effect which can be the blocked by cAMP antagonist Rp-cAMP (Hui, Nourparvar et al. 2003). In RINm5F cells, induction of fatty acid induced apoptosis with palmitate was markedly reduced by agents that increased levels of cyclic AMP such as GLP-1 and exendin-4. Furthermore, GLP-1 inhibited palmitate mediated caspase-3 activation, which demonstrates that GLP-1 has a protective effect against fatty

acid induced apoptosis (Kwon, Pappan et al. 2004). The antiapoptotic effect of GLP-1 is associated with the down-regulation of active caspase-3 and the up-regulation of bcl-2 at both the mRNA and protein levels in human islets (Farilla, Bulotta et al. 2003). Additionally, it has been demonstrated that GLP-1 increases β-cell survival via an increase in CREB activity. This increase in CREB activity up-regulates the expression of IRS-2 and increases PKB phosphorylation, resulting in inhibition of apoptosis (Jhala, Canettieri et al. 2003). Whilst over-expression of a dominant negative CREB in islet β-cells exhibited a reduction in cell mass and increased β-cell apoptosis (Jhala, Canettieri et al. 2003). GLP-1R signalling directly modulates the endoplasmic reticulum (ER) stress response, leading to an increase in β-cell adaptation and survival (Yusta, Baggio et al. 2006). The mechanism by which GLP-1 receptor activation reduces ER stress is via a reduction in eIF2α phosphorylation which decreases the expression of the pro-apoptotic regulators CHOP (DNA damage/CEBP homology protein) and ATF-4 (activating transcription factor 4) (Yusta, Baggio et al. 2006). Therefore, following induction of ER stress, exendin-4 treatment of INS-1 cells inhibited translational downregulation of insulin and improved cell survival *in vitro*. Exendin-4 also significantly potentiated the induction of ATF-4 by ER stress in a PKA-dependent manner. Therefore, GLP-1R agonists reduce ER stress associated β-cell death in a PKA-dependent manner which acts to promote β-cell function and survival during continued ER stress (Yusta, Baggio et al. 2006).

1.4.5 GLP-1 Actions on β-cell Differentiation and Neogenesis

A major mechanism for increasing β-cell number/mass is neogenesis or differentiation of new β-cells from precursor cells within the adult pancreatic ducts. When administered to rats, exendin-4 stimulates both the differentiation of β-cells from ductal progenitor cells (neogenesis) and proliferation of β-cells (Xu, Stoffers et al. 1999). In a partial pancreatectomy rat model of type 2 diabetes, the daily administration of exendin-4 for 10 days post-pancreatectomy attenuates the development of diabetes (Xu, Stoffers et al. 1999). GLP-1 and exendin-4 promote differentiation of the pancreatic duct cell line, AR42J or Capan-1 cell line to an endocrine phenotype as assessed by the expression of glucagon and insulin (Zhou, Wang et al. 1999; Zhou, Pineyro et al. 2002). Differentiated cells exhibited increased expression of β-cell genes such as GLUT-2 and glucokinase and the GLP-1 treated cells were also capable of secreting insulin in a glucose dependent manner (Zhou, Wang et al. 1999). The differentiation of these cells in response to GLP-1 was blocked by the addition of the MEK inhibitor PD98059 or by the PKA inhibitor PKI

(Zhou, Wang et al. 1999). Additionally, inhibition of the classical and novel PKC isoforms also completely blocked GLP-1 induced differentiation (Zhou, Wang et al. 1999). Differentiation can also be induced by activin which are members of the transforming growth factor (TGF) family that bind to TGF receptors and induce the phosphorylation and activation of SMAD transcription factors, in particular SMAD2 and 3. Furthermore, activation of the GLP-1R by exendin-4 was associated with release of TGF-β and changes in the expression of SMAD proteins. Exendin-4 treatment resulted in an increase in the levels of SMAD3 mRNA but decreased the levels of SMAD2. siRNA knock-down of SMAD2 resulted in a decrease in exendin-4 stimulated expression of insulin and PDX-1 mRNA as well as SMAD3 expression (Yew, Prasadan et al. 2004). Therefore, modulation of SMAD2 expression significantly impacted the extent of endocrine differentiation (Yew, Prasadan et al. 2004).

1.5 Extrapancreatic Effects of GLP-1

The main target of action of GLP-1 is the β-cell, where the hormone stimulates insulin secretion, promotes β-cell proliferation and neogenesis, and inhibits glucagon secretion. However, GLP-1 receptors are also expressed on other tissues/cells including the brain, heart, kidney and gastrointestinal tract (**Figure 1.5**).

1.5.1 GLP-1 Actions on Gastrointestinal Tract:

GLP-1 has been shown to inhibit gastric emptying and to inhibit gastric acid secretion (O'Halloran, Nikou et al. 1990). The inhibitory effect of GLP-1 on gastrin-induced acid secretion contributes to the inhibitory actions of pancreatic polypeptide (PYY) which is released from the L-cells in parallel to GLP-1. This interaction between GLP-1 and PYY almost abolishes gastrin secretion, indicating that GLP-1 and PYY have an important role in the physiologic control of gastric acid secretion (Wettergren, Maina et al. 1997). Additional studies demonstrated that GLP-1 inhibits gastric emptying by a mechanism dependent on vagel stimulation (Wettergren, Petersen et al. 1994). Further studies showed that following vagal afferent denervation the actions of GLP-1 when administrated centrally and peripherally on gastric emptying was blocked (Imeryuz, Yegen et al. 1997).

1.5.2 GLP-1 Actions on Pancreatic α and δ cells:

In the pancreas, GLP-1 also inhibits glucagon secretion from the α-cells and stimulates somatostatin secretion from the δ-cells (Orskov, Holst et al. 1988). The mechanism by

which GLP-1 inhibits glucagon secretion is not fully understood. It is generally thought that insulin released as a consequence of enhanced glucose induced insulin secretion inhibits glucagon secretion from the α-cells in paracrine fashion. However, in subjects with type 1 diabetes, which have diminished levels of insulin, GLP-1 is still capable of inhibiting glucagon secretion (Creutzfeldt, Kleine et al. 1996), suggesting other mechanisms may also be involved. Another possible mechanism for the inhibitory actions of GLP-1 on glucagon secretion is that GLP-1 also stimulates somatostatin secretion which has been reported to inhibit glucagon secretion (Fehmann, Goke et al. 1995). Somatostatin interacts with membrane somatostatin receptors located on the α-cells. In islets somatostatin potently inhibited glucagon release up to 85%, whereas the effect was reduced to 27% in islets generated from somatostatin receptor 2 knock-out mice (Strowski, Parmar et al. 2000).

1.5.3 GLP-1 Actions in the Heart:

GLP-1 receptors are expressed in the rodent and human hearts. In rat cardiac myocytes, GLP-1 has been shown to elevate cAMP levels but it displayed a mildly negative inotropic effect (Zhou, Montrose-Rafizadeh et al. 1999). In GLP-1R knock out mice, the animals exhibited reduced resting heart rate and elevated left ventricular end diastolic pressure (Gros, You et al. 2003). During cardiac injury, GLP-1 has been shown to increase cardiac output and reduce ventricular end diastolic pressure in association with improved myocardial insulin sensitivity and glucose uptake in dogs with pacing induced heart failure (Nikolaidis, Mankad et al. 2004).

1.5.4 GLP-1 Actions in the Brain:

An additional physiological role of GLP-1 is its central action to inhibit food intake. The GLP-1R is located in the neurons of the brain in particular the brain stem and hypothalmus which receive afferent information from the GI tract. In rats, intracerebroventricular injection of GLP-1 inhibited food intake. However, peripheral GLP-1 administration had little effect (Tang-Christensen, Larsen et al. 1996). In addition, administration of the GLP-1R antagonist, exendin(9-39) amide reversed the effect on food intake (Tang-Christensen, Larsen et al. 1996).

1.5.5 GLP-1 Actions on Peripheral tissues:

The GLP-1R agonist exendin-4 has also been reported to enhance insulin sensitivity in adipocytes (Idris, Patiag et al. 2002). For example, exendin-4 increases insulin stimulated

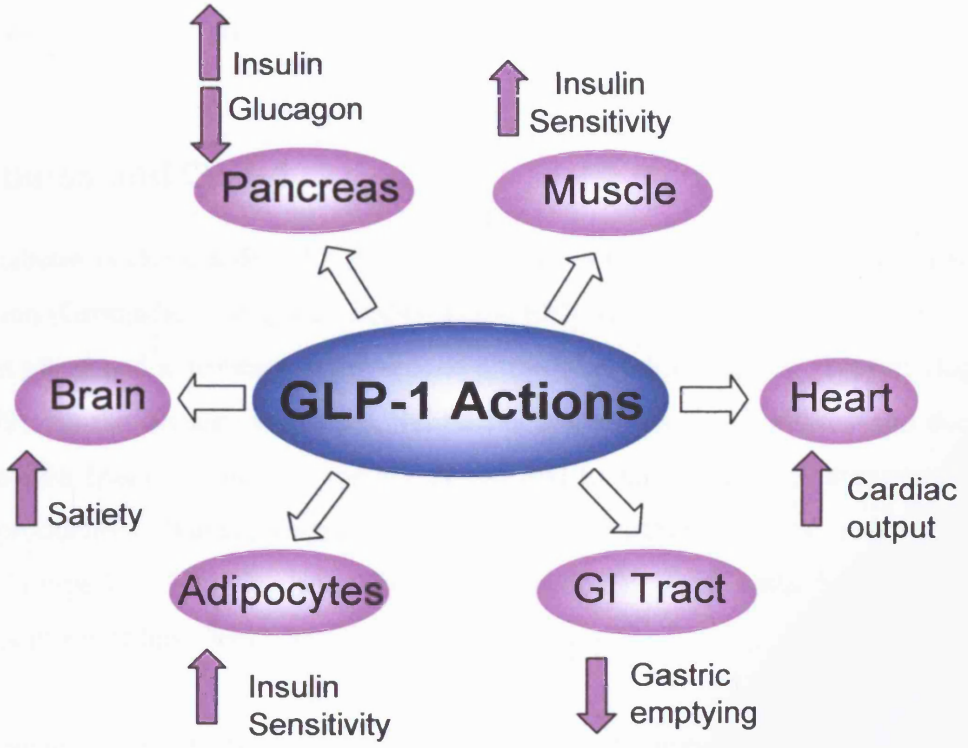


Figure 1.5: GLP-1 Actions on Peripheral tissues

Schematic representation of GLP-1 actions on target tissues. See text for details.

glucose uptake in muscle and fat derived cells. This effect was shown to be inhibited by blocking the PI3K pathway with LY294002, indicating that the enhanced insulin sensitivity occurs via a PI3K dependent mechanism (Idris, Patiag et al. 2002). Additionally GLP-1 is able to stimulate glycogen synthase in rat skeletal muscle and adipose tissue (Villanueva-Penacarrillo, Alcantara et al. 1994; Perea, Vinambres et al. 1997).

1.6 Diabetes and GLP-1

Type 2 diabetes is characterised by the development of insulin resistance and impaired β-cell function (Gromada, Brock et al. 2004). In the early stages of the disease, there is a loss of the first phase and a decrease in the second phase of insulin secretion (Weyer, Bogardus et al. 1999). As the disease progresses, the further loss of insulin sensitivity and declining β-cell function leads to fasting hyperglycaemia due to the inability to suppress hepatic glucose production (Weyer, Bogardus et al. 1999). Furthermore, the reduced insulin secretion in type 2 diabetes is also related to a reduction in β-cell mass due to accelerated rates of apoptosis (Matveyenko and Butler 2006).

The glucose lowering effects of GLP-1 suggests that GLP-1 may be used as a treatment for type 2 diabetes and the administration of exogenous GLP-1 is able to restore insulin secretion to near normal levels in Type 2 diabetic patients (Todd and Bloom 2007). Therefore, the insulinotropic action of GLP-1 is preserved in patients with Type 2 diabetes. Indeed, continuous infusion of exogenous GLP-1 increases insulin secretion and normalises both fasting and postprandial blood glucose. GLP-1 is also able to restore the first phase of insulin secretion (Ahren 1998; Todd and Bloom 2007). Besides its glucose lowering effects, GLP-1 also promotes insulin biosynthesis and insulin gene expression to replenish insulin stores for secretion. Additionally, GLP-1 has positive trophic effects on the β-cell by enhancing β-cell proliferation, growth and neogenesis from precursor cells (Holst, Deacon et al. 2008). GLP-1 exerts many of its intracellular effects via an increase in cAMP and elevations in cAMP protect β-cells from cytokine or fatty acid induced cell death (Hui, Nourparvar et al. 2003). CREB also contributes to the anti-apoptotic effects of GLP-1. Indeed, CREB knock-out mice develop diabetes as a consequence of increased apoptosis (Jhala, Canettieri et al. 2003). Furthermore, GLP-1 suppresses glucagon secretion, which together with the enhanced insulin secretion acts to reduce glucose production in the liver (Holst, Deacon et al. 2008). Additional effects exerted by GLP-1 include delaying of gastric emptying which reduces postprandial glucose excursions as

well as suppression of food intake and appetite. (Drucker 2001; Holst, Deacon et al. 2008). Therefore, these multiple actions of GLP-1 on the β-cell make GLP-1 an excellent therapeutic candidate for the treatment of type 2 diabetes. However, since GLP-1 is rapidly metabolised within a few minutes by the enzyme dipeptidyl peptidase-IV (DPP-IV), GLP-1 itself is not an ideal therapy for the treatment of type 2 diabetes. However, GLP-1R analogues such as Exendin-4, a naturally occurring GLP-1R agonist which was originally isolated from the venom of *Heloderma suspectum* lizard, (Glia lizard) (Pohl and Wank 1998) is resistant to DPP-IV and is currently being used as a therapeutic agent for the treatment of Type 2 diabetes (Wang and Brubaker 2002). An additional approach to enhancing the actions of GLP-1 involves the inhibition of DPP-IV, the GLP-1 degrading enzyme. Studies have shown that DPP-IV knock-out mice have an increased glucose tolerance, elevated levels of GLP-1 as well as increased insulin sensitivity compared to their wild type counterparts (Conarello, Li et al. 2003).

1.7 Summary and Thesis Aims

A number of kinases are activated in response to GLP-1R activation, including extracellular regulated kinases (Erk1/2), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB) and mammalian target of rapamycin mTOR, all of which contribute in regulating various aspects of β-cell function. However, the mechanism by which GLP-1 activates these signalling pathways in pancreatic β-cells is not fully understood. Therefore, the aims of this thesis were to identify the signalling pathways by which GLP-1 signals to Erk1/2, PKB and ribosomal protein S6 (rpS6) and define essential components of these pathways in pancreatic β-cells (islets of Langerhans and MIN6 cells, a clonal pancreatic β-cell line).

Chapter 2: Materials and Methods

2.1 General Reagents and Materials

Unless stated, all chemicals were of analytical grade and were routinely purchased from Sigma, Fisher or Melford. Restriction enzymes were purchased from New England Biolabs (NEB), T4 DNA ligase purchased from Promega. Bacterial cell culture reagents were from Melford. Disposable plastics (1.5ml microfuge tubes, non-filtered pipette tips) were obtained from Sarstedt. Filtered tips were purchased from Axygen. PD184532, LY294002, wortmannin, H89 MDL-12330A, rapamycin, AG1478, Ro318220, Tpl2 inhibitor and forskolin were all purchased from Calbiochem. Nifedipine, diazoxide, Phorbol 12-myristate 13-acetate (PMA) and GLP-1 were all purchased from Sigma. Siguazodan, Ro-20-1724 and 8-pCPT-2'-*O*-Me-cAMP were purchased from Tocris. FK506 (Caymen Chemicals). [γ^{32}P] ATP was purchased from GE Health Care. Recombinant adenoviruses encoding MKP3, constitutively active AMPK and WT-HA-PKB were generously provided by Prof. P Pratt, University of Wisconsin, USA, Dr Carling, Imperial College London and Dr. C. Sutherland, University of Dundee respectively.

2.2 Mammalian Cell Culture

Cell lines used in this study were Human Embryonic Kidney 293 (HEK-293), RAW264.7 macrophages (provided by Dr Bernard Burke, University of Leicester) Mouse Insulinoma 6 cells (MIN6), which respond to physiological changes in glucose concentrations (Miyazaki, Araki et al. 1990) were kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University Medical School, Japan.

Tissue culture plates and flasks were from NUNC or TPP. Tissue culture pipettes were from Greiner or Corning.

2.2.1 Maintenance of Cell Lines

HEK-293 cells were used at approximately 80% confluence and were grown in full Dulbecco's Modified Eagle's Medium (DMEM; Sigma) containing 25mM glucose supplemented with 10% heat-inactivated foetal calf serum (FCS; Gibco BRL), 100μg/ml

streptomycin and 100units/ml penicillin sulphate (Sigma), equilibrated with 5% CO_2, 95% air at 37°C. Cells were split every 2-3 days to prevent over confluence.

RAW264.7 cells were used at ~80% confluence and split every 3-5 days. RAW264.7 were maintained in RPMI 1640 medium containing 11mM glucose supplemented with 10% heat-inactivated FCS, 100μg/ml streptomycin and 100units/ml penicillin sulphate, equilibrated with 5% CO_2, 95% air at 37°C.

MIN6 cells were used at approximately 80% confluence between passages 16 to 40. MIN6 cells were grown in DMEM containing 25mM glucose supplemented with 15% heat-inactivated FCS, 100μg/ml streptomycin, 100units/ml penicillin sulphate, 40mM $NaHCO_3$ and 75μM β-mercaptoethanol, equilibrated with 5% CO_2, 95% air at 37°C. The medium was changed every 2-3 days.

2.2.2 Cell Splitting

When ~ 80% confluence was reached, cells were washed once in 1X phosphate buffered saline (PBS; Sigma) before incubation with 1X trypsin/EDTA (0.5%) (Gibco BRL) for 2-5 minutes at 37°C. Cells were resuspended in DMEM as soon as they started to detach from the plate. Resuspended MIN6 cells were split 1:3 to 1:4 for maintenance, or as required for experiments; 293 cells were split 1:5 to 1:10 for maintenance, or as required for experiments and RAW264.7 were split 1:3 for maintenance or as required for experiments.

2.2.3 Islet Isolation and Culture

Pancreatic islets were isolated from 200g to 250g male Wistar rats by collagenase digestion and Histopaque density gradient centrifugation by a modification of the method of Guest and Rhodes *et al* (Guest, Rhodes et al. 1989). Briefly, six male wistar rats were anesthetized and the pancreas was inflated by injecting 6ml of medium (RPMI 1640 medium containing 11mM glucose) containing 1mg/ml collagenase (Serva, Heidelberg, Germany) through the common pancreatic duct. After the animals were killed by incision of the heart, the pancreas was removed. The excised pancreata were then incubated at 37°C for 17 minutes. Following incubation the pancreata were then individually hand shaken for 1 minute. The partially disaggregated tissue was then centrifuged for 3 minutes at 200 x *g* at 4°C. The pelleted material was then resuspended in RPMI 1640 and subjected to another

cycle of resuspension and centrifugation. The pelleted material was then resuspended in RPMI containing 5% fetal calf serum and then filtered through a 6.5cm-diameter plastic tea strainer. Each filtrate was centrifuged for 3 minutes at 200 x *g* at 4°C and the pellets were resuspended in 10ml of Histopaque 1077 (Sigma) and over-layered with 10ml of RPM1 1640. The tubes were centrifuged for 20min at 1600 x *g* at 4°C and islets were recovered from the RPMI/Histopaque-1077 interface and washed once in RPMI containing 5% fetal calf serum. The islets were then hand-picked under a stereomicroscope to ensure high purity of the preparation. The islets were then cultured at 37°C 5% CO_2, 95% air in CMRL 1066 (Gibco) medium containing 5.6mM glucose and 1% bovine serum albumin instead of 10% fetal calf serum, to prevent flattening of the islets on the bottom of the dish. Unless otherwise specified, the islets were cultured overnight prior to treatments.

2.2.4 Calcium Phosphate Mediated DNA Transfection

2x Hepes Buffered Saline (HBS)

8g NaCl

0.2g Na_2HPO_4-$7H_2O$

6.5g HEPES

pH to 7.0 and make up to 500ml with distilled H_2O. Re-pH, filter-sterilise, aliquot and store at -20°C.

Cells were split to 20-30% confluence 6-10 hours prior to transfection. For a 6cm diameter plate, a solution containing 5μg of DNA (GFP tagged constructs were used or if plasmid did not contain GFP they were co-transfected with plasmids encoding pEGFP) and 244mM $CaCl_2$ with a final volume of 180μl was prepared. This solution was added drop-wise to 180μl 2XHBS, mixing gently upon addition. Air was bubbled through the DNA/$CaCl_2$/HBS mixture to break up any precipitate. The DNA/$CaCl_2$/HBS mixture was then added directly to the cells by dropping slowly and evenly into the medium. After 16-24 hours of incubation at 37°C/5% CO_2, the medium was removed, the cells were washed with PBS and fresh medium was added to the cells. Incubation was resumed for a further 24-48 hours prior to experimentation. Transfection efficiency was assessed by monitoring the levels of GFP expression, using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transfection was typically between 70-90% at the time of experimentation.

2.2.5 Infection of Cell Lines with Recombinant Adenoviruses

Adenoviral mediated transduction of cell lines was performed as follows. Growth medium was aspirated from cells and replaced with 400µl of DMEM (for a 4cm diameter plate) supplemented with antibiotics, minus FCS. High titre viral stock was added to the cells (for details see figure legends) and incubated at 37°C/5% CO_2. The plates were rocked gently every 5-10 minutes. After one hour, 1.2ml of complete medium (containing FCS) was added to the cells, and the incubation continued for a further 24-48h prior to experimentation. Adenoviral infection efficiency was assessed by monitoring the levels of GFP expression using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transduction was typically between 80-95% at the time of experimentation.

2.3 Experimentation

Krebs Ringer Bicarbonate buffer (KRB)

115mM NaCl

5mM KCl

10mM $NaHCO_3$

2.5mM $MgCl_2$

2.5mM $CaCl_2$

supplemented with 20mM HEPES pH 7.4 and 0.1% w/v BSA

2.3.1 Treatment of Cell Lines

Prior to treatment, the medium was removed and the cells washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (KRB buffer). The cells were then incubated for 1h at 37°C/5% CO_2 in KRB buffer supplemented with glucose and or amino acids (see figure legends for details) prior to incubation in KRB buffer cells were further incubated in test substances for the times indicated in the figure legends (details of treatments are provided in the figure legends). When cells were treated with elevated extracellular K^+ concentration, the K^+ concentration in the KRB was increased to 50mM and the Na^+ concentration decreased to 70mM to maintain isotonicity. In the calcium-free experiments, after pre-incubation the cells were incubated for 15 min in a nominal calcium KRB buffer and stimulated in the same buffer. All treatments were stopped by the addition of ice-cold MIN6 lysis buffer containing 1% Triton, 10mM β-glycerophosphate, 50mM Tris-HCl, pH

7.5, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 1mM benzamidine HCl, 0.2mM phenylmethylsulfonyl fluoride, 1μg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol, and 50mM sodium fluoride. The lysates were then centrifuged for 10 min at 16,000 × *g* (see section 2.7.3).

2.3.2 Treatment of Islets of Langerhans

For treatment, islets were counted into microfuge tubes containing KRB. Islets were then washed in the medium they were to be pre-incubated in, and were collected by centrifugation at 800rpm for 1 minute. The supernatant was removed and the wash step repeated and islets were then transferred into 4cm diameter Sterilin plates. Treatments were performed as described in the figure legends. After treatment, islets were collected and transferred back into microfuge tubes and collected by centrifugation 16,000 × *g* for 30 seconds at 4°C. The supernatant was discarded and islets lysed by the addition of ice cold MIN6 lysis buffer followed by one freeze/thaw cycle. Lysates were then analysed as described in section 2.7.

Amino Acid Composition: In some experiments, the KRB was supplemented with amino acids. KRB buffer was supplemented with MEM amino acids solution (50X), MEM non-essential amino acids solution (100X), and L-glutamine (100X).
For these experiments, the 0.5× concentration of amino acids was defined as the following in mM: L-arginine 0.36, L-cystine 0.1, L-glutamine 1.0, L-histidine·HCl·H_2O 0.1, L-isoleucine 0.2, L-leucine 0.2, L-lysine HCl 0.25, L-methionine 0.05, L-phenylalanine 0.1, L-threonine 0.2, L-tryptophan 0.025, L-tyrosine 0.1, L-valine 0.2, L-alanine 0.05, L-asparagine 0.05, L-aspartic acid 0.05, L-glutamic acid 0.05, glycine 0.05, L-proline 0.05, L-serine 0.05.

2.4 Molecular Biology

2.4.1 Bacterial Strains

Strain	Resistance	Supplier
BJ5183	Streptomycin	Gift from He, T.C. (He *et al.*, 1998)
Chemically competent DH5α (Subcloning Efficiency)	N/A	Invitrogen
Rosetta 2 (DE3)pLysS Competent Cells	Chloramphenicol	Novagen

2.4.2 Cloning Vectors

Bacterial Culture Media and Supplements

Luria-Bertani media (LB)

1% w/v Tryptone

0.5% w/v Yeast Extract

1% w/v NaCl

1.5% w/v Bacteriological Agar (for solid media only)

SOB

2% w/v Tryptone

0.5% w/v Yeast Extract

10mM NaCl

2.5mM KCl

SOC

2% w/v Tryptone

0.5% w/v Yeast Extract

10mM NaCl

2.5mM KCl

10mM $MgCl_2$

10mM $MgSO_4$

20mM *D*-glucose

Ampicillin (Filter sterilised)

Used at a final concentration of 100μg/ml

Kanamycin (Filter sterilised)

Used at a final concentration of 50μg/ml

2.4.3 Preparation of Competent Cells

Preparation of Electrocompetent BJ5183 E. coli Containing the Adenoviral Backbone Vector, pAdEasy-1

BJ5183 cells were transformed with the adenoviral backbone vector pAdEasy-1 by electroporation as described in section 2.4.4.2. Briefly, 50μl of BJ5183 cells were thawed on ice and mixed with 100ng of pAdEasy-1 in a cold microfuge tube. The mixture was

then transferred to a pre-chilled electroporation cuvette (Molecular Bioproducts) and pulsed at 1.8kV in a Bio-Rad *E. coli* gene pulser. The cuvette was removed immediately and 0.5ml of warm SOC medium was added. Cells were transferred to sterile tubes and recovered by shaking at 225rpm at 37°C for 30 minutes. Cells were then plated on to LB plates supplemented with ampicillin and grown overnight at 37°C. One positive clone was picked into 5ml SOB medium supplemented with ampicillin and streptomycin, and was incubated overnight at 37°C. The following day, 500ml of SOB medium was inoculated with 5ml of the overnight culture and was incubated at 37°C with constant shaking at 225rpm until the OD_{600} reached 0.6. The cells were then transferred to cold centrifuge bottles and spun down at 5,000rpm in a Sorvall GSA rotor at 4°C for 15 minutes. The supernatant was discarded and cells were resuspended in a total of 400ml of ice-cold 10% glycerol to wash them. The cells were then spun again, supernatants discarded and the wash step repeated with 400ml of 10% glycerol. The pellet was then resuspended in 250ml of ice-cold 10% glycerol and spun as before. The supernatant was discarded and the cells were resuspended in 2ml ice-cold 10% glycerol. Cells were dispensed into 50µl aliquots and snap-frozen in a dry-ice ethanol bath. Aliquots were stored at -80°C.

2.4.4 Transformation of Competent Cells

2.4.4.1 Chemically Competent Cells

Competent DH5α cells were purchased from invitrogen. 50-100µl of competent cells were thawed on ice for 5 minutes, and then added gently to ~ 100ng of DNA in a pre-chilled tube. The cells and DNA were mixed by gently pipetting once up and down. Cells were then incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 25-30seconds, then left on ice for a further 2 minutes. Cells which had been transformed with plasmids conferring ampicillin resistance were plated directly on to LB-agar containing ampicillin. Cells which had been transformed with plasmids allowing resistance to kanamycin were recovered in 500µl of warm LB for 1 hour at 37°C with constant shaking at 225rpm. Cells were then centrifuged for 1 minute at 3,000rpm in an Eppendorf bench top microfuge and 400µl of the LB was discarded. The cells were resuspended gently in the remaining LB and plated on to LB-agar plates containing kanamycin. Plates were incubated overnight, upside down at 37°C.

2.4.4.2 Electrocompetent Cells

100ng of DNA was pre-chilled in a microfuge tube (where possible the total volume of DNA used in electroporation did not exceed 2µl). Electroporation cuvettes (1mm gap electroporation cuvettes; Molecular Bioproducts) and the safety chamber for electroporation were also pre-chilled on ice. 50µl aliquots of BJ5183 *E. coli* containing the adenoviral backbone of AdEasy were thawed on ice for 5 minutes and then cells were added to the DNA gently. The bacteria/DNA suspension was incubated on ice for 1 minute before being transferred into chilled electroporation cuvettes. Cuvettes were placed into the safety chamber and placed between the contacts of an *E. coli* gene pulser (Bio-Rad). Cells were pulsed at 1.8 kilovolts, then immediately recovered in 1ml of warm SOC medium for 30 minutes with constant shaking at 225rpm at 37°C. After recovery cells were centrifuged for 1 minute at 800 x *g* and 900µl of SOC medium discarded. Cells were resuspended in the remaining medium and plated on to selective LB-agar plates. Plates were incubated at 37°C upside down overnight.

2.4.5 Polymerase Chain Reaction (PCR)

Pfu Turbo DNA polymerase (Stratagene) and specific oligonucleotides (detailed in section 2.4.16) were used to amplify specific sequences of DNA. A 50µl reaction mix typically included: ~ 50ng of template DNA (for plasmids) or 1µl of cDNA reaction, 5µl 10X reaction buffer, 4µl dNTP mix (final concentration, 200µM each), 50pmol forward primer, 50pmol reverse primer and 0.5µl Pfu Turbo polymerase (1.25 units). Reaction mixtures were transferred to thin-walled PCR tubes (ABgene) and were cycled in an Eppendorf Mastercycler personal thermal cycler. Cycles varied according to the Tm of the oligos and the length of DNA to be amplified. Generally, there was an initial denaturing period at 94°C for 2 minutes followed by 30-35 cycles of denaturing at 94°C for 30 seconds, annealing (at a temperature determined by the Tm of the oligos) for 30 seconds, and an extension period at 72°C for two minutes for every kb to be amplified. There was a final seven minute extension period at 72°C and then the reaction mixture was kept at 4°C. PCR reactions were analysed on agarose gel electrophoresis.

2.4.6 Buffers and Reagents for Cloning into Plasmid Vectors

1X Tris-EDTA (TE)

10mM Tris pH 7.5

1mM EDTA

1X TAE (Tris-Acetate EDTA)

40mM tris-acetate

1mM EDTA pH 8

6X DNA loading buffer

50% v/v glycerol

0.1M EDTA pH 8

1% w/v SDS

1mg/ml bromophenol blue

1mg/ml xylene cyanol

2.4.7 TAE-Agarose Gel Electrophoresis

DNA samples were prepared by the addition of DNA loading buffer. Agarose (Melford) (0.7%-1.5%) was dissolved in 1XTAE by heating in a microwave for ~ 2 minutes. Once cooled to ~ 60°C, 0.1μg/ml ethidium bromide was added and the gel allowed to set. DNA samples were loaded alongside either 100bp or 1kb DNA ladders (New England Biolabs) as appropriate. Agarose gels were run horizontally immersed in 1X TAE at 90 volts for ~ 45 minutes or as required for resolution of bands.

2.4.8 Excision and Purification of DNA from Agarose Gels

DNA bands were visualized by exposure to UV light on a UV transilluminator. The DNA fragments of interest were determined by size comparison with DNA ladders and were excised from the gel. Gel slices were transferred to microfuge tubes and the DNA isolated using Geneclean II kit (Q-Biogene) according to manufacturer's instructions. Briefly, the gel was melted in 3 volumes of NaI at 55°C, vortexing regularly to aid melting of the agarose. Typically 5μl of glassmilk (silica matrix) was added and the NaI/glassmilk/DNA mixture was incubated for 5-15 minutes at room temperature with regular vortexing to allow the DNA to bind the silica matrix. The mixture was centrifuged at full speed for 15 seconds and the supernatant removed. The glassmilk/DNA complex was then washed by addition of 1ml of New Wash solution and centrifuged again at full speed for 15 seconds. The supernatant was discarded and the wash step repeated. The glassmilk/DNA pellet was

air-dried, and the DNA eluted by the addition of sterile distilled H_2O. The glassmilk was pelleted by centrifugation at full speed (16,000 x *g*) for 1 minute, and the supernatant containing the DNA kept and stored at -20°C.

The amount of eluted DNA was quantified by agarose gel electrophoresis of 1µl of the purified DNA against DNA ladders containing known quantities of DNA.

2.4.9 DNA Ligation

Ligation of insert DNA into plasmid vectors was performed according to manufacturer's instructions using T4 DNA ligase (Promega). Briefly, insert DNA was mixed with vector DNA in a 3:1 insert to vector ratio using 50-100ng of vector DNA. Reactions contained 1µl 10X T4 ligase buffer, appropriate quantities of vector/insert DNA and 1µl T4 DNA ligase (3 units) in a total volume of 10µl. Control ligations containing only the vector were performed in parallel. Ligation reactions were left overnight in iced water at ~ 15°C and then stored at -20°C until required.

2.4.10 Plasmid DNA Purification

Plasmid DNA was prepared from overnight bacterial cultures (containing appropriate antibiotics) following manufacturer's instructions. Plasmid minipreps were prepared from 5ml of overnight culture using the Genelute Plasmid Miniprep kit (Sigma). Miniprep DNA was eluted in 100µl of sterile distilled H_2O. DNA maxipreps were prepared from 100ml of overnight culture using the Ultraclean Endotoxin-Free Plamid Maxi prep Kit (Qiagen) and the DNA eluted in 1X TE. Maxiprep DNA was stored in 1X TE. All DNA minipreps/maxipreps were stored at -20°C until required.

2.4.11 Plasmid DNA Purification by Caesium chloride density gradients

Plasmid DNA was also purified by centrifugation through a caesium chloride (CsCl) density gradient. Plasmid DNA was prepared from 500ml of overnight bacterial cultures (containing appropriate antibiotics). The bacteria were then pelleted by centrifugation for 10 minutes at 7700 x *g* at 4°C. The pellet was then resuspended in 10ml buffer P1 (10mM EDTA, 50mM Tris-HCl pH8.0). The cells were then lysed by the addition of 20ml freshly prepared buffer P2 (0.2M NaOH, 1% SDS) followed by the addition of 15ml of buffer P3

(3M Potassium acetate pH5.5). The lysate was then cleared by centrifugation for 15 minutes at 12,000 x *g* at 4°C. The DNA was precipitated by the addition of an equal volume of ice-cold isopropanol, followed by incubation at -20°C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 12,000 x *g* for 10 minutes at 4°C. The DNA pellet was resuspended in a 5.5ml of TE buffer containing 6g of caesium chloride and 5mg/ml ethidium bromide, which is loaded into polyallomer tubes and subjected to ultracentrifugation at 100,000rpm in a TLA 100.3 rotor for 16-20 hours. The ethidium bromide stained plasmid DNA bands, equilibrated within the caesium chloride density gradient after ultracentrifugation, are visualized under long wave UV light and the lower band is removed with a 21 gauge needle on a 1ml syringe. The DNA band is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol by adding 2.5 volumes of cold 95% ethanol. Centrifuge at 10,000rpm for 45 minutes in the Sorvall GSA rotor to recover the DNA. Gently decant the supernatant, add 80% ethanol, centrifuge as before, decant, and air dry the DNA pellet. Resuspend the DNA in 1X TE buffer. Store at -20°C.

2.4.12 Ethanol Precipitation of DNA

DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate pH 5.2 (to make 0.3M final concentration) and 2 volumes of ice-cold ethanol, followed by incubation at -20°C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 16,000 x *g* for 10 minutes at 4°C. The pellet was washed in 75% ethanol and repelleted by centrifugation at 16,000 x *g* at 4°C. The supernatant was discarded and the pellet was dried under vacuum for 1 minute. The DNA pellet was then resuspended in an appropriate volume of de-ionised water or 1X TE and store at -20°C.

2.4.13 Restriction Digestion of DNA

All restriction endonucleases were obtained from New England Biolabs (NEB). Final reaction volume was determined such that the glycerol concentration in the reaction did not exceed 5%. Restriction digests contained an appropriate quantity of the DNA to be digested, 1X NE Buffer (as recommended by NEB), 1X bovine serum albumin (where appropriate) and restriction enzyme (2-10 units). Double digests were performed at the same time where possible; otherwise digests were performed sequentially according to NEB guidelines. Digests were typically incubated for up to 2 hours in a water bath at 37°C.

2.4.14 DNA Sequencing

DNA sequencing was performed by Cogenics (www.cogenics.com) using primer extension (silver service). Primers used were specific to the sequencing reactions.

2.4.15 Site Directed Mutagenesis

Site directed mutagenesis was performed using the QuickChange™ Site Directed Mutagenesis kit (Stratagene) as per manufacturer's instructions. Mutagenic primers are listed in section 2.4.16. PCR reactions contained ~ 50ng of plasmid template, 125ng of forward primer, 125ng of reverse primer, 5µl of 10X Pfu reaction buffer, 200µM of each dNTP and 1µl of Pfu Turbo DNA polymerase (2.5 units). Reactions varied according to the number of mutations to be introduced. Typically there was an initial denaturation step at 95°C for 30 seconds, then 16-18 cycles of a 30 second at 95°C denaturation step, 30 seconds at 55°C for annealing of primers, then 2 minutes per kb of plasmid at 68°C for extension of primers. Following this, the reaction was subjected to a final extension period of 7 minutes at 68°C, and then cooled at 4°C. 1µl of DpnI was added directly to the reaction, and reactions were incubated at 37°C for 1h to digest the methylated parental wild-type DNA. The PCR product undigested nicked mutated plasmid was then transformed into competent DH5α as described in section 2.4.4. Mutated plasmids were checked initially by restriction digest (if a restriction site was introduced or destroyed by mutagenesis), then by sequencing.

2.4.16 PCR Primers

Name	Sequence
HA-Rap1aA17For	5' GGC GTT GGG AAG GCT GCT CTG ACA GTT CAG 3'
HA-Rap1aA17Rev	5' CTG AAC TGT CAG AGC AGC CTT CCC AAC GCC 3'
HA-Rap1aN17For	5' ACT AAG CTT ATG GCT TAC CCA TAC GAT G 3'
HA-Rap1aN17Rev	5' GTT GAT ATC CTA GAG CAG CAG ACA TGA 3'
Myc-CaMKIIδ2For	5' CTT AGA TCT ATG GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG ATG GCT TCG ACC ACC ACC TGC 3'
Myc-CaMKIIδ2Rev	5' GAT GCG GCC GCT GAG GAC AGA ATG AAG ACG 3'
Myc-CaMKIVFor	5' AG CA CAA TGG CA CCC CGT GC 3'
Myc-CaMKIVRev	5' CCA GTT AAC AAC AAC AAT TGC 3'

2.4.17 Cloning Strategies

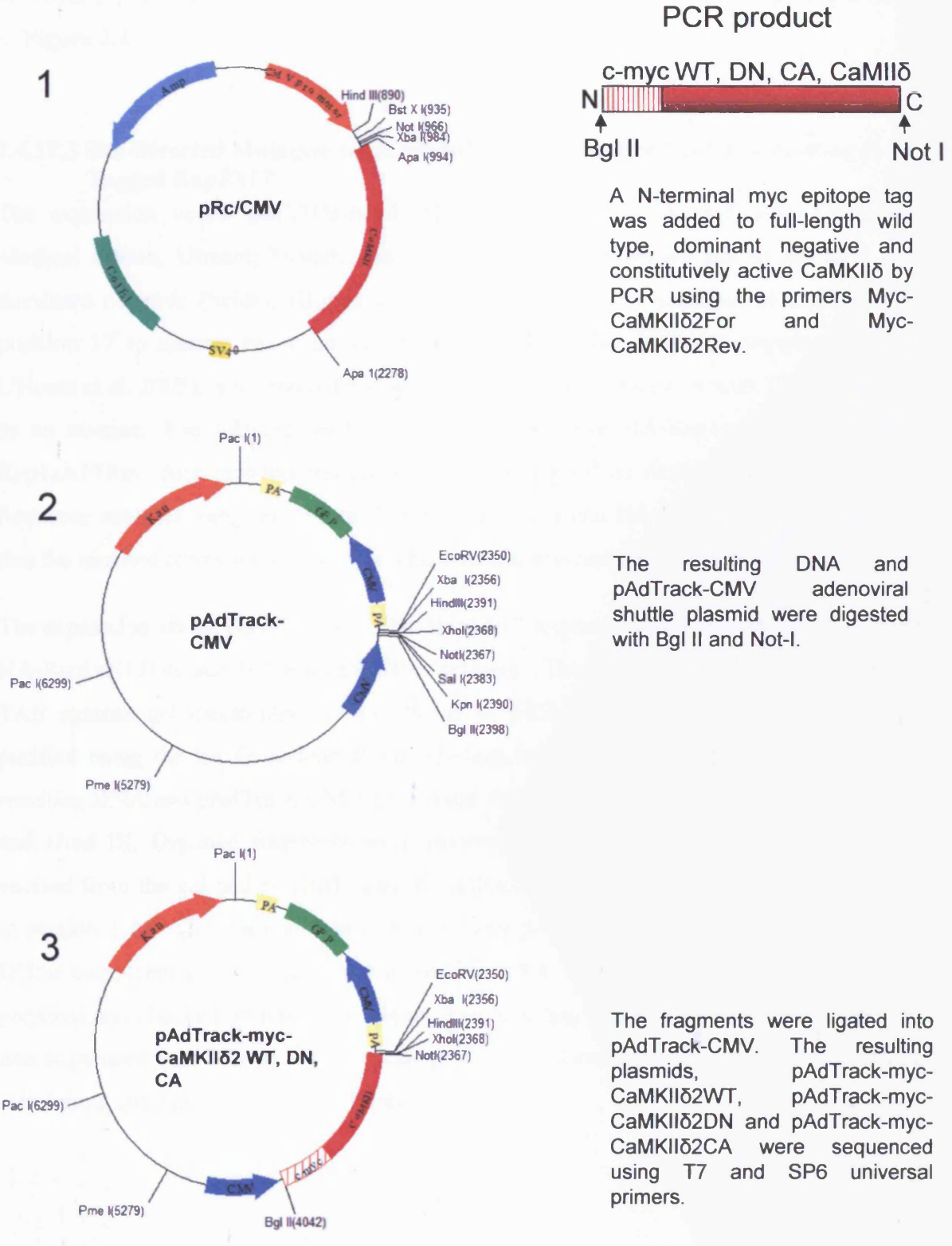
2.4.17.1 Construction of Adenoviral Shuttle Plasmids Encoding Myc-tagged CaMKIIδ_2 (Wild Type, Dominant Negative or Constitutively Active)

Mammalian pRc/CMV expression vectors encoding wild type, dominant negative (Ala substitution for Lys^{43} in the K43A ATP-binding defect mutant) or constitutively active (Amino acid substitution of Thr^{287} to aspartic acid generated a constitutively active mutant) CaMKIIδ_2 were kindly provided by Prof. Harold A. Singer (Center for Cardiovascular Sciences, Albany Medical College, Albany, New York). A N-terminal myc epitope tag was added to full-length wild type, dominant negative and constitutively active mutants by PCR using the primers Myc-CaMKIIδ2For and Myc-CaMKIIδ2Rev. The PCR products were resolved by TAE agarose gel electrophoresis and bands of 1650bp were excised from the gel and purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The resulting DNA and pAdTrack-CMV adenoviral shuttle plasmid were digested with Bgl II and Not I. Digested fragments were resolved on agarose gel and the fragments excised as before. The fragments were ligated into pAdTrack-CMV and transformed in DH5α competent cells as described in section 2.4.4. Minipreps of plasmid DNA were prepared and checked by restriction digest. The resulting plasmids, pAdTrack-myc-CaMKIIδ_2^{WT}, pAdTrack-myc-CaMKIIδ_2^{DN} and pAdTrack-myc-CaMKIIδ_2^{CA} were sequenced using the T7 and SP6 universal primers and found to be correct. An overview of the cloning strategy is provided in **Figure 2.1.**

2.4.17.2 Construction of Adenoviral Shuttle Plasmids Encoding Myc-tagged Constitutively Active CaMKIV

A mammalian PME185 expression vector encoding constitutively active (amino acid substitution of Thr^{287} to aspartic acid), was kindly provided by Prof. Edo Vellenga (from the Divisions of Hematology and Clinical Immunology, Department of Internal Medicine, University of Groningen, The Netherlands). An N-terminal myc epitope tag was added to full-length constitutively active CaMKIV by PCR using the primers Myc-CaMKIVFor and Myc-CaMKIVRev. The PCR reactions were resolved by TAE agarose gel electrophoresis and bands of 1626bp were excised from the gel and purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The resulting DNA and pAdTrack-CMV adenoviral shuttle plasmid were digested with Kpn I and Xba I. Digested fragments were resolved on an agarose gel and the fragments excised from the gel and purified using the

Figure 2.1: Overview of the cloning strategy for the construction of adenoviral shuttle plasmids encoding myc-tagged CaMKIIδ2 (wild type, dominant negative and constitutively active)



kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The fragments were ligated into pAdTrack-CMV and transformed with DH5α competent cells as described in section 2.4.4. Minipreps of plasmid DNA were prepared and checked by restriction digest. The resulting plasmid, pAdTrack-myc-CaMKIV^{CA} was sequenced using the T7 and SP6 universal primers and found to be correct. An overview of the cloning strategy is provided in **Figure 2.2**.

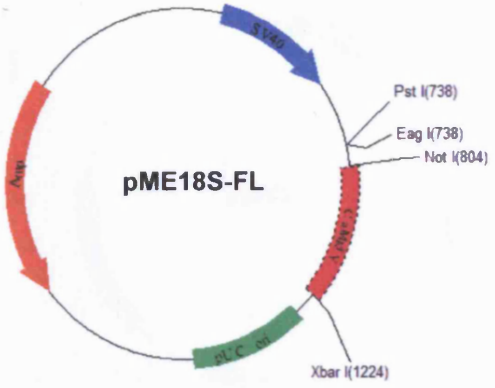
2.4.17.3 Site-Directed Mutagenesis of Mammalian Expression Vectors Encoding HA-Tagged Rap1N17

The expression vector pMT2Ha-Rap1N17 was provided by Dr. J. L. Bos, University Medical Centre, Utrecht, Netherlands. Rap1N17 has been shown not to act as a true dominant negative (Seidel, Klinger et al. 1999). However, a mutation of the serine at position 17 to alanine has been shown to be an effective dominant negative (Dupuy, L'Hoste et al. 2005). Site directed mutagenesis was used to mutate residue 17 of Rap1N17 to an alanine. The primers used for this reaction were HA-Rap1aA17For and HA-Rap1aA17Rev. Site directed mutagenesis was performed as detailed in section 2.4.15. Sequence analysis using the primers HA-Rap1aN17For and HA-Rap1aN17Rev confirmed that the mutated construct was correct. This plasmid was named pMT2Ha-RapA17.

The expression vector encoding pMT2Ha-Rap1A17 sequence was amplified by PCR using HA-Rap1aN17For and HA-Rap1aN17Rev primers. The PCR products were resolved by TAE agarose gel electrophoresis and bands of 585bp were excised from the gel and purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The resulting DNA and pAdTrack-CMV adenoviral shuttle plasmid were digested with EcoRV and Hind III. Digested fragments were resolved on an agarose gel and the fragments excised from the gel and purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The fragments were ligated into pAdTrack-CMV and transformed with DH5α competent cells as described in section 2.4.4. Minipreps of plasmid DNA were prepared and checked by restriction digest. The resulting plasmid, pAdTrack-Ha-RapA17, was sequenced with T7 and SP6 universal primers and found to be correct. An overview of the cloning strategy is provided in **Figure 2.3**.

Figure 2.2: Overview of the cloning strategy for the construction of adenoviral shuttle plasmids encoding myc-tagged CaMKIV constitutively active

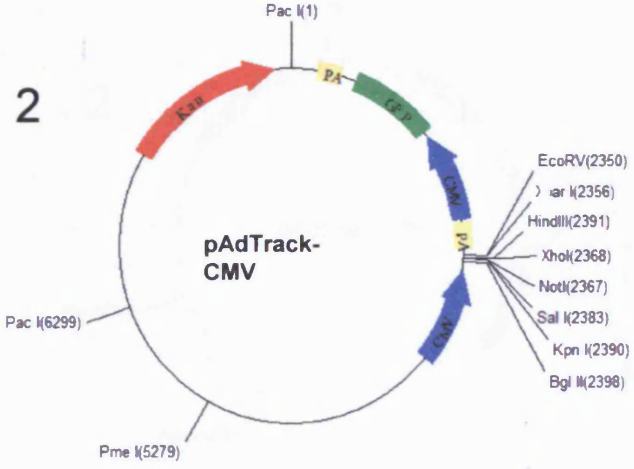
1



PCR product

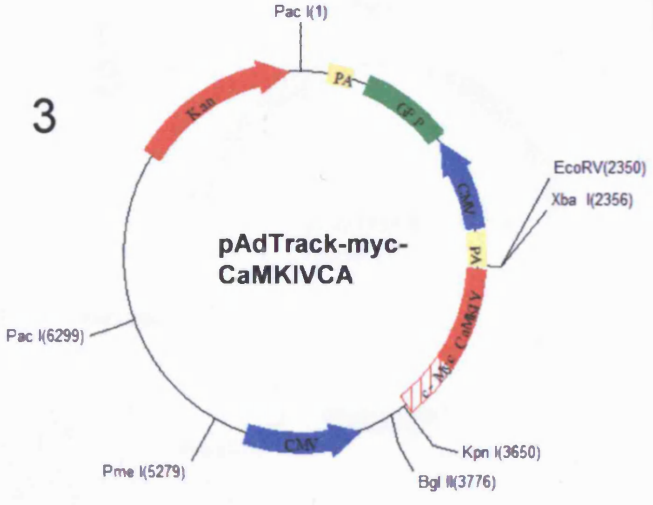
A N-terminal myc epitope tag was added to full-length constitutively active CaMKIV by PCR using the primers Myc-CaMKIVFor and Myc-CaMKIVRev

2



The resulting DNA and pAdTrack-CMV adenoviral shuttle plasmid were digested with Kpn I and Xba I

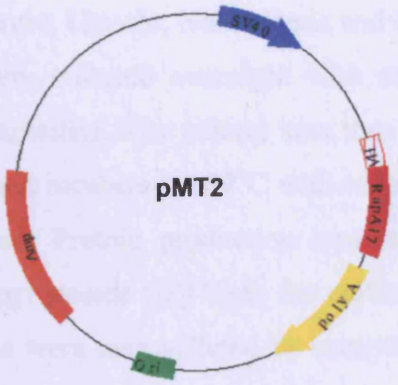
3



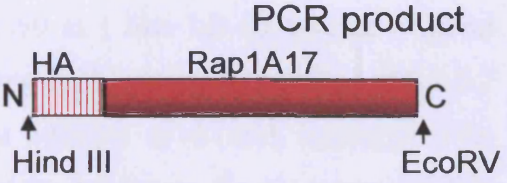
The fragments were ligated into pAdTrack-CMV. The resulting plasmid pAdTrack-myc-CaMKIVCA were sequenced using T7 and SP6 universal primers.

Figure 2.3: Overview of the cloning strategy for the construction of adenoviral shuttle plasmid HA-Rap1A17

1

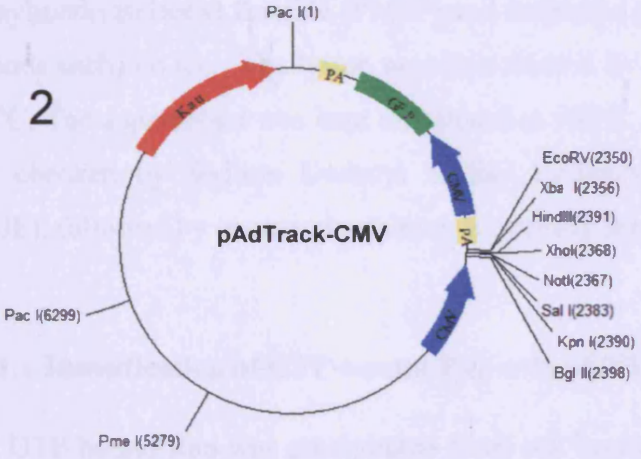


Site directed mutagenesis was used to mutate residue 17 of RapN17 to an alanine. The primers used for this reaction were HA-Rap1aA17For and HA-Rap1aA17Rev.



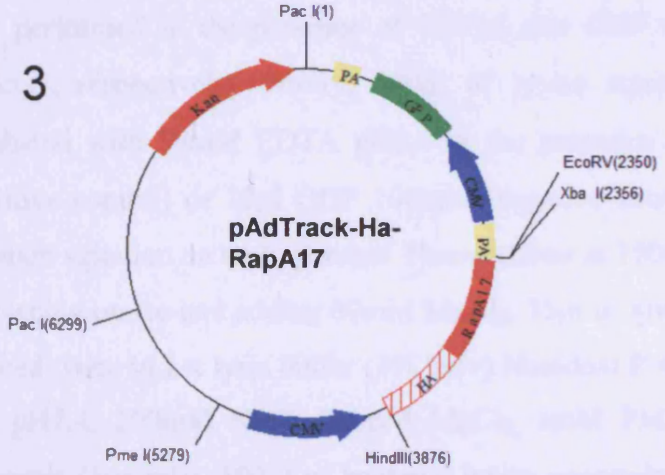
The expression vector encoding pMT2Ha-RapA17 was amplified by PCR using HA-Rap1aN17For and HA-Rap1aN17Rev primers.

2



The resulting DNA and pAdTrack-CMV adenoviral shuttle plasmid was digested with EcoRV and Hind III.

3



The DNA fragment was ligated into pAdTrack-CMV. The resulting plasmid, pAdTrack-Ha-RapA17, was sequenced with T7 and SP6 universal primers and found to be correct.

2.5 Protein Expression

2.5.1 Expression of GST-tagged RalGDS-RBD

The GST-tagged RalGDS-RBD construct was obtained from Dr. J. L. Bos, University Medical Centre, Utrecht, Netherlands and transformed into *Escherichia coli* (strain BL21). Bacteria were cultured overnight with constant shaking at 225rpm in the presence of 75μg/ml ampicillin. The culture was then diluted 1:50 in 1 litre LB containing 75μg/ml ampicillin and incubated at 37°C with constant shaking at 225rpm until an OD_{600} of 0.6-0.7 was obtained. Protein production was initiated by addition of 0.1mM isopropyl β-D-thiogalactopyranoside (IPTG) to the culture followed by incubation for 2-3 hours at 37°C. The bacteria were then pelleted by centrifugation for 10 minutes at 7700 x *g* at 4°C. The pellet was then resuspended in 25ml ice cold lysis buffer (20% (w/v) sucrose, 10% (v/v) glycerol, 50mM Tris pH8.0, 2mM dithiothreitol (DTT), 2mM $MgCl_2$, 1mM phenylmethylsulfonyl fluoride (PMSF) and leupeptin (1μg/ml)) and sonicated 10 times (30 seconds each) on ice. The lysate was then cleared by centrifugation for 1 hour at 12,000g at 4°C. The supernatant was kept and stored at -80°C. The presence of GST fusion protein was checked by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), followed by coomassie staining to identify the protein (see section 2.7.6).

2.5.1.1 Identification of GTP-bound Rap using GST-Tagged RalGDS-RBD

The GTP-bound Rap was precipitated from cell lysate using the activation specific probe (GST-tagged RalGDS-RBD) pre-coupled to glutathione beads. Pull-downs for Rap were also performed in the presence of GTPγS and GDP to serve as positive and negative controls, respectively. Briefly, 100μl of lysate equivalent to 100μg of protein was incubated with 10mM EDTA pH8.0 in the presence of either 10μl of GTPγS 0.1mM (positive control) or 10μl GDP 100mM (negative control) at 30°C for 30 minutes with constant agitation on an Eppendorf Thermomixer at 1000rpm. The reaction was terminated by placing on ice and adding 60mM $MgCl_2$. 75μl of glutathione beads (bed volume) were washed twice in 1 x lysis buffer (1% 9v/v) Nonident P-40, 10% (v/v) glycerol, 50mM Tris Hcl pH7.4, 200mM NaCl, 2.5mM $MgCl_2$, 1mM PMSF, 2mM sodium orthovavadate, leupeptin (1μg/ml)). 100μl of bacterial lysate expressing GST-RalGDS was added to the glutathione beads for 60 minutes at 4°C on a rotator. The beads were then washed with 1ml of 1 x lysis buffer four times. The beads were then combined with 100μl cell lysates

equilvant to 200μg of protein and incubated for 45 minutes at 4°C on a rotator. The lysate/glutathione beads were washed four times in 1 ml of 1 x lysis buffer and resuspended in 20μl each of 2X Laemmli's sample buffer. Samples were boiled for 3 minutes, and then loaded straight on to a 12.5% SDS-PAGE gel, followed by western blotting with an anti-Rap1 antibody (BD Biosciences).

2.5.2 Expression of GST-MEK

The GST-MEK construct was transformed into *Escherichia coli* (strain Rosetta BL21). Bacteria were cultured overnight in the presence of 100μg/ml ampicillin at 37°C with constant shaking at 225rpm. The culture was then diluted 1:10 in 1 litre LB containing 100μg/ml ampicillin and incubated at 37°C with constant shaking at 225rpm until an OD_{600} of 0.9 was obtained. Cells were then cooled on ice for 15 minutes before protein production was initiated by addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG) to the culture followed by incubation overnight at 25°C. The bacteria were then pelleted by centrifugation for 15 minutes at 5,000 x *g* at 4°C. The cells were then resuspended in at 2–5 ml of lysis buffer per gram wet weight (5% glycerol (v/v), 1M KCl, 20mM Tris Hcl pH7.5, 3mM $MgCl_2$, 5mM β-mercaptoethanol, 20mM Imidazol pH7.9, 5mM NaF, 1mM PMSF, leupeptin (1μg/ml), pepstatin (1μg/ml), 0.1% triton). At low concentrations of imidazole (20mM) was added to the lysis buffer in order to minimize binding of untagged contaminating proteins. The lysate was then incubated on ice in the presence of 1mg/ml lysozyme for 30 minutes, and then sonicated 6 times (10 seconds pulse each). The lysate is then centrifuged 16000 x *g* for 15 minutes at 4°C. The fusion protein was affinity purified on a His GraviTrap column (GE Healthcare) according to manufactures instructions. Briefly, the column was equilibrated with 10ml of binding buffer (20mM sodium phosphate, 500mM NaCl, 20mM imidazole pH7.9). The sample was then applied to the column and allowed to pass via gravity flow. The column was then washed with 10ml binding buffer and the purified proteins eluted by the addition of 2 x 3ml elution buffer (20mM sodium phosphate, 500mM NaCl, 500mM imidazole pH7.9). 1ml fractions were collected and run on SDS PAGE to determine protein expression. The fractions containing the protein were determined, pooled together and dialysed in order to remove excess salt and achieve a higher protein concentration. Briefly, pooled fractions were injected into a dialysis cassette 0.5-3ml capacity, 3.5K molecular weight cut off (Pierce) and dialysed 2 hours at 4°C in 1.5 litres of dialysis buffer (5% glycerol (v/v), 100mM KCl, 20mM Tris HCl pH7.5, 5mM β-mercaptoethanol, 5mM NaF, 1mM PMSF, leupeptin (1μg/ml),

pepstatin (1μg/ml)) followed by a further overnight incubation at 4°C in 1.5ml of fresh dialysis buffer. Once the sample was removed from the cassette, the total protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as standard (see section 2.7.4). The sample was then aliquoted and stored at -80°C until required.

2.5.3 Expression of GST-rpS6

The GST-rpS6 construct was transformed into *Escherichia coli* (strain Rosetta BL21). Bacteria were cultured overnight in the presence of 100μg/ml ampicillin at 37°C with constant shaking at 225rpm. The culture was then diluted 1:10 in 1 litre LB containing 100μg/ml ampicillin and incubated at 37°C with constant shaking at 225rpm until an OD_{600} of 0.9 was obtained. Cells were then cooled on ice for 15 minutes before protein production was initiated by addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG) to the culture followed by incubation for 3h at 37°C. The bacteria were then pelleted by centrifugation for 15 minutes at 5,000 x *g* at 4°C. The cells were then resuspended in at 2–5 ml of lysis buffer per gram wet weight (5% glycerol (v/v), 1M KCl, 20mM Tris HCl pH8.0, 3mM $MgCl_2$, 5mM β-mercaptoethanol, 5mM NaF, 1mM PMSF, leupeptin (1μg/ml), pepstatin (1μg/ml), 0.1% triton). The lysate was then incubated on ice in the presence of 1mg/ml lysozyme for 30 minutes, and then sonicated 6 times (10 seconds pulse each). The lysate is then centrifuged 16000 x *g* for 15 minutes at 4°C. The fusion protein was affinity purified using glutathione sepharose beads (Amersham) according to manufacturer's instructions. Briefly, an appropriate volume of resin (use 1-2mls for a normal expression of up to 10 mg of protein) was dispensed into a 50ml tube and wash three times with PBS. The sample was then applied to the resin and incubated for one hour at 4°C on a rotator, to allow binding of the GST protein to the GST-Sepharose. The protein was eluted with 1.5ml of Elution Buffer (lysis buffer containing 20mM reduced glutathione), adding and collecting 0.5ml at a time. The fractions containing the protein were determined, pooled together and dialysed in order to remove excess salt and achieve a higher protein concentration. Briefly, pooled fractions were injected into a dialysis cassette 0.5-3ml capacity, 3.5K molecular weight cut off (Pierce) and dialysed 2 hours at 4°C in 1.5 litres of dialysis buffer (5% glycerol (v/v), 100mM KCl, 20mM Tris HCl pH7.5, 5mM β-mercaptoethanol, 5mM NaF, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin) followed by a further overnight incubation at 4°C in 1.5ml of fresh dialysis buffer. Once the sample was removed from the cassette, the total protein concentration was determined by the

Bradford assay (Bio-Rad) using bovine serum albumin as standard (see section 2.7.4). The sample was then aliquoted and stored at -80°C until required.

2.6 Recombinant Adenoviral Techniques

2.6.1 Production of Recombinant Adenoviruses

Recombinant adenoviruses were produced using the AdEasy system (He, Zhou et al. 1998). pAdTrack-CMV shuttle plasmids containing $CaMKII^{WT}$, $CaMKII^{DN}$, $CaMKII^{CA}$, $CaMKIV^{CA}$, RapGAPII, RapA17 were linearised by digestion with PmeI. The linearised plasmids were run out on 1% TAE agarose gels and gel bands excised under low UV. The DNA was then purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8, and 100ng of the linearised plasmid was used to transform the pAdEasy-1 transformed BJ5183 cells by electroporation (see section 2.4.4.2). The BJ5183 cells then allowed recombination between the shuttle vector and the pAdEasy-1 adenoviral backbone vector. Transformed cells were plated on to LB-kanamycin plates and incubated overnight upside down at 37°C. This gave two types of colonies: small which represent potential recombinants, and large colonies which represent bacteria transformed with uncut shuttle plasmids. The smallest colonies were grown overnight at 37°C in 5ml LB supplemented with kanamycin and minipreps of the plasmids were prepared. Plasmids were then digested with PacI to screen for positives. Positive clones should yield fragments at ~ 30kb and either 3kb or 4.5kb. Positive plasmids were then used to transform chemically competent DH5α cells (see section 2.4.4.1) and minipreps of plasmids were prepared. The presence of insert DNA was further confirmed by restriction digestion, PCR analysis and protein expression in HEK-293 cells. 2.5-5μg of recombinant DNA was then used to transfect T25 flasks of HEK-293 cells (low passage) for amplification of the virus. Briefly, the plasmid DNA is digested with Pac-1 then purified using the kit Geneclean II kit (Q-Biogene) as described in section 2.4.8. The resulting DNA was then transfected of DNA by the calcium phosphate methodology (see section 2.4.4).

2.6.2 Harvesting Virus from HEK-293 cells

The viruses were ready for harvesting 7 to 10 days post-transfection of the recombinant adenoviral plasmids. Cells were washed off the flasks, transferred to 50ml conical tubes and pelleted at 1800 x *g* for 5 minutes at 4°C in an Eppendorf 5810R centrifuge. The

pellets were resuspended in 1ml PBS, frozen in a dry ice/ethanol bath then thawed in a 37°C water bath and vortexed. This freeze/thaw/vortex cycle was repeated 3 more times to fully lyse the cells. The samples were spun again at 3200 x *g* for 10 minutes at 4°C to pellet cell debris. The pellet was discarded and the resulting viral supernatant was stored at -80°C.

2.6.3 Generation of High Titre Adenovirus Stocks

90% of the adenoviral supernatant was used to re-infect two confluent T-25 flasks of HEK-293 cells. When GFP was present, transfections and viral productions were monitored by GFP expression on a Nikon fluorescence microscope fitted with a mercury lamp. Approximately 3-5 days post-infection, when 70-90% of the cells were floating, the cells were harvested and subjected to four cycles of freeze/thaw/vortex as described above. The virus supernatant from two T-25 flasks was used to infect two T-75 flasks. Supernatant from this round of amplification was then used to infect 5 T-75 flasks. Finally high titre virus was produced when the virus supernatant from 5 T-75 flasks was used to infect 20 T-75 flasks.

2.7 Protein Techniques

2.7.1 Buffers and Reagents

10X Tris-Glycine buffer (for 1l)
30g Tris base (Melford)
144g Glycine (Melford)
SDS-PAGE Running Buffer
1X Tris-Glycine buffer
0.1% w/v SDS

Semi-Dry Transfer Buffer
1X Tris-Glycine buffer
0.01% w/v SDS
20% v/v Methanol

10X PBS (Phosphate Buffered Saline; 1l)

3g KCl

100g NaCl

14g Na_2HPO_4

3g KH_2PO_4

pH to 7.4 and make up to 1l

PBS-Tween (PBST)

1X PBS

0.1% v/v Tween-20 (Sigma)

Laemmli Sample buffer (4X)

0.25M Tris pH 6.8

4% w/v SDS

40% v/v Glycerol

10% v/v β-mercaptoethanol

20μg/ml Bromophenol blue

Coomassie Blue Stain

45% v/v Methanol

10% v/v Acetic acid glacial

0.625g Coomassie Blue R (Sigma)

Filtered through Whatmann 3MM

Destain/Fixing Solution

50% v/v Methanol

10% v/v Acetic acid

MIN6 Lysis Buffer

1% v/v Triton X-100

10mM β-glycerophosphate pH 7.4

50mM Tris-HCl pH 7.5

1mM EDTA pH 8

1mM EGTA

1mM Sodium orthovanadate

1mM Benzamidine

0.2mM PMSF

1μg/ml Leupeptin

1μg/ml Pepstatin A

0.1% v/v β-mercaptoethanol

50mM NaF

2.7.2 Antibodies

Antibody	Obtained from:	Primary Dilution	Secondary antibody	Application
Anti-phospho ribosomal protein S6 (Ser^{235} Ser^{236})	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho ribosomal protein S6 (Ser^{240} Ser^{244})	New England Biolabs	1/1000	Rabbit	WB
Anti-ribosomal protein S6 (New England Biolabs)	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho Akt/PKB (Ser^{473})	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho Akt/PKB (Thr^{308})	New England Biolabs	1/1000	Rabbit	WB
Anti-Akt/PKB	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho (Thr^{172}) AMPK antibody	New England Biolabs	1/1000	Rabbit	WB
Anti-AMPK antibody	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho p44 p42 (Thr^{202} Tyr^{204})	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho MEK	New England Biolabs	1/1000	Rabbit	WB
Anti-p70S6 Kinase	Provided by Ann Willis, University of Nottingham	1/1000	Rabbit	WB
Anti-S6K2	Provided by I Gout, Ludwig Institute for Cancer Research	1/100	Rabbit	IP
Anti-4EBP1	Provided by Ann Willis, University of Nottingham	1/1000	Rabbit	WB
Anti-Rap1	BD biosource	1/1000	Mouse	WB
Anti-Erk2 antisera	New England Biolabs	1/1000	Rabbit	WB
Monoclonal anti-*c-myc* clone 9E10 antibody	Sigma	1/1000	Mouse	WB
Anti-GFP antibody for use in western blotting	Cambridge BioScience	1/1000	Rabbit	WB
Anti-Cot / Tpl2	Santa Cruz	1/200	Rabbit	WB
Anti-HA	Provided by Andy Tee, Cardiff University	1/1000	Mouse	WB
Anti-phospho p70S6Kinase (Thr^{389})	New England Biolabs	1/1000	Rabbit	WB
Anti-phospho-p70S6Kinase (Thr^{229})	R & D Systems	1/1000	Rabbit	WB
Anti-B Raf	Provided by Dr Catrin Pritchard, University of Leicester	1/1000	Mouse	WB
Anti-C Raf	Provided by Dr Catrin Pritchard, University of Leicester	1/1000	Mouse	WB
Anti-A Raf	Provided by Dr Catrin Pritchard, University of Leicester	1/1000	Rabbit	WB

Anti-Tpl2 (70mer)	Provided by Dr Steve Ley, National Institute for Medical Research, London	1/100		IP
Anti-B Raf	Provided by Dr Catrin Pritchard, University of Leicester	1/100	Mouse	IP
Anti-HA	Provided by Andy Tee, Cardiff University	1/100	Mouse	IP
HRP-coupled anti-Rabbit	New England Biolabs	1/3000	-	WB
HRP-coupled anti-Mouse	New England Biolabs	1/3000	-	WB

WB = Western Blotting IP = Immunoprecipitation

2.7.3 Sample Preparation

Following experimentation, cells were scraped off of 4cm or 6cm diameter plates in ~ 100µl or 200µl respectively of ice cold triton lysis buffer and transferred to a clean microfuge tube. The lysate was vortexed for 10 seconds, and centrifuged at 16000 x *g* at 4°C for 10 minutes. The post nuclear supernatant was removed and transferred to a fresh microfuge tube. Cell lysates were then stored at -80°C until required.

2.7.4 Bradford Assay

The Bradford protein assay was used to determine protein content of cell lysates. Bradford reagent (Bio-Rad) was diluted with distilled water 1/5 for use. 2µl of protein samples of unknown protein content was mixed with 1ml of diluted Bradford reagent and incubated for 5 minutes at room temperature. The A_{595} of the reaction mixture was measured on a WPA spectrophotometer, and the protein content of unknowns was determined by linear regression against a standard curve of BSA protein standards (0 to 20µg). Protein contents of individual lysates were adjusted to the sample with the lowest protein content using the appropriate lysis buffer used in the experiment.

2.7.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.7.5.1 Gel Formulation

The running gel is poured between glass plates and then immediately overlaid with distilled water. When the running gel has set (~ 30 minutes) the water is removed and the stacking gel poured on top. The comb is inserted immediately after and the gel left to set for ~ 30 minutes.

SDS-PAGE (Enough for two ATTO system running gels and 4 stacking gels)

Acrylamide: Bis acrylamide ratio of 37.5:1

SOLUTION	7.5%	10%	12.5%	15%	17.5%	20%	STACKING
40% Acrylamide (BDH)	2.925ml	3.825ml	4.725ml	5.625ml	6.525ml	7.275ml	1.24ml
2% Bis-Acrylamide[1]	1.56ml	2.04ml	2.52ml	3ml	3.48ml	3.75ml	0.65ml
1.5M Tris-HCl, pH 8.8	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	-
1M Tris-HCl, pH 6.8	-	-	-	-	-	-	1.25ml
H_2O	6.54ml	5.165ml	3.78ml	2.4ml	1.02ml	0ml	6.7ml
10% SDS	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.1ml
TEMED[2]	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	10µl
10%APS[3] (Add just before pouring)	75µl	75µl	75µl	75µl	75µl	75µl	75µl

1: N,N'-methylene bis-acrylamide (BDH/Bio-Rad)

2: NNN'N' tetramethylethylenediamine

3: Ammonium persulphate

2.7.5.2 Running of Gels

SDS-PAGE gels were immersed in SDS-PAGE running buffer in ATTO system gel tanks. Protein samples prepared in section X are added to Laemmli sample buffer (4X) to give a 1X final. Samples were then vortexed and centrifuged at 16000 x *g* at 4°C for 1 minute. Protein samples were boiled for 3 minutes, then centrifuged briefly at full-speed and loaded alongside prestained protein markers (broad range; NEB). SDS-PAGE gels were run vertically at 180 volts for ~ 90 minutes until the bromophenol blue just ran off the edge of the gel.

2.7.6 Coomassie Staining of Proteins on SDS-PAGE Gels

SDS-PAGE gels were immersed in Coomassie blue stain and shaken for 30 minutes. After staining, the Coomassie solution was discarded, and gels placed into destain/fixing solution. The gels were shaken until the destain solution was blue. The destain solution was discarded and replaced with fresh solution. This step was repeated until stained

proteins could be visualised easily. Gels were then placed on to Whatman 3MM paper, covered with Saran wrap and then dried on a Scie-Plas vacuum gel dryer.

2.7.7 Autoradiography of Radiolabelled Proteins

Following resolution of protein samples on 12.5% SDS-PAGE gels, gels were immersed in destain/fixing solution and shaken for 15 minutes. The fixative was removed and the fixing step repeated. [^{32}P]-labelled protein gels were dried immediately after destaining as described in section 2.7.6 and exposed to X-ray film at either -80°C or room temperature for as long as required.

2.7.8 Western Blotting

Protein samples were run on SDS-PAGE gels as described in section 2.7.5. Proteins were transferred on to Immobilon-P PVDF filters (Millipore) using a Semi-Dry Transfer cell (Bio-Rad). Briefly, membranes were soaked in 100% methanol to wet them. 6 Whatman 3MM papers per gel, the membrane and the gel were then equlibrated in semi-dry transfer buffer for 5 minutes. 3 Whatman papers were placed on the cell and air bubbles removed. The membrane was then layered onto these papers. The gel was placed on top of the membrane, and 3 more Whatman papers placed on top. Any air bubbles were removed and excess transfer buffer removed. The transfer was then performed at 15 volts for 38 minutes. Transfer efficiency was assessed by transfer of the prestained protein markers.

Following transfer, the membrane was blocked in 5% milk PBST/TBST (depending on which buffer the primary antibody was diluted in) for one hour at room temperature. Where antibodies were diluted in 5% PBST/TBST milk the blocking step was followed by an overnight incubation at 4°C in the specific antibody. If primary antibodies were diluted in 5% BSA PBST/TBST, the membrane was washed 3 times for 5 minutes in PBST/TBST prior to incubation. Antibody concentrations were adjusted according to manufacturer's instructions. The following day, the membrane was washed three times for 5 minutes in PBST/TBST. The membrane was then incubated in the appropriate secondary antibody (secondary antibodies from NEB was diluted 1:3000 and made in 5% milk PBST/TBST) for 1 hour at room temperature. The membrane was then washed three times for 10 minutes with PBST/TBST. Detection of proteins was performed by enhanced chemiluminescence (ECL; Amersham Biosciences). Expose membrane to X-ray film

(GRI) for 1 minute to 1 hour, depending on protein signal and chemiluminesence method. Proteins of interest were identified by comparison of their size to broad range protein markers.

2.7.9 Immunoprecipitation

15µl of Protein-G Sepharose beads per sample (bed volume) were washed twice in 500µl of MIN6 lysis buffer, centrifuged at 800 x *g* for 1 minute and the supernatant discarded. The total volume of Protein-G Sepharose beads was resuspended in 100µl of MIN6 lysis buffer per sample and incubated with the relevant antibody for one hour at room temperature on a rotator, to allow binding of the antibody to the Protein-G Sepharose beads. The beads were then centrifuged at 400 x *g* and the supernatant containing any unbound antibody were discarded and resupended in MIN6 lysis buffer. 100µl of the beads were then added to a volume of the sample lysate equivalent to 100µg of protein. Immunoprecipitations were then performed for 2 hours at 4°C on a rotator. After incubation, the Protein-G Sepharose beads were centrifuged at 2000rpm for two minutes. The supernatants were removed and stored at -80°C. The Protein-G Sepharose beads were then washed 3 times in 1ml of MIN6 lysis buffer, centrifuged at 800 x *g* for one minute and resuspended in 25µl each of 2X Laemmli's sample buffer. Samples were boiled for 3 minutes, then loaded straight on to SDS-PAGE gels, or were stored at -80°C until required.

2.7.10 Fractionation on Resource S (Cation-exchange) or Resource Q (anion exchange) Column.

Prior to fractionation, cells were plated out on 10cm diameter plates. Prior to treatment, the medium was removed and the cells washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (115mM NaCl, 5mM KCl, 10mM $NaHCO_3$, 2.5mM $MgCl_2$, 2.5mM $CaCl_2$, 20mM HEPES, pH 7.4) containing 0.5% bovine serum albumin (KRB buffer). The cells were then incubated for 1 h at 37 °C in KRB buffer containing 1mM glucose prior to incubation in KRB buffer containing 1mM (control) or 16.7mM glucose plus 10nM GLP-1(stimulated). Treatments were stopped by the addition of 1ml fresh ice-cold lysis buffer containing 1% Triton, 10mM β-glycerophosphate, 50mM Tris-HCl, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 1mM benzamidine HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol, and 50mM sodium fluoride. The lysates were then centrifuged for 10 min at 16,000 × *g* at 4°C. The supernatants were kept, and total protein concentrations were

determined by the Bradford assay (Bio-Rad) using bovine serum albumin as standard. The columns were prepared by washing with two column volumes of start buffer (20mM Tris-HCl, pH7.4). The columns were then equilibrated with five column volumes of start buffer. 3mg of protein was applied to a 1ml Resource S (Amersham Biosciences) cation-exchange column or a 1ml Resource Q (Amersham Biosciences) anion-exchange column. The columns were then washed with 3 column volumes of TE buffer pH 7.4 containing 1mM DTT. The samples were eluted with a linear gradient of 0–1M NaCl at a flow rate of 0.5 ml/min in a total volume of 10ml. Fractions 0.5-ml were collected and protease inhibitors (1mM benzamidine HCl, 0.2mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin) added directly to each fraction to prevent protein degradation. Each fraction was then assayed for kinase activity.

2.7.10.1 Fractionation on Heparin-Sepharose

For fractionation on a heparin-Sepharose column (1ml) (Amersham Biosciences). The columns were prepared by washing with two column volumes of start buffer (20mM Tris-HCl, pH 7.4). The columns were then equilibrated with five column volumes of start buffer. 3mg of protein lysate was applied to the column. The column was washed with 2 column volumes of TE buffer pH 7.4 containing 1mM DTT and eluted with a linear gradient of 0–2M NaCl at a flow rate of 1ml/min in a total volume of 10ml. Fractions of 1ml were collected and protease inhibitors added directly as before. Each fraction was then assayed for kinase activity.

2.7.11 MEK Kinase Assay

Following treatment, cells were lysed in MIN6 lysis buffer. MEK kinase assays were performed using total cell lysate or fractions collected from resource S, Q or heparin columns. 20μg of total lysate or 14μl of each fraction were incubated in a total volume of 20μl containing 20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM ATP and ~ 4μg of recombinant GST-MEK. 1μCi [γ-^{32}P]-ATP was added and the reaction was incubated immediately at 30°C for 60 minutes in an Eppendorf Thermomixer at 1000rpm. The kinase reactions were stopped by addition of 6.6μl of 4X Laemmli sample buffer and the samples boiled 3min at 100 °C. The proteins were separated on a 12.5% SDS-PAGE gel. The gel was fixed and dried as described in section 2.7.6 and the phosphorylation state of GST-MEK was revealed by autoradiography.

2.7.12 Tpl2 MEK Kinase Assay

To assay Tpl2 MEK kinase activity, RAW264.7 were used as a positive controls for this assay. Details of the MIN6 cell treatments are provided in the figure legend. RAW264.7 cells were plated in 6cm diameter dishes and cultured overnight in RPMI containing, 10% FCS 100μg/ml streptomycin and 100units/ml penicillin sulphate at 37°C/5% CO_2. RAW264.7 cells were then stimulated with Lipopolysaccharide (LPS) (1μg/ml) for 15 minutes. Cells were washed once in PBS prior to lysis in kinase lysis buffer containing 0.5% NP-40, 5mM Na β-glycerophosphate, and 0.1% β-mercaptoethanol, 50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 50mM NaF, 1mM Na_3VO_4, 100nM okadaic acid (Sigma), 2mM $Na_4P_2O_7$ plus a protease inhibitors cocktail (sigma). Lysates were immunoprecipitated overnight at 4°C with anti-TPL-2 (70mer) antibody coupled to protein A-Sepharose. Specific antibody-coupled beads were washed once in kinase lysis buffer and twice with kinase buffer (50mM Tris pH 7.5, 5mM β-glycerophosphate, 0.1mM sodium vanadate, 100nM okadaic acid, 10mM $MgCl_2$, 0.1mM EGTA, 0.03% Brj35, 0.1% β-mercaptoethanol). The beads were resuspended in 25 μl of kinase buffer supplemented with 1mM ATP, 6.5μg of GST-MEK/ml, and 100μg of GST-ERK/ml and incubated for 30 min at room temperature on an Eppendorf Thermomixer at 1000rpm. After the beads were pelleted by centrifugation, 5μl of the supernatant was added to 45μl of kinase buffer containing 0.33 mg/ml of myelin basic protein (MBP) (Sigma), 0.1mM ATP, and 2.5μCi of [γ-^{32}P]ATP (Amersham Biosciences) and incubated at room temperature for 10 min. The assay was terminated by adding 16μl of 4X Laemmli sample buffer. The proteins were separated on a 12.5% SDS-PAGE gel. The gel was fixed and dried as described in section 2.7.6 and the phosphorylation state of MBP was revealed by autoradiography.

2.7.13 B-Raf Kinase Assay

Details of the MIN6 cell treatments are provided in the figure legend. Protein lysates were prepared as described by Marais *et al* (Marais, Light et al. 1997). Briefly, B-Raf protein was immunoprecipitated for 2 h at 4 °C from 100 μg of cell lysates using 4 μg of anti-B-raf rabbit polyclonal antibody coupled to Protein G-Sepharose. The beads were subjected to one wash of 500μl wash buffer (30mM Tris·HCl, 0.2mM EDTA, 0.3% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5mM NaF, 0.2mM Na_3VO_4, pH7.5) containing 1.0M KCl, one wash with 500 μl wash buffer containing 0.1M KCl, and one wash with 500μl wash buffer. The beads were resuspended in a total volume

of 20µl containing 20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM ATP and ~ 4µg of recombinant GST-MEK. To start the reaction 1µCi [γ-^{32}P]-ATP was added and the reaction was incubated immediately at 30°C for 60 minutes on an Eppendorf Thermomixer at 1000rpm. The kinase reactions were stopped by addition of 6.6µl of 4X Laemmli sample buffer and boiled 3min at 100 °C. The proteins were separated on a 12.5% SDS-polyacrylamide gel. The gel was fixed and dried as described previously in section 2.7.6 and the phosphorylation state of GST-MEK was revealed by autoradiography.

2.7.14 cAMP Assay

MIN6 cells were plated out in 24 well plates. Cells were washed twice and finally incubated in 450µl Krebs-Ringer bicarbonate buffer (115mM NaCl, 5mM KCl, 10mM $NaHCO_3$, 2.5mM $MgCl_2$, 2.5mM $CaCl_2$, 20mM HEPES, pH 7.4) containing 0.5% bovine serum albumin (KRB buffer) supplemented with 1mM glucose and 1mM IBMX. Cells were incubated at 37°C for 10 min before the addition of 50µl of agonist (details in figure legend) or buffer for 10min. Reactions were terminated by aspiration of buffer and addition of 400µl ice-cold 0.5M trichloroacetic acid. Samples were collected and mixed with 50µl 10mM EDTA and 500µl of a mixture of 1,1,2-trichlorofluoroethane and tri-n-octylamine (1:1 v/v), vortexed and left for 15 min at room temperature. Samples were centrifuged at 16,000 x *g* for 4 min and 200µl of the upper phase was removed and transferred to fresh tubes containing 60mM NaHCO3. Amounts of cAMP were determined using a binding assay previously described by Brown *et al* (1971). Standard concentrations of cAMP (2.5 - 200nM) were diluted in blank buffer (0.5M TCA extracted as described above for samples). Fifty microlitres of these standards or samples were added to fresh tubes containing 100µl [^{3}H]-cAMP (4nM). Reactions were started by the addition of 150µl cAMP binding protein extracted from bovine adrenal glands (see Brown et al., 1971) and samples were incubated on ice for 90 min. Reactions were terminated by the addition of 250µl ice-cold charcoal (0.25g/ml) and BSA (0.1g/ml) diluted in assay buffer (50mM Tris-HCl, 4mM EDTA, pH 7.5). Samples were incubated on ice for 12 min, centrifuged at 16,000g for 4 min at 4°C and then 400µl of supernatant was transferred to scintillation vials. Samples were mixed with 4.2ml Safe Fluor scintillation cocktail and counted by liquid scintillation spectrometry.

2.7.15 Insulin Secretion Assay

Insulin secretion was measured in islets. Briefly, islets were pre-incubating islets in KRB supplemented with 1mM glucose for 1hour. Islets were incubated with KRB containing 20mM glucose or 20mM glucose plus 10nM GLP-1 for 60 minutes at 37°C. Media were collected and centrifuged at 2300 x *g* for 10 min at 4°C. The supernatant was stored at -20°C until assaying the insulin content by an insulin ELISA kit (DRG Instruments GmbH, Germany) with rat insulin as a standard in accordance with the manufacturer's instructions. Briefly, 50µl of either the standards or samples were dispensed into the strips of a 96 well plate. 50µl of Anti-insulin-HRP conjugate was then added into all the wells. The plate was then incubated for 2 hours at 30°C on a horizontal shaker set at 700± 100rpm. The plate was then washed 6 times with wash buffer then 200µl of the freshly prepared revelation solution into each well and the plate was incubated for 15 min on a horizontal shaker set at 700 ±100 rpm at room temperature. The reaction was terminated by dispensing 50µl of stopping reagent into each well. The absorbance was read at 450nM on a Novostar plate reader (BMG Labtech).

2.7.16 PKB Kinase Assay

Recombinant adenovirus Ad-WT-HA-PKB containing WT-PKB (a gift Dr. C. Sutherland) (Kotani, Ogawa et al. 1999) was amplified as described in section 2.5.3. MIN6 cells were infected with recombinant adenovirus expressing WT-HA-PKB. 48hours post infection MIN6 cells were treated as described in the figure legend. Protein lysates were prepared as described in section 2.6.3. PKB protein were immunoprecipitated for 2 h at 4 °C with a monoclonal HA antibody coupled to protein G-Sepharose (Sigma). The beads were then centrifuged at 400 x *g* and the supernatant was removed. The immunocomplexes were washed three times with 1ml of Buffer A containing 0.5M NaCl (50mM Tris-HCl pH 7.5, 0.1% Triton X-100, 1mM EDTA, 1mM EGTA, 50mM sodium fluoride, 10mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 1mM activated sodium orthovanadate, 0.1% (v/v) β-mercaptoethanol) then two times with 1ml of Buffer B (50mM Tris-HCl, pH 7.5, 0.03% (w/v) Brij-35, 0.1mM EGTA and 0.1% 2-mercaptoethanol) and once with 1ml of ADB (assay dilution buffer) (20mM MOPS, pH7.2, 25mM β-glycerolphosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol). The immunocomplexes were resuspended and incubated in a total volume of 50µl containing 10µl of ice cold ADB, 10µM PKI (PKA-specific inhibitor peptide) to inhibit any cAMP-dependent Protein

Kinases that may non-specifically bind to the complex, 10µl of Crosstide substrate peptide (GRPRTSSFAEG) (~30mM). The reaction was started by adding 10µl of diluted [γ-32P] ATP stock solution (A cocktail of 500µM ATP and 75mM magnesium chloride in ADB to a final concentration = 1µCi/µl). The reactions were incubated for 10 minutes at 30°C with continuous shaking on an Eppendorf Thermomixer at 1000rpm. The immunocomplexes were centrifuged 2000rpm for 30 seconds and 25µl of the supernatant was spotted onto the center of a 2cm x 2cm P81 phosphocellulose paper to terminate the reaction. The assay squares were washed three times with 0.75% phosphoric acid for five minutes per wash at room temperature followed by one wash in acetone. The squares were then transferred into vials and 3ml of scintillation cocktail added and counted by liquid scintillation spectrometry.

2.7.17 CaMKII Kinase Assay

MIN6 cells were infected with recombinant adenovirus expressing Ad-WT-CaMKII, Ad-DN-CaMKII and Ad-CA-CaMKII as described in section 2.2.5. 48hours post infection MIN6 cells were treated as described in the figure legend. MIN6 cells were lysed in 4cm diameter dishes with 300µl of lysis buffer (50mM MOPS, pH8.6, 100mM $Na_4P_2O_7$, 100mM NaF, 250mM NaCl, 2mM Na_3VO_4, 3mM EGTA, and 1% NP-40). Protein lysates were scraped and centrifuged at 16,000 x g for 10 min at 4°C. The Protein content in the lysates was determined using the Bio-Rad Protein assay as described previously in section 2.6.4. Total CaMKII activity was assayed in the presence of saturating Ca^{2+} and 400nM calmodulin (Upstate) and autonomous CaMKII activity was assayed without added activators. The assay was performed using autocamtide II, a specific peptide substrate. The total CaM kinase II activity in the lysate was assayed in 25µl containing 10mM MOPS (pH 7.4), 10mM magnesium chloride, 3mM EGTA, 4mM calcium chloride, 400nM calmodulin, 0.2mM [γ-32P] ATP (400–1000cpm/pmol), 20mM autocamtide-2 (KKALRRQETVDAL) as substrate (Hanson, Kapiloff et al. 1989), and 0.5mg of lysate protein. To determine the Ca^{2+}/CaM-independent (autonomous) activity in the same lysates $CaCl_2$ and calmodulin were omitted from the kinase assay mixture. The reactions were carried out at 30°C on an Eppendorf Thermomixer at 1000rpm for 3 min. The reactions were centrifuged 2000rpm for 30 seconds and 25µl of the supernatant was spotted onto the center of a 2cm x 2cm on Whatman P81 phosphocellulose paper to terminate the reaction. The assay squares were washed three times with 0.75% phosphoric acid for five minutes

per wash at room temperature followed by one wash in acetone. The squares were then transferred into vials and 3ml of scintillation cocktail added and counted by liquid scintillation spectrometry. Autonomous (Ca^{2+}/CaM independent) kinase activity was expressed as a percent of total Ca^{2+}/CaM dependent activity from the same samples.

2.7.18 PI3K Kinase Assay

PI3K kinase assay was assessed by a modification of the method from Hawkins *et al* (Hawkins, Welch et al. 1997). PI3K activity was measured in MIN6 cells as follows. On the day of the experiment, cells were washed twice with PBS containing 100nM Na_3VO_4 and lysed in PBS extraction buffer containing (50mM HEPES, 137mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM sodium pyrophosphate, 10mM NaF, 2mM EDTA, 2mM Na_3VO_4, 2mM PMSF, and 10mM benzamidine, 10% glycerol (v/v), 1% NP-40 (v/v), 10mg/ml aprotinin, and 10µg/m leupeptin). The protein content in the lysates was determined using the Bio-Rad Protein assay as described previously in section 2.7.4. An aliquot of each lysate (0.5-1mg protein) was incubated with 1.6µg anti-phosphotyrosine monoclonal antibody (Santa Cruz PY99) and 40ul of a 1:1 v/v suspension of packed beads/lysis buffer made from Protein A-Sepharose CL4B beads (Pharmacia; pre-equilibrated for 2h on ice in lysis buffer) and rotated, 2h, 0°C. Immunoprecipitates were washed successively in *1*) PBS containing 1% NP-40 and 100µM Na_3VO_4; *2*) 100mM Tris·HCl (pH 7.5), 500µM $LiCl_2$, and 100µM Na_3VO_4; and *3*) 100µM Tris·HCl (pH 7.5), 100µM NaCl, 1mM EDTA, and 100µM Na_3VO_4. PI3K activity associated with phosphotyrosine was measured by resuspending the immunoprecipitates in a solution of 10mM Tris·HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 15mM $MgCl_2$, 100 µM Na_3VO_4, 20mg phosphatidylinositol, 1mM EGTA, and 440µM ATP (containing [γ-^{32}P]ATP). The reaction mixtures were incubated with gentle agitation at 37°C for 5min before the addition of 450ul of Chloroform/Methanol (1:2 v/v) to stop the reaction. The organic phase was removed by centrifugation and applied to silica gel thin-layer chromatography using a developing solution of 42.9ml methanol, 30ml chloroform, 7.65ml ammonia (29.1%, 15.15M) and 9.45ml H_2O. The plates were then dried and the results were visualized by autoradiography.

2.7.19 S6K1 and S6K2 Kinase Assay

MIN6 cells were lysed in lysis buffer containing 50mM HEPES pH 7.5, 150mM NaCl, 1% (vol/vol) Nonidet P-40, 2mM EDTA, 50mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 50μg of leupeptin, 1mM phenylmethylsulfonyl fluoride, and 3mM benzamidine. Whole-cell extracts were centrifuged at 10,000*g* for 10min at 4°C, and endogeneous S6K1 or S6K2 were immunoprecipitated with the anti-S6K1 (NEB) or anti-S6K2 (Provided by Dr I Gout) antibody immobilized on protein G-Sepharose beads (Sigma). Immune complexes were washed three times with lysis buffer followed by a single wash with kinase assay buffer (50mM HEPES pH 7.5, 10mM $MgCl_2$, 1mM dithiothreitol, 10mM β-glycerophosphate. The kinase reaction was initiated by resuspending the beads in 25μl of kinase assay buffer supplemented with 1μM PKI (Calbiochem), 50μM ATP, 5μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech), and 6μg of GST-rpS6. The reaction was carried out at 30°C for 10min and terminated by the addition 4X Laemmli sample buffer and boiled 3min at 100 °C. Samples were subjected to SDS-12.5% PAGE, and the amount of ^{32}P incorporated into the rpS6 protein was assessed by autoradiography.

2.7.20 Quantification and Statistical Analysis

Immunoblot band intensities were quantified using the SynGene GeneGnome System with gene tools software. All data are given as mean ± SEM, and statistical analysis were performed using a One-way or Two-way ANOVA plus Bonferroni's post hoc test and regarded as significant if $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

Chapter 3: Regulation of Erk1/2 Phosphorylation by Glucagon-Like Peptide-1 in Pancreatic β-cells

3.1 Introduction

3.1.1 Mammalian MAP Kinase Pathway

In mammalian cells, three MAPKs have been characterized: the classical MAPK (also known as ERK), C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38 kinase. These MAP kinases lie within protein kinase cascades consisting of no fewer than three enzymes that are activated in series: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK) (**Figure 3.1**). MAPK pathways relay, amplify and integrate signals from a diverse range of stimuli and elicit an appropriate physiological response by transducing extracellular signals in to cellular responses. The JNK/SAPK pathway is activated in response to inflammatory cytokines and stresses such as DNA damage and oxidative stress (Force, Pombo et al. 1996; Hirai, Noda et al. 1998). The mammalian p38 MAPK family are activated by cellular stress including UV irradiation, heat shock, high osmotic stress, lipopolysaccharides, protein synthesis inhibitors and pro-inflammatory cytokines (Ichijo 1999). The Erk1/2 signalling pathway is activated by a large number of ligands which include growth factors, serum, phorbol esters and ligands of the heterotrimeric G protein-coupled receptors and cytokines. The MAPK pathways play important roles in cell proliferation, differentiation, survival and apoptosis.

3.1.1.1 The Erk1/2 Kinase Pathway

Extracellular regulated kinase 1 (Erk1) and 2 (Erk2) were identified as protein kinases activated in response to growth factors and phosphorylate microtubule associated protein-2 (MAP-2) and myelin basic protein (MBP) (Boulton and Cobb 1991). Erk1 and Erk2 have 83% amino acid identity and are expressed to various extents in all tissues (Pearson, Robinson et al. 2001). Erk1/2 are serine/threonine protein kinases with a strikingly large number of substrates (Yoon and Seger 2006). The dual specificity kinase that phosphorylates Erk1/2 is MEK (Mitogen/Extracellular-signal regulated kinase kinase). MEK is a 45KDa protein and exists in two forms, MEK1 and MEK2, both of which are ubiquitously expressed (Zheng and Guan 1993). MEK1 and MEK2 phosphorylate Erk1 and Erk2 within the Thr-Glu-Tyr motif in the activation loop via phosphorylation of

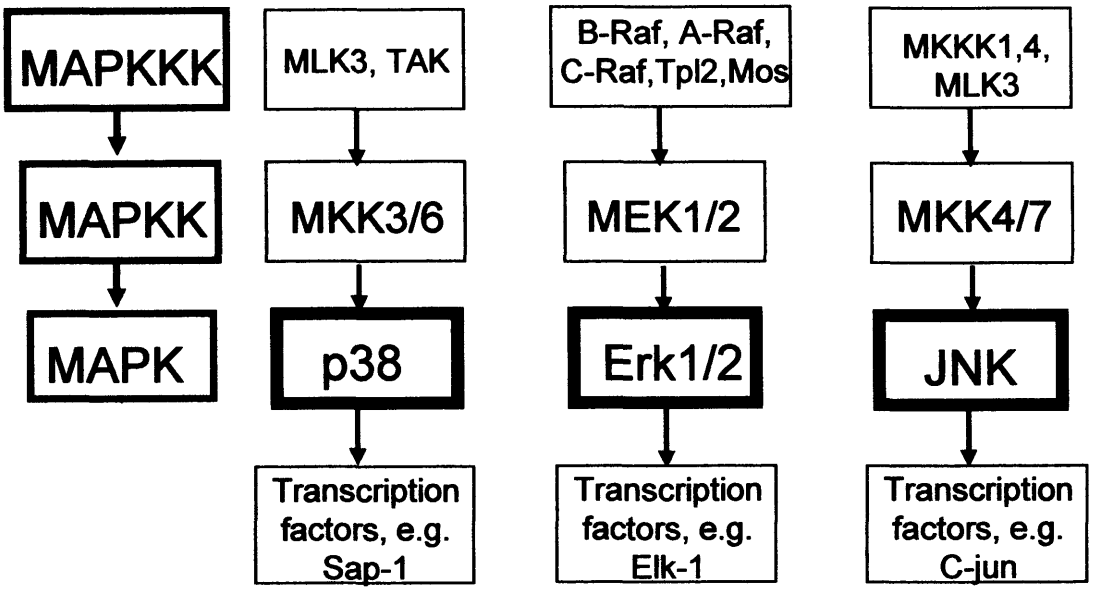


Figure 3.1: Schematic Diagram of the Mitogen Activated Protein Kinase Signalling Cascades

This cascade contains three tiers of evolutionarily conserved, sequentially acting kinases, a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK).

Thr202/Tyr204 and Thr185/Tyr187, respectively (Dhanasekaran and Premkumar Reddy 1998). MEK itself is activated via phosphorylation on Ser218 and Ser222 within the kinase domain by upstream activator kinases, including the Raf family of kinases, Tpl2 and Mos. Mutation of Ser218 and Ser222 to alanines prevents activation of MEK whilst substitution of Ser218 and Ser222 to glutamic acids, which act as phosphomimic, activates MEK independently of phosphorylation (Yan and Templeton 1994).

3.1.1.2 Raf Isoforms

The Raf kinase family consists of three isoforms, A-Raf, B-Raf and, the most extensively studied, C-Raf (Raf-1). All activated Ras proteins can interact directly with the three Raf kinases and its interaction is a crucial first step for Raf activation. The mechanism for converting the Raf kinases from inactive to active enzymes is a highly complex process, involving membrane recruitment, a series of phosphorylation/dephosphorylation steps and protein interactions. Based on the difference in expression pattern and their ability to activate Erk1/2, the Raf isoforms appear to perform distinct roles within cells. C-Raf is ubiquitously expressed, whereas A-Raf is located in muscle and urogenital tissue and B-Raf is found to be strongly expressed in fetal brain and adult cerebrum (Chong, Vikis et al. 2003). The Rafs not only differ in their tissue expression profiles. The phenotypes of Raf knock-out mice also differ indicating differential functions. Mice deficient in A-Raf survive birth but exhibit intestinal and neurological abnormalities, whilst B-Raf knock out mice are embryonic lethal and die before birth due to vascular defects (Chong, Vikis et al. 2003). In C-Raf knock-out mice, embryos are growth retarded and die at midgestation with anomalies in the placenta and in the fetal liver (Mikula, Schreiber et al. 2001). Furthermore, C-Raf deficient fibroblasts are more sensitive than wild-type cells to specific apoptotic stimuli, such as actinomycin D (Mikula, Schreiber et al. 2001). MEK/Erk activation is normal in C-Raf deficient cells and embryos, and is probably mediated by B-Raf. Therefore, in cells where C-Raf is knocked out, B-Raf is able to compensate for the loss and to activate MEK (Chong, Vikis et al. 2003). However, C-Raf knock-out mice are more susceptible to apoptotic stimuli even in the presence of A and B-Raf (Mikula, Schreiber et al. 2001).

The Rafs all share a conserved structure consisting of a large regulatory N-terminal domain that consists of the two conserved regions, CR1 and CR2 and a kinase domain located in the C-terminus which is located within the third conserved region CR3 (**Figure 3.2**) (Chong, Vikis et al. 2003). Raf binding of Ras via its Ras binding domain (RBD) and

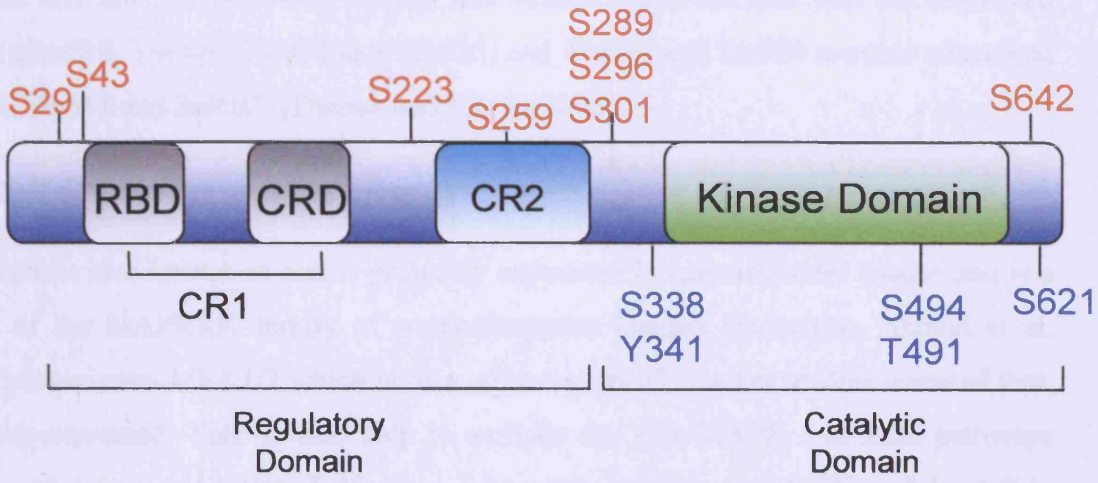


Figure 3.2: Schematic Diagram of C-Raf Protein Kinase Structure.
The three regions conserved in all Raf proteins (CR1, CR2 and CR3) are shown. CR1 is composed of a Ras binding domain (RBD) and a cysteine-rich domain (CRD). The kinase domain is referred to as the CR3 domain which contains the negative-charge regulatory region (N-region) and activation segment. Phosphorylation of Ser43, just N-terminal to this region, blocks C-Raf binding to Ras. Phosphorylation of residues marked in blue are required for C-Raf activation whereas phosphorylation of the residues marked in red blocks its activation. CR2 contains one of the 14-3-3 binding sites, which encompasses Ser259.

cysteine rich domain (CRD) within CR1 leads to the recruitment of Ras to the plasma membrane and its activation (Chong, Vikis et al. 2003).

Phosphorylation is essential for Raf activation and most studies have focused on C-Raf. The C-Raf isoform requires phosphorylation on four sites Ser338, Tyr341, Thr491 and Ser494 for activation (reviewed by Dumaz and Marais 2005). All four sites are conserved in A-Raf (Ser298, Tyr301, Thr452 and Thr455) and Thr491 and Ser494 are also conserved in B-Raf (Thr598 and Ser602) (Dumaz and Marais 2005).

3.1.1.3 Tpl2 (Tumor Progression Locus 2)

Tpl2, which is also known as cot, is primarily expressed in hematopoietic tissues and is a member of the MAPKKK family of serine/threonine kinases (Salmeron, Ahmad et al. 1996). Tpl2 activates MEK1/2 which in turn activates Erk1/2. Earlier studies revealed that, when over-expressed, Tpl2 is also able to activate the p38 MAPK and JNK pathways (Patriotis, Makris et al. 1994). Tpl2 plays a key role in activating MEK and Erk1/2 in macrophages stimulated by lipopolysaccharides (LPS) and cytokines and therefore plays a key role in regulating immune responses (Eliopoulos, Das et al. 2006). Activation of Tpl2 is dependent on several steps. Tpl2 is associated with p105, the nuclear factor kappa B (NF-kB) precursor protein, which also functions as IκB molecules (Waterfield, Zhang et al. 2003). This interaction stabilizes Tpl2 and inhibits its kinase activity (Beinke, Deka et al. 2003). Stimulation with LPS or TNFα promotes the release and its degradation of p105 from Tpl2 via phosphorylation by IKKβ (Waterfield, Jin et al. 2004). In this unbound state, Tpl2 is active but unstable and is subject to rapid degradation by the proteasome. Tpl2 also undergoes phosphorylation at Thr290 by IKKβ in response to LPS which parallels an increase in its activity (Cho, Melnick et al. 2005). The signalling role of Tpl2 depends on both the cell type and stimulus. In Tpl2 knock-out mice, LPS stimulated Erk1/2 activation is severely reduced whilst signalling by p38 and JNK pathways are largely unaffected (Das, Cho et al. 2005). However, in mouse embryonic fibroblasts, Tpl2 is required for the activation of both Erk1/2 and JNK by TNFα (Das, Cho et al. 2005).

3.1.1.4 Mos

Another known MEK kinase is Mos, which functions to control MAPK activation during meiotic maturation of oocytes. Mos activates the MAPK cascade in vertebrate oocytes via the phosphorylation of MEK. The Mos protein is required for the activation and stabilization of M phase-promoting factor MPF, which plays an important role in cell cycle

progression (Kosako, Gotoh et al. 1994). The activation and inactivation of Mos thus enables the control of the cell cycle at a crucial period of development. Mos expression is regulated at several levels, including mRNA polyadenylation and translation, as well as stabilization by phosphorylation, which protects Mos from degradation by the ubiquitin pathway (Gebauer, Xu et al. 1994; Gebauer and Richter 1996; Gebauer and Richter 1997). In immature oocytes, Mos mRNA is present but not translated into protein. Steroid stimulation increases Mos polyadenylation which leads to increased Mos expression, which in turn promotes the activation of MEK1 and subsequently of Erk1/2 (reviewed by Raman, Chen et al. 2007). Mos has been shown to activate the MAPK pathway through two opposite pathways: activation of MEK1 and inhibition of a phosphatase (Verlhac, Lefebvre et al. 2000).

3.1.2 Protein Associations in the MAPK pathway

Scaffolding proteins interact with several components of the MAPK cascades to tether both enzymes and substrates specifically to achieve accurate signal transduction which promotes the formation of spatially distinct pools of MAPK pathway components. These scaffolding proteins associate with several components of the ERK signalling pathway and enhance ERK activation. The first ERK scaffolding proteins identified were Kinase Suppressor of Ras (KSR) and MEK partner 1 (MP1) (Schaeffer, Catling et al. 1998). MP1 tethers Erk1/2 and MEK together, and favours the activation of Erk1 over Erk2 (Schaeffer, Catling et al. 1998). KSR links Raf, MEK and Erk together. It acts as a scaffolding protein that coordinates the assembly of a membrane localized, multiprotein MAP kinase complex and plays a vital step in Ras-mediated signal transduction (Reviewed by Morrison 2001).

3.1.3 Inactivation of MAPK

One conserved property of MAPK signalling is that the magnitude and duration of MAPK activation is a crucial determinant of the biological outcome and is a balance between signal activation and inactivation mechanisms. Erk1/2 activation is regulated by modification of both the threonine residue and the tyrosine residue within the activation motif. Dephosphorylation of either residue inactivates these enzymes. This can be achieved by tyrosine-specific phosphatases, serine-threonine phosphatases or dual-specificity (Thr/Tyr) phosphatases. The largest group of phosphatases dedicated to the regulation of MAPK signalling is the dual-specificity MAPK phosphatases (MKPs) (for a detailed review see Dickinson and Keyse 2006). The protein tyrosine phosphatases PTP-SL (brain-specific PTPases), STEP (striatum enriched phosphatase), and HePTP (mitogen-activated

protein kinase-targeted hematopoietic tyrosine phosphatase) have also emerged as major regulators of MAP kinase functions (Blanco-Aparicio, Torres et al. 1999). It has also been shown that PKA phosphorylates PTP-SL on Ser231, thus preventing the binding and tyrosine dephosphorylation of Erk1/2 (Blanco-Aparicio, Torres et al. 1999).

3.1.4 Mechanisms of Erk1/2 activation

3.1.4.1 Activation of Erk1/2 by cAMP

In many cell types, hormones that increase intracellular cAMP inhibit Erk1/2 signalling (Schmitt and Stork 2001). However, in other cell types, such as PC12 and neuronal cells, increased intracellular cAMP leads to an increase in Erk1/2 activation (Vossler, Yao et al. 1997). cAMP activation or inhibition of Erk1/2 is thought to occur through the activation of either protein kinase A (PKA) or cAMP-regulated guanine nucleotide exchange factors (Epac 1 or 2). cAMP activated PKA phosphorylates C-Raf at three sites (Ser43, Ser233 and Ser259) and by doing so blocks C-Raf activation when cAMP levels are elevated (Dumaz and Marais 2005). It has also been suggested that the small G-protein Rap1 is activated in a PKA dependent manner, which acts to sequester C-Raf and prevent its activation by Ras (Stork and Schmitt 2002). Rap1 activation is regulated by guanine nucleotide exchange factors (GEFs) such as Epac and C3G (Crk Src homology domain 3 guanine nucleotide exchange factor) which are regulated by cAMP or PKA respectively. In many cell types, the role of Epac in Erk1/2 activation is unclear. In PC12 cells, cAMP activates Rap1 via Epac in a PKA independent manner and Rap1 then stimulates B-Raf and Erk1/2 activation (York, Yao et al. 1998). However, the activation of Epac using a cAMP analogue, 8-CPT-2Me-cAMP, which selectively activates Epac but not PKA, activates a distinct pool of Rap1 which does not lead to Erk1/2 activation (Wang, Dillon et al. 2006). However, relocalization of Epac to the plasma membrane by a membrane targeting motif enables cAMP to activate Erk1/2 via Rap1/B-Raf, suggesting that the spatial localization of Epac is important in its signalling (Wang, Dillon et al. 2006). Activation of Rap1 by C3G has been shown to promote Erk1/2 phosphorylation (Wang, Dillon et al. 2006). C3G is recruited to the plasma membrane via CrkL adaptor protein, a process which is facilitated by the scaffold protein Cbl. C3G and CrkL are recruited to Cbl following the phosphorylation of a tyrosine residue on Cbl that acts as a docking site for the SH2 domain of CrkL (Sakkab, Lewitzky et al. 2000). This tyrosine phosphorylation of Cbl requires Src and PKA activates Src by phosphorylating it on Ser17 (Schmitt and Stork

2002). This phosphorylation is essential for PKA to activate Rap1 which is then able to signal downstream to Erk1/2 (**Figure 3.3a**).

3.1.4.2 Activation of Erk1/2 by Receptor Tyrosine Kinases

The best characterized pathway leading to the activation of Erk1/2 is via receptor tyrosine kinases (RTKs) (Reviewed by Hunter 1995). RTKs are membrane-spanning, cell surface proteins that contain an N-terminal extracellular ligand binding domain and a C-terminal intracellular tyrosine kinase domain. Receptor activation as a consequence of ligand binding results in receptor dimerization and autophosphorylation of multiple tyrosine residues on the intracellular region of the receptor. The tyrosine residues act as docking sites for SH2 (Src homology region 2) and PTB (phosphotyrosine-binding) domains located within target proteins such as Grb2 and Shc which mediate protein-protein interactions involved in many signal transduction pathways (Reviewed by Schlessinger 1994). The recruitment of SH2 containing proteins such as Grb2 facilitates the binding and recruitment to the guanine nucleotide exchange factor (GEF) son of sevenless (mSOS) which promotes the exchange of GDP to GTP on the small membrane associated G-protein Ras. Once in its GTP bound state, Ras represents an important signalling branch point as it activates several signalling pathways through a number of effectors, including the Raf isoforms (Moodie, Willumsen et al. 1993). Activated Ras binds and recruits the Raf kinases to the plasma membrane where Raf activation takes place. The increase in Raf activity results in the phosphorylation of the dual specific protein kinase MEK which in turn activates Erk1/2 via phosphorylation of threonine and tyrosine residues (Moodie, Willumsen et al. 1993)(**Figure 3.3b**).

3.1.4.3 Activation of Erk1/2 by GPCR

Signalling via G-protein coupled receptors (GPCR) is coupled to the activation of heterotrimeric G-proteins which transmit extracellular signals into intracellular effects. Signalling responses elicited via GPCR include proliferative responses which are believed to be mediated via the MAPK pathway in particular Erk1/2 (Werry, Sexton et al. 2005). Signals generated from GPCR's coupled to Gαs are dependent on the stimulation of adenylyl cyclase and the production of cAMP. The mechanisms by which cAMP regulate Erk1/2 activation is discussed in section 3.1.4.1. Signalling via G_{q11} coupled receptors also leads to the activation of Erk1/2. This mechanism is dependent on the activation phospholipase C (PLC) which hydrolyses phosphatidylinositol (4,5) bisphosphate to form

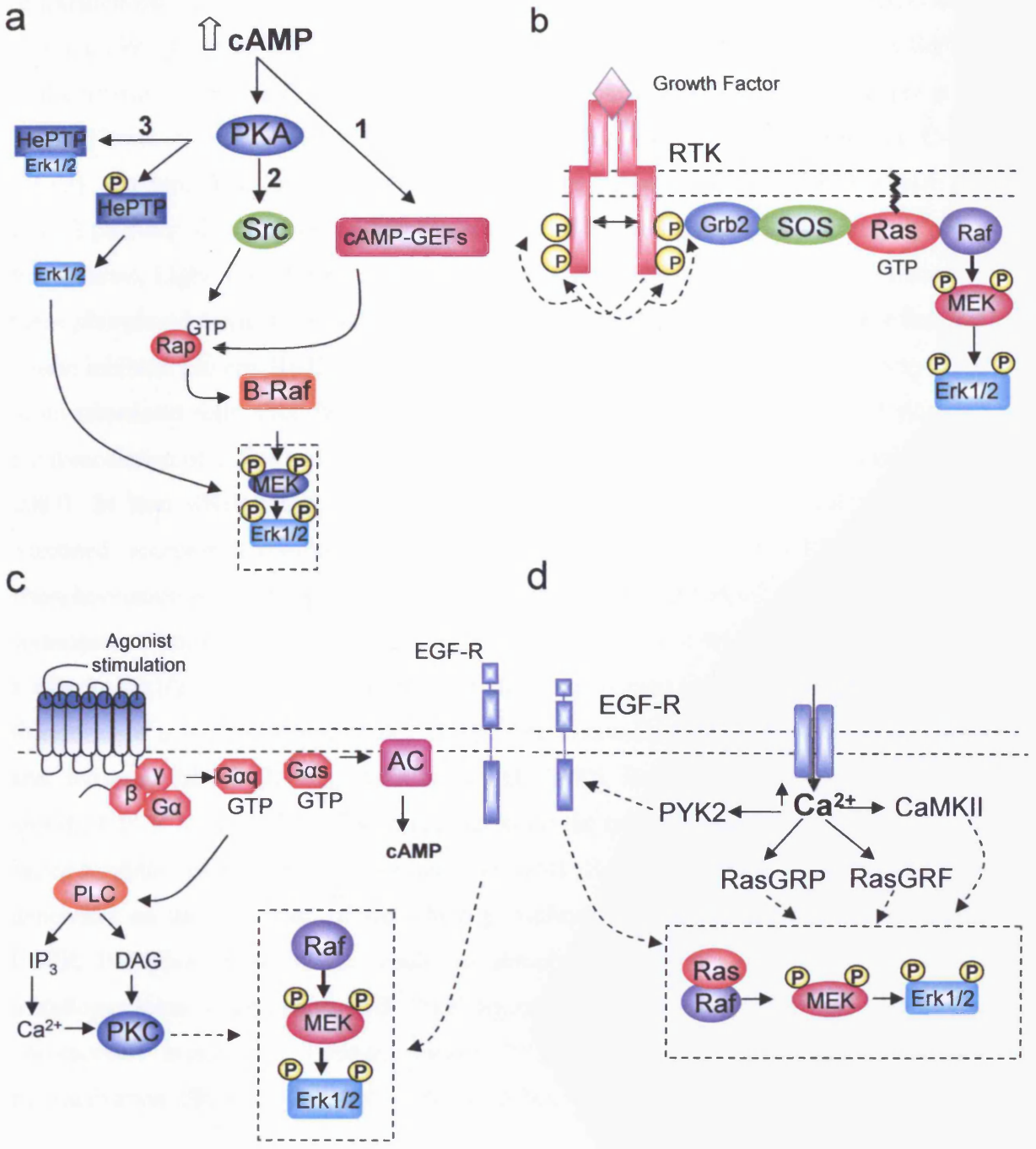


Figure 3.3: Schematic Diagram of the Modes of Erk1/2 Activation

a) cAMP activates two GEFs, Epac and C3G which lead to Erk1/2 activation (1), PKA leads to the activation of Src which activates Erk1/2 (2) or PKA phosphorylation of the Erk1/2 phosphatase releases Erk1/2 from inhibition (3). b) Receptor tyrosine kinase c) GPCR and d) Calcium regulation of Erk1/2 signalling pathways. See text for details.

two products, inositol (1,4,5) trisphosphate (IP_3) which via the IP_3-R stimulates the release of intracellular calcium, and diacylglycerol which activates protein kinase C (PKC) (Rhee and Bae 1997; Rhee 2001). Early reports suggested that PKC could phosphorylate Ser499 in the activation loop of C-Raf, which then increases its activity (Kolch, Heidecker et al. 1993). However, mutation of Ser499 to alanine had no effect on PKC stimulated C-Raf activity (Barnard, Diaz et al. 1998). Subsequent studies showed that PKC activates the Erk1/2 pathway via a Ras dependent mechanism rather than direct phosphorylation of C-Raf (Marais, Light et al. 1998). Although C-Raf is phosphorylated by PKC, the nature of these phosphorylations in regulating C-Raf activity is unclear. The recently identified Raf kinase inhibitor protein, RKIP binds to C-Raf and inhibits C-Raf mediated phosphorylation in un-stimulated cells. PKC has been reported to phosphorylate RKIP on Ser152 allowing the dissociation of C-Raf and subsequent MEK and Erk1/2 activation (Corbit, Trakul et al. 2003). In turn RKIP binds to GRK2 to prevent receptor desensitization resulting in sustained receptor signalling (Rozengurt 2007). In PC12 cells, PKC dependent phosphorylation of RKIP results in the prolonged activation of Erk1/2 in response to NGF stimulation (Santos, Verveer et al. 2007). β-Arrestins have also been demonstrated to play a role in Erk1/2 activation. Stimulation of the Gαq-coupled receptor PAR2 leads to the formation of a multiprotein complex comprising internalized receptor, β-arrestin, C-Raf and activated Erk1/2 (DeFea, Zalevsky et al. 2000). Recent studies have shown that specific GPCR's can mediate Erk1/2 activation via the transactivation of epidermal growth factor receptor (EGFR) (Kalmes, Daum et al. 2001; Rozengurt 2007). This mechanism is dependent on the activation of src which phosphorylates the intracellular region of the EGFR. Phosphorylation by src results in autophosphorylation or by the activation of metalloproteases which cleave EGF "like" ligands which act as ligands at the EGFR. The non-receptor proline-rich tyrosine kinase PYK2 has also be implicated in EGFR transactivation (Shah and Catt 2002) (**Figure 3.3c**).

3.1.4.4 Erk1/2 Activation in Response to Calcium

Several mechanisms for Ca^{2+} induced Erk1/2 activation have been reported. Agents such as angiotensin II, endothelin and phenylephrine which act to mobilize calcium have been shown to activate Erk1/2 (Abraham, Benscoter et al. 1996). These agents act by binding to their specific GPCR. Signals generated from receptors coupled to Gαq activate membrane bound phospholipase C (PLC) which hydrolyses phosphatidylinositol (4,5) bisphosphate to form two products, inositol (1,4,5) trisphosphate which goes onto release intracellular calcium and diacylglycerol which activates protein kinase C (PKC) which in turn leads to

the activation of Erk1/2. In vascular smooth muscle cells it has been reported that the family of calcium/calmodulin dependent protein kinases (CaMKs) are activated by an increase in intracellular Ca^{2+} and that CaMKII can stimulate the activation of Erk1/2 in response to calcium mobilizing agents (Abraham, Benscoter et al. 1997). PYK2, which is a Ca^{2+} dependent kinase has been shown to be important in the Ca^{2+} dependent activation of Erk1/2. It was recently reported that CaMKII can activate PYK2 and Erk1/2 activation in vascular smooth muscle cells (Ginnan and Singer 2002). Subsequent studies revealed that GPCR mediated Ca^{2+} release activates both CaMKIIδ and PKCδ which mediate the transactivation of the EGFR resulting in increased Erk1/2 activation (Ginnan, Pfleiderer et al. 2004). CaMKIV has been reported to participate in Erk1/2 activation in cortical neurons. CaMKIV can phosphorylate the small G Protein Rap1 which increases Erk1/2 activation via B-Raf (Sahyoun, McDonald et al. 1991). Additional mechanisms for Ca^{2+} mediated Erk1/2 activation have been reported and include the activation of specific guanine nucleotide exchange factors for the G-protein Ras which include Ras-GRP and Ras-GRF. Ras-GRF is activated by the binding of Ca^{2+} and calmodulin (CaM). Once bound, Ras-GRF facilitates the GTP exchange and Ras activation. Ca^{2+}/CaM also regulate the Erk1/2 pathway by regulating synGAP which is a neuron-specific Ras GTPase-activating protein (GAP). synGAP can be inhibited by CaMKII mediated phosphorylation (Oh, Manzerra et al. 2004) (**Figure 3.3d**).

3.1.5 Downstream Targets of Erk1/2

The Erk1/2 pathway regulates a number of substrates including, i) transcription factors, ii) protein kinases and phosphatases, iii) cytoskeletal and scaffold proteins, iv) receptors and signalling molecules and v) apoptosis related proteins (for a detailed review of these substrates see Yoon and Seger 2006). A brief overview of some of the best characterised Erk1/2 substrates will be discussed.

3.1.5.1 Nuclear Substrates of Erk1/2

Once activated, Erk1/2 translocates into the nucleus where it plays an important role in regulating gene expression by phosphorylating transcription factors to regulate their activities. Erk1/2 phosphorylates and alters the activities of several classes of transcription factors. The AP-1 (activating protein-1) family of leucine zipper transcription factors including c-jun and c-fos which, following activation, form homodimers and heterodimers that bind to DNA. c-fos enters the nucleus following translation where it becomes phosphorylated by Erk1/2 on Ser374 and RSK on Ser362 at the C-terminus (Murphy,

MacKeigan et al. 2004). These phosphorylations stabilize the protein but also prime c-fos for phosphorylation on Thr325 which further stabilizes c-fos and results in an increase in its transcriptional activity.

Another class of transcription factors which are Erk1/2 substrates include the ternary complex factors (TCFs) which include Elk. Elk-1 is the best studied target of Erk1/2. It mediates transcription from serum response elements (SREs) in promoters of genes such as c-fos. Following growth factor stimulation, Erk1/2 phosphorylates Elk-1 on several distinct sites which include, Thr353, Thr368, Ser383, Ser389, Thr417, Ser324, Thr336 and Ser422 (Cruzalegui, Cano et al. 1999). Phosphorylation of these sites enhances DNA binding and hence increases the transcriptional activity and the phosphorylation of Ser383 is crucial for Elk-1 activity (Buchwalter, Gross et al. 2004).

3.1.5.2 Cytosolic Substrates of Erk1/2

Many of Erk1/2 substrates also reside in the cytoplasm. Protein kinases form a large subset of Erk1/2 targets. These include the RSK family of protein kinases of which there are four isoforms and they play an important role in transducing Erk1/2 signalling in the cytoplasm and nucleus. RSK contains two catalytically active kinase domains, the C-terminal domain induces autophosphorylation and is important for activation by Erk1/2 and the N-terminal domain is required to phosphorylate its substrates (Roux and Blenis 2004). RSK contains six phosphorylation sites essential for activity which include, Ser221, Ser359, Ser363, Ser380, Thr573 and Ser732 (Dalby, Morrice et al. 1998). Erk1/2 phosphorylates Thr573 in the activation loop and Ser363. The phosphorylation of Ser363 leads to the activation of the C-terminal kinase domain at Ser380 which provides a docking site for PDK-1 to phosphorylate Ser221 (Frodin, Jensen et al. 2000). Once RSK is activated, it is able to phosphorylate a number of substrates which include the transcription factor CREB.

Erk1/2 also phosphorylates MAPKAPKs, including MNK1/2 (MAP kinase-interacting kinase 1/2) and MSK1/2 (Mitogen- and Stress-activated protein Kinases 1 and 2). MNK1 is activated by Erk1/2 and p38 MAPK by phosphorylation at Thr255 and Thr385 (Waskiewicz, Flynn et al. 1997; Roux and Blenis 2004). MSK1 and MSK2 share the major structural characteristics of the RSKs, including two distinct catalytic domains and have also been shown to be phosphorylated by Erk1/2 (Roux and Blenis 2004).

3.1.6 Role of Erk1/2 in Pancreatic β-cells

In MIN6 and INS-1 pancreatic β-cell lines and human islets, Erk1/2 has been shown to be activated by glucose and that this activation is potentiated by GLP-1 (Benes, Poitout et al. 1999; Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003; Briaud, Lingohr et al. 2003; Trumper, Ross et al. 2005). The activation of Erk1/2 by glucose and GLP-1 in MIN6 and INS-1 cells has been shown to be dependent on a rise in intracellular free Ca^{2+} levels via Ca^{2+} influx through L-type voltage-gated calcium channels (L-type VGCC) (Gomez, Pritchard et al. 2002) and the release of intracellular Ca^{2+} (Arnette, Gibson et al. 2003; Gibson, Lawrence et al. 2006) respectively. In MIN6 cells, GLP-1 stimulated Erk1/2 activation was shown to require an influx of extracellular calcium since EGTA, a Ca^{2+} chelator, inhibited GLP-1 stimulated Erk1/2 activation. However, treatment with the Ca^{2+} ionophore, ionomycin, which acts to artificially raise intracellular Ca^{2+} concentrations, had no effect on GLP-1 stimulated Erk1/2 activation indicating that a global increase in Ca^{2+} entry is not sufficient to activate Erk1/2 (Gomez, Pritchard et al. 2002). Furthermore, it was demonstrated that the mode of Ca^{2+} entry is important since treatment with nifedipine (an L-type VGCC blocker) blocks both GLP-1 and glucose stimulated Erk1/2 activation (Gomez, Pritchard et al. 2002). Depolarising concentrations of potassium also increases Erk1/2 phosphorylation via Ca^{2+} influx through L-Type VGCC demonstrating that Ca^{2+} entry is sufficient for Erk1/2 activation (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). Additionally, activation of the L-Type VGCC with Bay K8644 is also able to activate Erk1/2, indicating that L-type VGCC is sufficient for signalling to Erk1/2 (Gomez, Pritchard et al. 2002). Therefore, it is likely that the increase in Ca^{2+} influx is an important determinant by which GLP-1 induces Erk1/2 activation. The Ca^{2+} dependency of glucose and GLP-1 stimulated Erk1/2 activation appears to be dependent on calmodulin, as W7 (calmodulin inhibitor) inhibits glucose and GLP-1 stimulated Erk1/2 activation in MIN6 and INS-1 cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). Interestingly, calmodulin is required for the activation of a phosphatase, calcineurin and pharmacological inhibition of calcineurin using FK506 blocks both glucose and GLP-1 stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). Calmodulin is also required for the activation of CaMKs and pharmacological inhibition of CaMKII using KN62 and KN93 inhibits GLP-1 stimulated Erk1/2 activation in MIN6 cells and INS-1 cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003).

Given that GLP-1 promotes the production of cAMP, many groups examined the role of cAMP in glucose and GLP-1 stimulated Erk1/2 activation. The best characterized cAMP

effector is the cAMP dependent protein kinase (PKA). Pharmacological inhibition of PKA using H89, which has a limited specificity, inhibits glucose and GLP-1 stimulated Erk1/2 activation in MIN6 and INS-1 cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003; Gibson, Lawrence et al. 2006).

A study by Briaud *et al.* demonstrated in INS-1 cells that glucose stimulated Erk1/2 activation was dependent on increases in both Ca^{2+} and cAMP acting via PKA (Briaud, Lingohr et al. 2003). They hypothesised that the increase in Ca^{2+} following membrane depolarization leads to the activation of Ca^{2+} sensitive adenylyl cyclase such as isoform 3 which has been reported to be expressed in pancreatic β-cells (Tian and Laychock 2001; Delmeire, Flamez et al. 2003). However, in pancreatic β-cells, another cAMP target which has been shown to be important in regulating many β-cell functions is the guanine nucleotide exchange factor Epac1 and 2, with Epac2 showing prominent expression in the pancreatic islets of langerhans (Kashima, Miki et al. 2001). Epac is a cAMP binding protein that exhibits intrinsic guanine nucleotide exchange factor (GEF) activity towards the small G-protein Rap (Holz, Kang et al. 2006). In PC12 cells, Erk1/2 activation is stimulated by Rap1 via B-Raf (York, Yao et al. 1998; Stork 2005). A recent study in human islets showed that glucose and GLP-1 promoted the association of Rap with B-Raf (Trumper, Ross et al. 2005).

Reports in the literature disagree as to the role of Ras and Raf in glucose and GLP-1 stimulated Erk1/2 activation in pancreatic β-cells. Adenoviral mediated expression of a dominant negative form of Ras (RasN17) had no effect on glucose stimulated Erk1/2 activation in INS-1 cells (Briaud, Lingohr et al. 2003) or glucose and GLP-1 stimulated Erk1/2 activation in MIN6 cells (Gomez, Pritchard et al. 2002). In addition, it was shown that GLP-1 stimulated Erk1/2 activation is independent of Raf since none of the three isoforms were activated following GLP-1 stimulation as assessed by an *in vitro* kinase assay (Gomez, Pritchard et al. 2002). In contrast, the expression of a different dominant negative Ras (RasG15A) and a dominant negative form of Raf-1 (RafC4B) blocked glucose stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). However, all studies agree that glucose stimulated Erk1/2 activation is dependent on the upstream activation of MEK (Benes, Poitout et al. 1999; Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003; Briaud, Lingohr et al. 2003).

3.1.6.1 Importance of Erk1/2 Signalling in Pancreatic β-cells

In pancreatic β-cells the MAPK cascade regulates a number of nuclear events which include the modulation of cell proliferation, differentiation and gene transcription. Glucose stimulated Erk1/2 activation up-regulates insulin gene transcription in MIN6 cells via an increase in insulin promoter activity (Lawrence, McGlynn et al. 2005). In INS-1 cells, glucose and GLP-1 have been shown to increase β-cell proliferation via a mechanism dependent on the activation of Erk1/2 (Briaud, Lingohr et al. 2003). Additionally, in MIN6 cells, glucose induced Erk1/2 activation has been shown to play an important role in regulating insulin exocytosis via the phosphorylation of synapsin 1 which increases insulin secretion (Longuet, Broca et al. 2005). Furthermore, the phosphorylation and protein level of CREB, which plays an important role in glucose-mediated pancreatic β-cell survival, are controlled by Erk1/2 in a PKA dependent manner (Costes, Broca et al. 2006). In pancreatic β-cells, CREB controls the expression of genes such as IRS-2 which promotes cell survival by mediating the phosphorylation of PKB which in turn potentiates insulin signalling (Jhala, Canettieri et al. 2003).

3.1.7 Aims

In pancreatic β-cells, Erk1/2 is activated by nutrients (glucose) and hormones (GLP-1) in a calcium dependent manner. However, the molecular mechanisms by which glucose and GLP-1 stimulate the phosphorylation of Erk1/2 in pancreatic β-cells are not fully understood. Therefore, the aims of this study were to identify the signal transduction pathway by which glucose and GLP-1 regulate Erk1/2 phosphorylation in pancreatic β-cells.

3.2 Results

3.2.1 The Role of CaMKII in GLP-1 Stimulated Erk1/2 Phosphorylation in Pancreatic β-cells

Our group had previously shown that GLP-1 stimulation of Erk1/2 requires an influx of extracellular calcium through L-type VGCC in β-cells. Based on inhibitor experiments the Ca^{2+} dependency of glucose and GLP-1 stimulated Erk1/2 activation appears to be dependent on calmodulin (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). It has also been demonstrated that pharmacological inhibitors of CaMKII, KN62 and KN93, inhibit GLP-1 stimulated Erk1/2 activation in MIN6 cells (Gomez, Pritchard et al. 2002) and glucose stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). However, the inhibitors KN62 and KN93 interfere with channel activity and both inhibitors have been shown to suppress Ca^{2+} influx following potassium induced membrane depolarization in pancreatic β-cells (Bhatt, Conner et al. 2000). Therefore it was important to re-examine the role of CaMKII in GLP-1 stimulated Erk1/2 activation. To these ends, three c-myc tagged constructs were produced and recombinant adenoviruses expressing wild type CaMKII (Ad-$CaMKII^{WT}$), dominant-negative CaMKII (Ad-$CaMKII^{DN}$) and constitutively active CaMKII (Ad-$CaMKII^{CA}$) were generated (materials and methods). The Ad-$CaMKII^{DN}$ virus expressed a dominant negative kinase produced by an amino acid substitution of a lysine at position 43 to an alanine (K43A), which prevents ATP binding. The Ad-$CaMKII^{CA}$ virus expressed a constitutively active kinase, produced by the amino acid substitution T287D, which mimics autophosphorylation at this site and results in autonomous activity in the absence of Ca^{2+}/calmodulin (**Figure 3.4a**). To initially test the functionality of the CaMKII constructs in pancreatic β-cells, MIN6 cells, a mouse clonal pancreatic β-cell line, which is able to synthesise and secrete insulin in response to physiological changes in glucose concentrations (Ishihara, Asano et al. 1993; Skelly, Schuppin et al. 1996), were infected with increasing amounts of either an empty virus (AdEmpty-GFP) or with Ad-$CaMKII^{WT}$, Ad-$CaMKII^{DN}$ and Ad-$CaMKII^{CA}$. 48h post-infection, the efficiency of infection was assessed to be 100% by green fluorescent protein (GFP) expression. Myc tagged CaMKII expression was determined by western blotting using anti-myc antisera (**Figure 3.4bi,ii,iii**). To determine the effects of expressing these constructs on CaMKII activity, *in vitro* kinase assays were performed using lysates from control or infected cells. To obtain total and autonomous kinase activity, CaMKII activity was assayed both with and without Ca^{2+} and calmodulin, respectively. Infection of cells with Ad-$CaMKII^{WT}$ resulted in a large increase in total CaMKII activity. Ad-$CaMKII^{DN}$

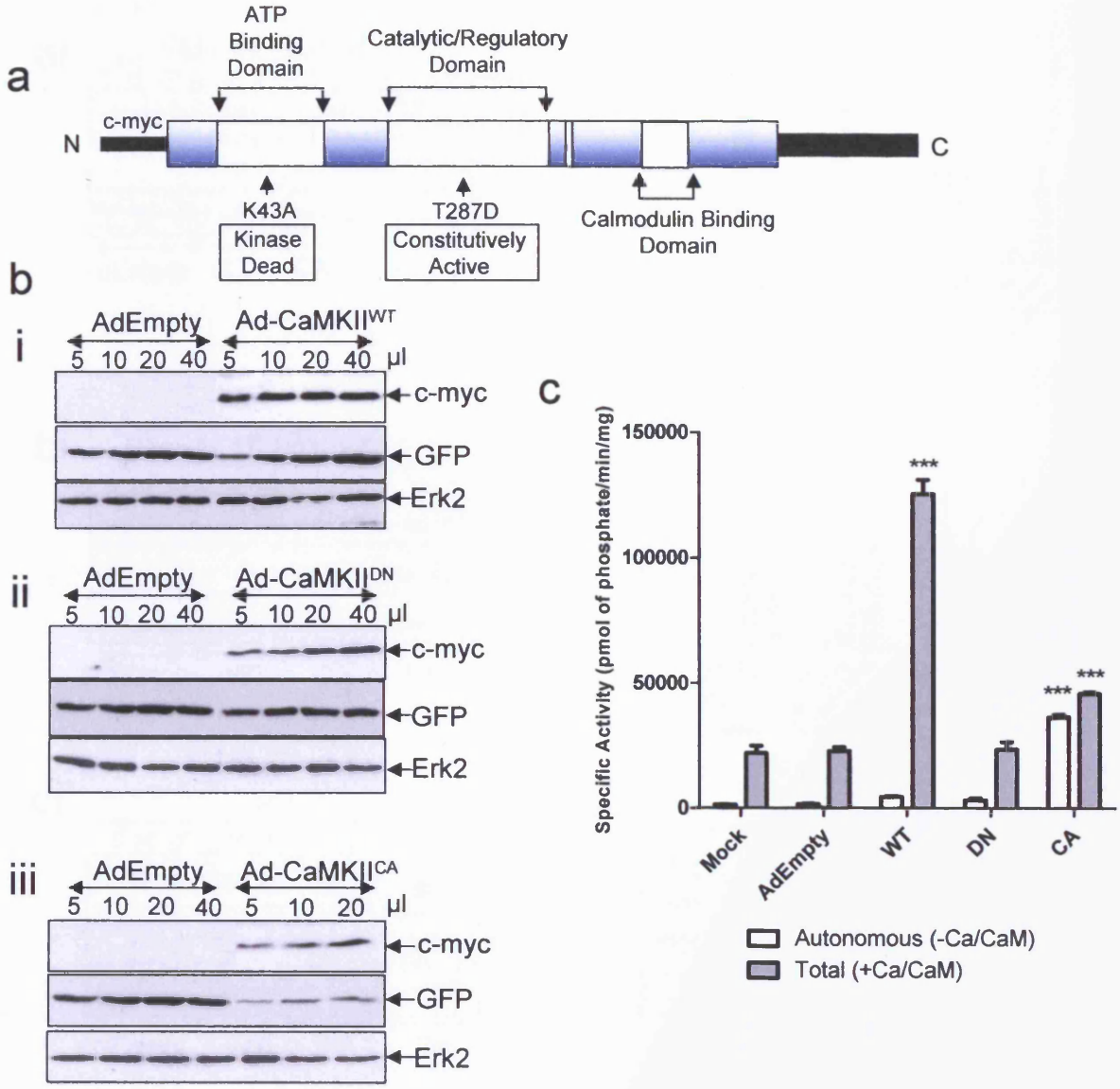


Figure 3.4: Expression of Calcium Calmodulin Dependent Protein Kinase II Wild Type and Mutants

a) Schematic representation of CaMKII mutants. Dominant negative CaMKII contains an amino acid substitution at K43A within the ATP binding site and constitutively active CaMKII was generated by an amino acid substitution T287D in the autoinhibitory domain. b) In order to assess the expression level of CaMKII (WT, DN and CA), MIN6 cells were infected with AdEmpty, Ad-CaMKII^{WT,} Ad-CaMKII^{DN} and Ad-CaMKII^{CA} for 48h. Following infection, cells were lysed and the level of expression determined by SDS-PAGE and Western blotted using antisera against myc (to see CaMKII expression) and GFP and Erk2 as a loading control. c) To determine CaMKII activity, MIN6 cells were mock infected or infected with AdEmpty, Ad-CaMKII^{WT}, Ad-CaMKII^{DN} and Ad-CaMKII^{CA} for 48h. Following infection, cells were lysed and total or autonomous CaMKII activity was assayed in the lysates using a selective peptide substrate, autocamtide II (for details see materials and methods). The results shown are a mean ± SEM n = 3 *** <0.001. P value obtained using a Two-way ANOVA compared to AdEmpty.

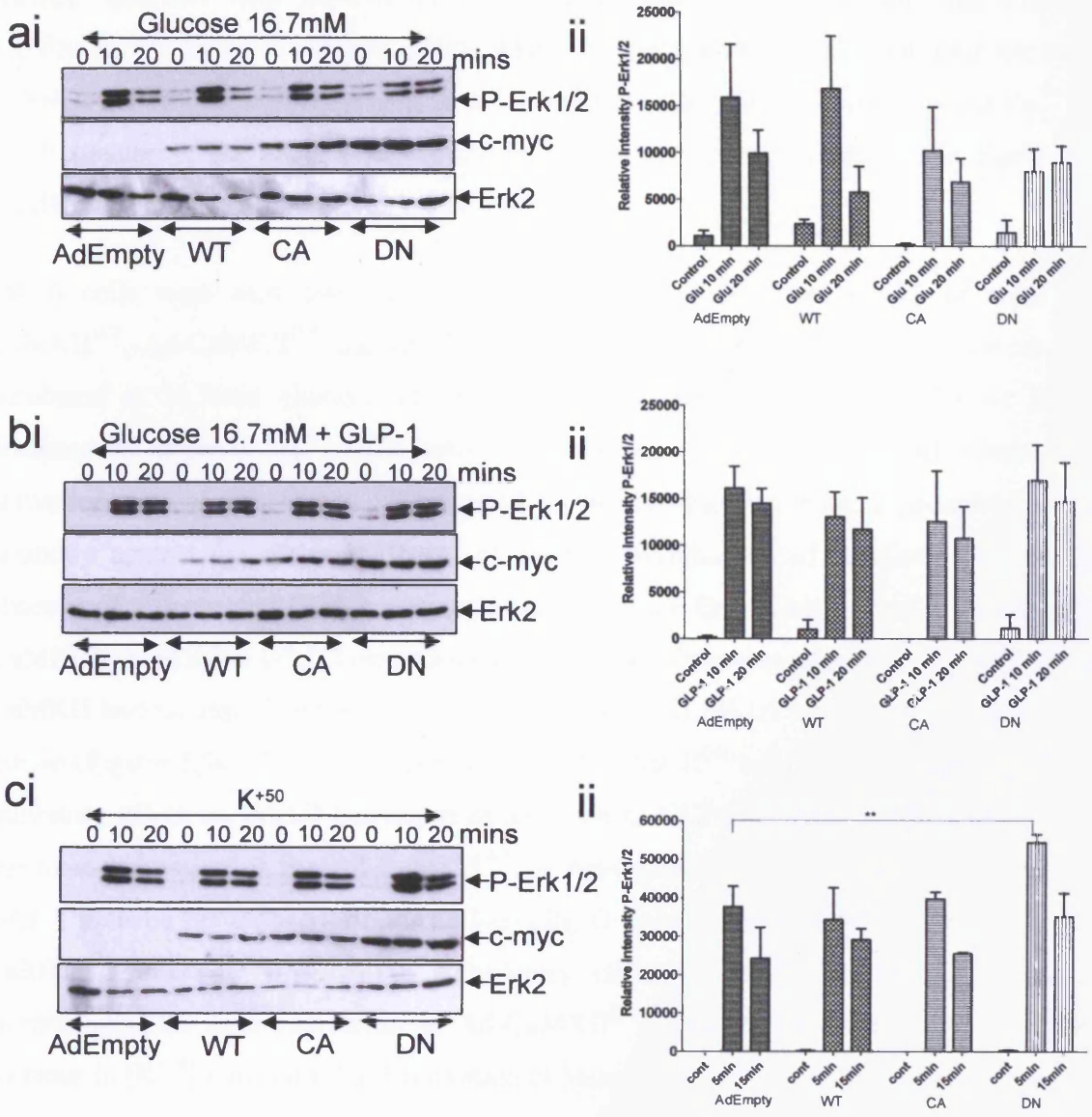


Figure 3.5: Role of CaMKII in Erk1/2 Phosphorylation in MIN6 Cells

MIN6 cells were infected with Ad-Empty (control), Ad-CaMKII^{WT}, Ad-CaMKII^{DN} and Ad-CaMKII^{CA} for 48h. Following infection, the cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated for 10 or 20min in KRB containing ai) 16.7mM glucose bi) 16.7mM glucose and GLP-1 (10nM) and ci) 2.8mM glucose in the presence of 50mM KCl (K^{+50}). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204), total myc and total Erk2 as a loading control. aii,bii,cii) Quantified data from phospho-ERK1/2 blots in *ai,bi,ci* shown as means ± SEM; $n = 3$. ** < 0.01, P value obtained using a Two-way ANOVA.

had no effect on total CaMKII activity resulting in similar CaMKII activity levels to control. Infection with Ad-CaMKII^{CA} increased both autonomous and total CaMKII activity in MIN6 cells (**Figure 3.4c**). Although the CaMKII^{DN} did not decrease total CaMKII activity *in vitro* compared to cells infected with AdEmpty, the levels of Ca^{2+} are much greater in the assay than would be expected *in vivo*, therefore it is likely that CaMKII^{DN} will act as a dominant negative *in vivo*.

MIN6 cells were then infected with an empty virus (AdEmpty-GFP) or with Ad-CaMKII^{WT}, Ad-CaMKII^{DN} and Ad-CaMKII^{CA}. 48h post-infection, MIN6 cells were then incubated at 16.7mM glucose, 16.7mM glucose in the presence of GLP-1, or in the presence of depolarising concentration of potassium [K^{+50}], for 10 and 20min. The activation state of Erk1/2 was investigated by western blotting using a phospho-specific antibody against the activated forms of Erk1/2. Introduction of Ad-CaMKII^{CA} in the absence of glucose and GLP-1 was unable to stimulate Erk1/2 activation, indicating that CaMKII cannot drive Erk1/2 activation (**Figure 3.5a**). Over-expression of WT, DN or CA CaMKII had no significant effect on glucose stimulated Erk1/2 activation at either 10 or 20min (**Figure 3.5a**). The over-expression of Ad-CaMKII^{DN} had no statistically significant inhibitory effect on Erk1/2 activation in response to GLP-1 (**Figure 3.5b**). Furthermore, the over-expression of the Ad-CaMKII^{WT} or Ad-CaMKII^{CA} had no detectable effect on GLP-1 induced Erk1/2 activation in MIN6 cells. Over-expression of Ad-CaMKII^{WT} or Ad-CaMKII^{CA} did not enhance the stimulatory effect of [K^{+50}] on Erk1/2 activation. Surprisingly, the over-expression of Ad-CaMKII^{DN} stimulated a statistically significant increase in [K^{+50}] induced Erk1/2 activation at 5min.

3.2.2 The Role of CaMKIV in GLP-1 Stimulated Erk1/2 Phosphorylation in Pancreatic β-cells

Over-expression of CaMKIV in PC12 cells has been shown to drive MAPK activation (Enslen, Tokumitsu et al. 1996). CaMKIV has been shown to be expressed in both primary β-cells and the insulin secreting cell line RINm5F (Ban, Yamada et al. 2000). Given that there is evidence that CaMKIV can activate Erk1/2 signalling pathways, I examined the role of CaMKIV in GLP-1 stimulated Erk1/2 phosphorylation. In order to do this a recombinant adenovirus expressing a myc-tagged constitutively active CaMKIV (Ad-CaMKIV^{CA}) was generated. It is a T287D mutant of CaMKIV which results in autonomous activity in the absence of Ca^{2+}/calmodulin. MIN6 cells were infected with increasing amounts of either an empty virus (AdEmpty-GFP) or Ad-CaMKIV^{CA}. After 48h, the efficiency of infection was assessed by green fluorescent protein (GFP) expression

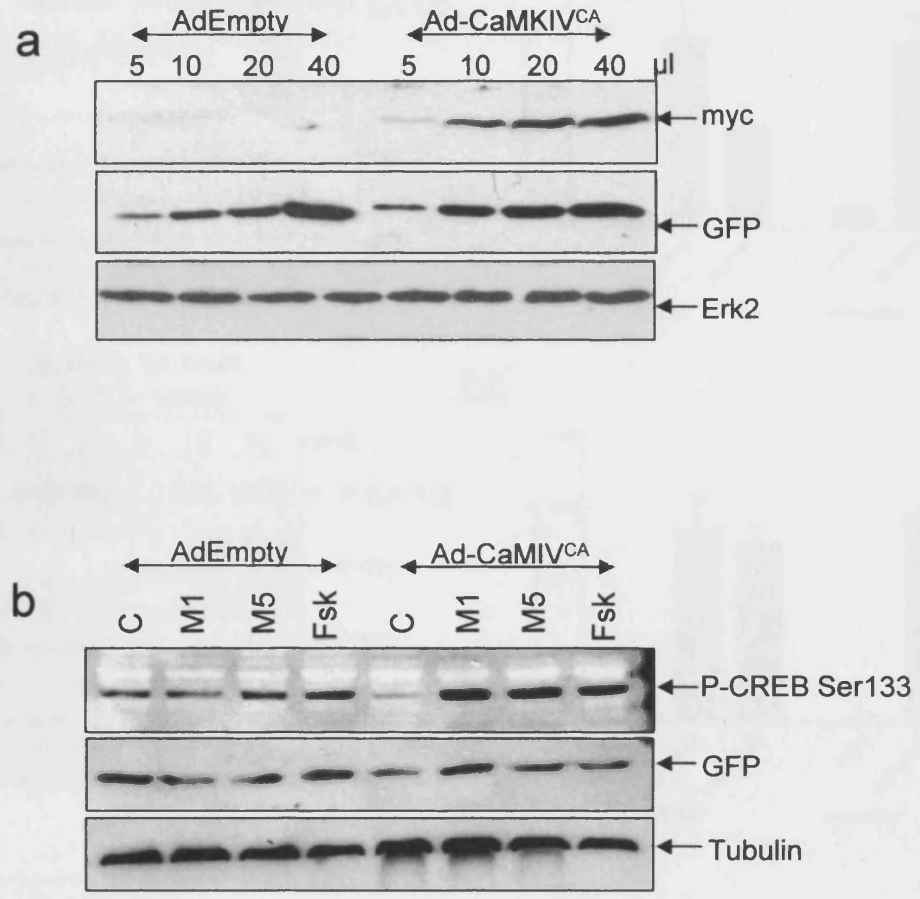


Figure 3.6: Expression of Calcium Calmodulin Dependent Protein Kinase IV Mutant

a) MIN6 cells were infected with AdEmpty and Ad-CaMKIV^{CA} for 48h. Following infection, the cells were lysed and the level of expression determined by SDS-PAGE and Western Blotting using antisera against total myc, total GFP or total Erk2 as a loading control. b) SH-SY5Y cells were infected with AdEmpty or with Ad-CaMKIV^{CA} for 48h. Following infection, the cells were pre-incubated in KRB for 30min. Cells were either left untreated (C) or treated with KRB in the presence of methacholine (1µM) for 1 and 5min (M1 and M5) or 10µM forskolin/500µM IBMX (Fsk) for 10min. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-CREB (Ser133), total GFP and total tubulin as a loading control (This experiment was performed by Dr E Rosethorne).

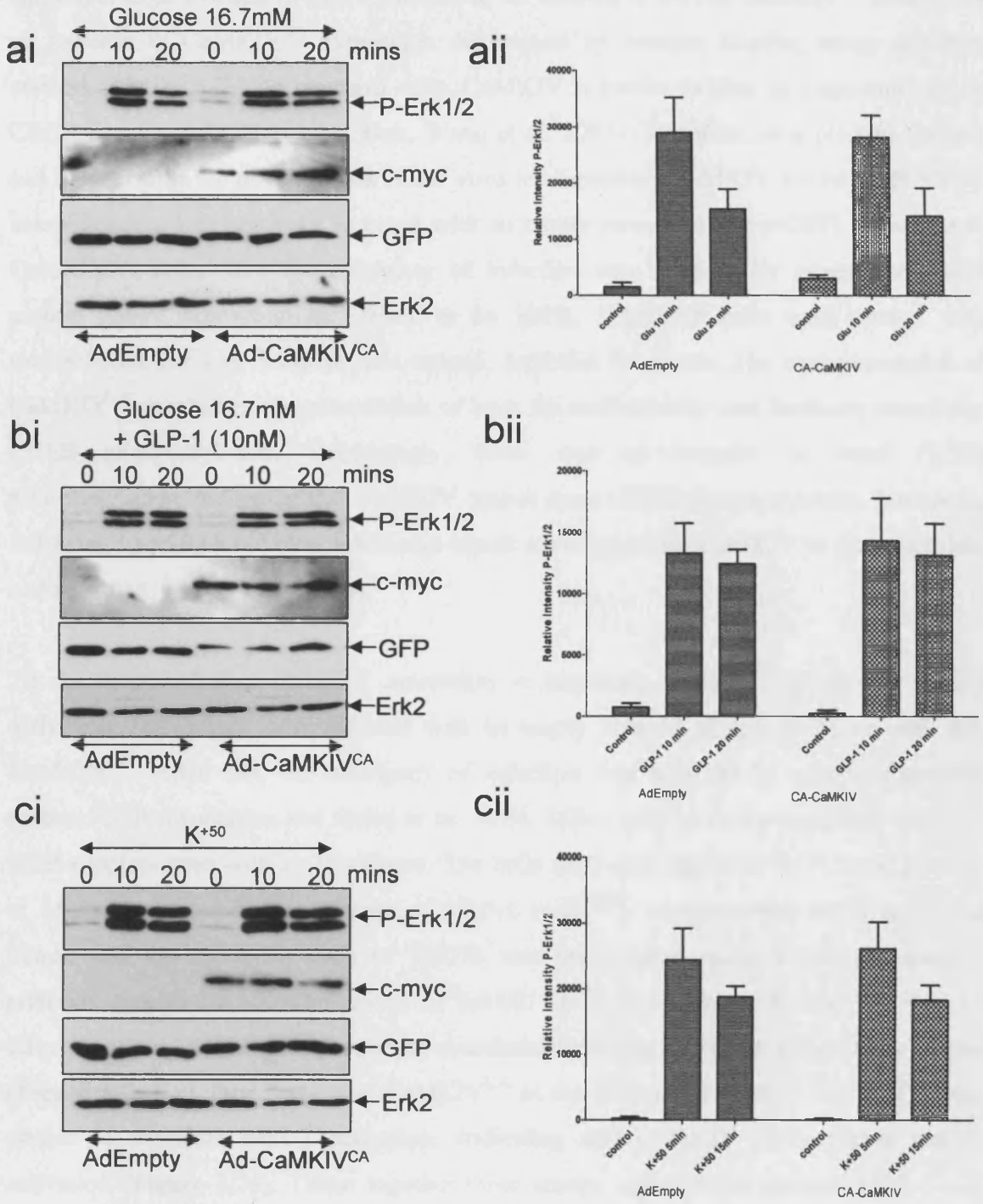


Figure 3.7: Role of CaMKIV in Erk1/2 Phosphorylation in MIN6 Cells

MIN6 cells were infected with Ad-Empty (control) or Ad-CaMKIV^{CA} for 48h. Following infection, the cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated for 10 or 20min in KRB containing ai) 16.7mM glucose bi) 16.7mM glucose and GLP-1 (10nM) and ci) KRB supplemented with 2.8mM glucose in the presence or absence of 50mM KCl (K+50). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204), total myc, total GFP or total Erk2 as a loading control. aii,bii,cii) Quantified data from phospho-ERK1/2 blots in *ai,bi,ci* shown as means ± SEM; $n = 3$.

and found to be essentially 100%. Increasing the amount of the Ad-CaMKIV^{CA} resulted in an increase in CaMKIV^{CA} expression determined by western blotting using anti-myc antisera (**Figure 3.6a**). In neuronal cells, CaMKIV is known to play an important role in CREB Ser133 phosphorylation (Bok, Wang et al. 2007). Therefore, as a positive control and to determine the effectiveness of the virus in stimulating CaMKIV activity, SH-SY5Y neuroblastoma cell line were infected with an empty virus (AdEmpty-GFP) or with Ad-CaMKIV^{CA}. After 48h, the efficiency of infection was assessed by green fluorescent protein (GFP) expression and found to be 100%. SH-SY5Y cells were treated with methacholine for 1 or 5min, or as a control, forskolin for 10min. The over-expression of CaMKIV^{CA} resulted in the potentiation of both the methacholine and forskolin stimulated CREB phosphorylation. Surprisingly, there was no increase in basal CREB phosphorylation, indicating that CaMKIV cannot drive CREB phosphorylation. Moreover, following agonist stimulation, additional inputs are required for CaMKIV to phosphorylate and activate CREB (**Figure 3.6b**).

To determine whether CaMKIV activation is important in GLP-1 stimulated Erk1/2 activation, MIN6 cells were infected with an empty virus (AdEmpty-GFP) or with Ad-CaMKIV^{CA}. After 48h, the efficiency of infection was assessed by green fluorescent protein (GFP) expression and found to be 100%. MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. The cells were then incubated in 16.7mM glucose or 16.7mM glucose in the presence of GLP-1 or [K^{+50}]. Samples were taken at 10 and 20min and the activation state of Erk1/2 was investigated using a phospho-specific antibody against the activated forms of Erk1/2. Over-expression of CaMKIV^{CA} had no effect on glucose, GLP-1 or potassium stimulated Erk1/2 activation at either 10 or 20min (**Figure 3.7a,b,c**). Introduction of CaMKIV^{CA} in the absence of glucose and GLP-1 was unable to stimulate Erk1/2 activation, indicating that CaMKIV cannot drive Erk1/2 activation (**Figure 3.7a**). Taken together these results indicate that glucose, GLP-1 and [K^{+50}] stimulated Erk1/2 activation is independent of CaMKIV.

3.2.3 Role of cAMP in Erk1/2 Activation

Glucagon like peptide-1 (GLP-1) is a G_s-coupled receptor agonist that exerts multiple effects on pancreatic β-cells. The GLP-1R couples to a $G_{\alpha s}$-containing heterotrimeric G-protein leading to the activation of adenylyl cyclase and the increase in the production of cAMP (Drucker, Philippe et al. 1987). Indeed, incubating MIN6 cells from 1mM to

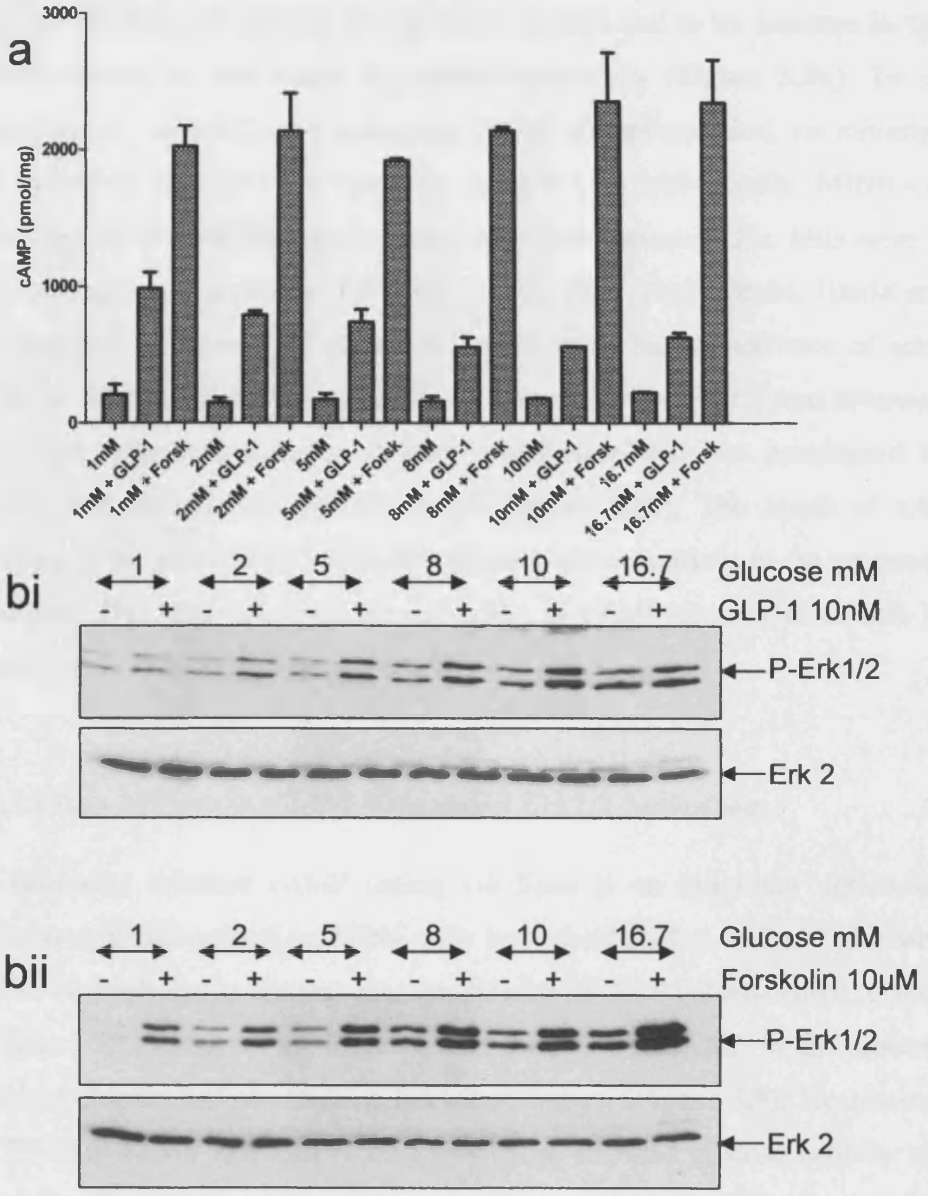


Figure 3.8: Role of cAMP in Erk1/2 Phosphorylation

a) MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose and IMBX (1μM) to prevent cAMP breakdown. Cells were then treated for a further 10min at 1, 2, 5, 8, 10 and 16.7mM glucose in the presence or absence of GLP-1 (10nM) or forskolin (10μM). The intracellular cAMP content in MIN6 cells was determined by RIA as described in materials and methods. The results shown are a mean ± SEM of 3 independent experiments. b) MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were then treated for a further 10min at 1, 2, 5, 8, 10 and 16.7mM glucose in the presence or absence of bi) GLP-1 (10nM) or bii) forskolin (10μM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control.

16.7mM glucose in the presence or absence of either GLP-1 or forskolin, an artificial activator of adenylyl cyclase as a positive control, led to an increase in the intracellular cAMP content as determined by radioimmunoassay (**Figure 3.8a**). To investigate the mechanism by which GLP-1 stimulates Erk1/2 phosphorylation, we initially characterized the activation of Erk1/2 in response to GLP-1 in MIN6 cells. MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. The cells were then incubated in increasing concentrations of glucose (1mM, 2mM, 5mM, 8mM, 10mM and 16.7mM) in the presence or absence of either GLP-1 or forskolin, an activator of adenylyl cyclase. Samples were taken at 10min and the activation state of Erk1/2 was assessed. Glucose led to a dose dependent increase in Erk1/2 activation that was potentiated by GLP-1 and forskolin at all glucose concentrations (**Figure 3.8b**). The levels of cAMP remained constant in the presence of increasing glucose concentrations in the presence of GLP-1 or forskolin. This data suggests that elevation in cAMP plays a tonic role in potentiating glucose stimulated Erk1/2 phosphorylation.

3.2.3.1 Role of Epac in GLP-1 Stimulated Erk1/2 Activation

To determine whether cAMP acting via Epac is an important determinant in GLP1-stimulated Erk1/2 activation, MIN6 cells were incubated at 16.7mM glucose alone or with GLP-1 or forskolin in the presence or absence of 8-CPT-2Me-cAMP, a specific activator of Epac. Treatment of MIN6 cells with 8-CPT-2Me-cAMP in the presence of 1mM or 16.7mM glucose had no effect on Erk1/2 activation (**Figure 3.9**). Treatment of MIN6 with 8-CPT-2Me-cAMP and GLP-1 or forskolin at 16.7mM glucose actually reduced Erk1/2 activation. This suggests that Epac activation cannot drive Erk1/2 activation or potentiate glucose stimulated Erk1/2 activation; therefore GLP-1 is unlikely to stimulate Erk1/2 via Epac. Collectively, these results indicate that GLP-1 stimulated Erk1/2 activation is not dependent on the activation of Epac.

3.2.4 Role of Rap in Glucose and GLP-1 Stimulated Erk1/2 Activation

A study in human islets showed that glucose and GLP-1 activate Rap and B-Raf which mediates Erk1/2 activation (Trumper, Ross et al. 2005). However, studies in MIN6 cells using a dominant negative Rap (RapN17) demonstrated that RapN17 had no effect on GLP-1 stimulated Erk1/2 activation (Gomez, Pritchard et al. 2002). Therefore, the role of Rap in GLP-1 stimulated Erk1/2 activation is unclear. Therefore, we re-examined the role

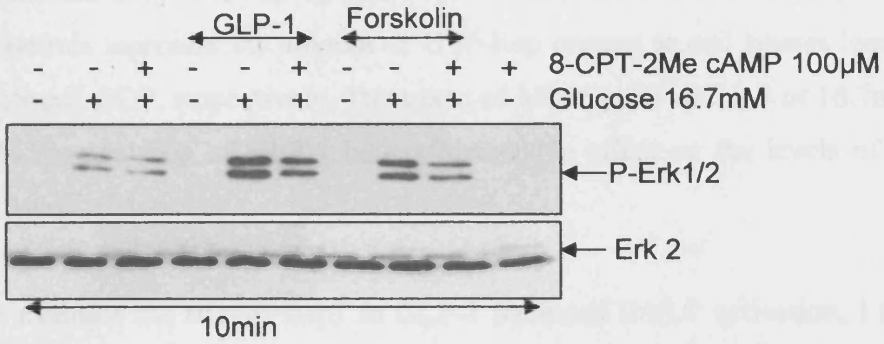


Figure 3.9: Role of Epac in GLP-1 Stimulated Erk1/2 Phosphorylation

MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were then treated with either 16.7mM glucose alone or in the presence of GLP-1 (10nM) or forskolin (10μM) in the presence or absence of 8-CPT-2Me-cAMP (100μM) for 10min. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control.

of Rap activation by measuring the levels of active GTP bound Rap in GLP-1 stimulated MIN6 cells. MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were then treated for 10min in KRB with 16.7mM glucose in the presence or absence of GLP-1. Rap1 was precipitated with the polyhistidine-tagged Rap binding domain of RalGDS (RBD) bound to nickel beads and identified by Western Blotting using a monoclonal antibody directed against Rap1 (see materials and methods). Positive and negative controls represent the amount of GTP-Rap present in cell lysates loaded *in vitro* with GTPγS and GDP, respectively. Treatment of MIN6 cells with 2.8 or 16.7mM glucose alone or in the presence of GLP-1 had no detectable effect on the levels of active Rap (**Figure 3.10a**).

To further examine the role of Rap1 in GLP-1 mediated Erk1/2 activation, I investigated the effect of a recombinant adenovirus expressing a dominant negative form of Rap. Previous studies using the dominant negative form of Rap, Rap1N17, showed that Rap was not involved in GLP-1 stimulated Erk1/2 activation in pancreatic β-cells (Gomez, Pritchard et al. 2002). However, Rap1N17 is not a true dominant negative as this mutant fails to prevent activation of wild type Rap1 suggesting that, upon over-expression, Rap1(S17N) does not block cellular signalling (Seidel, Klinger et al. 1999). Based on these findings, I have generated an adenovirus expressing Rap1(S17A). This mutant was shown to interfere and inhibit both cAMP-Epac and EGF-C3G pathways in a study to identify novel Rap1 dominant negative mutants (Dupuy, L'Hoste et al. 2005). MIN6 cells were mock-infected or infected with recombinant adenoviruses expressing an either empty vector (AdEmpty.EGFP) or Ad-Rap1A17. 48h post-infection, the cells were treated for 10min or 20min with 16.7mM glucose alone or 16.7mM glucose plus GLP-1 and the activation status of Erk1/2 determined. The over-expression of dominant negative Rap1A17 had no detectable inhibitory effect on the ability of GLP-1 to stimulate Erk1/2 activation (**Figure 3.10c**). Therefore, GLP1-stimulated Erk1/2 activation appears to be independent of the small G-protein Rap1.

We next examined the ability of B-Raf to stimulate Erk1/2 activity *in vitro* using immunocomplexed Raf kinase assays using GST-MEK. MIN6 cells were treated with 16.7mM glucose in the presence of GLP-1 for 10min. Protein lysates were prepared, and the Raf protein was immunoprecipitated using an antibody specific to B-Raf. The immunoprecipitates were incubated with GST-MEK and [γ-^{32}P] ATP and the activity of Raf assessed by monitoring the phosphorylation state of MEK. The results revealed that

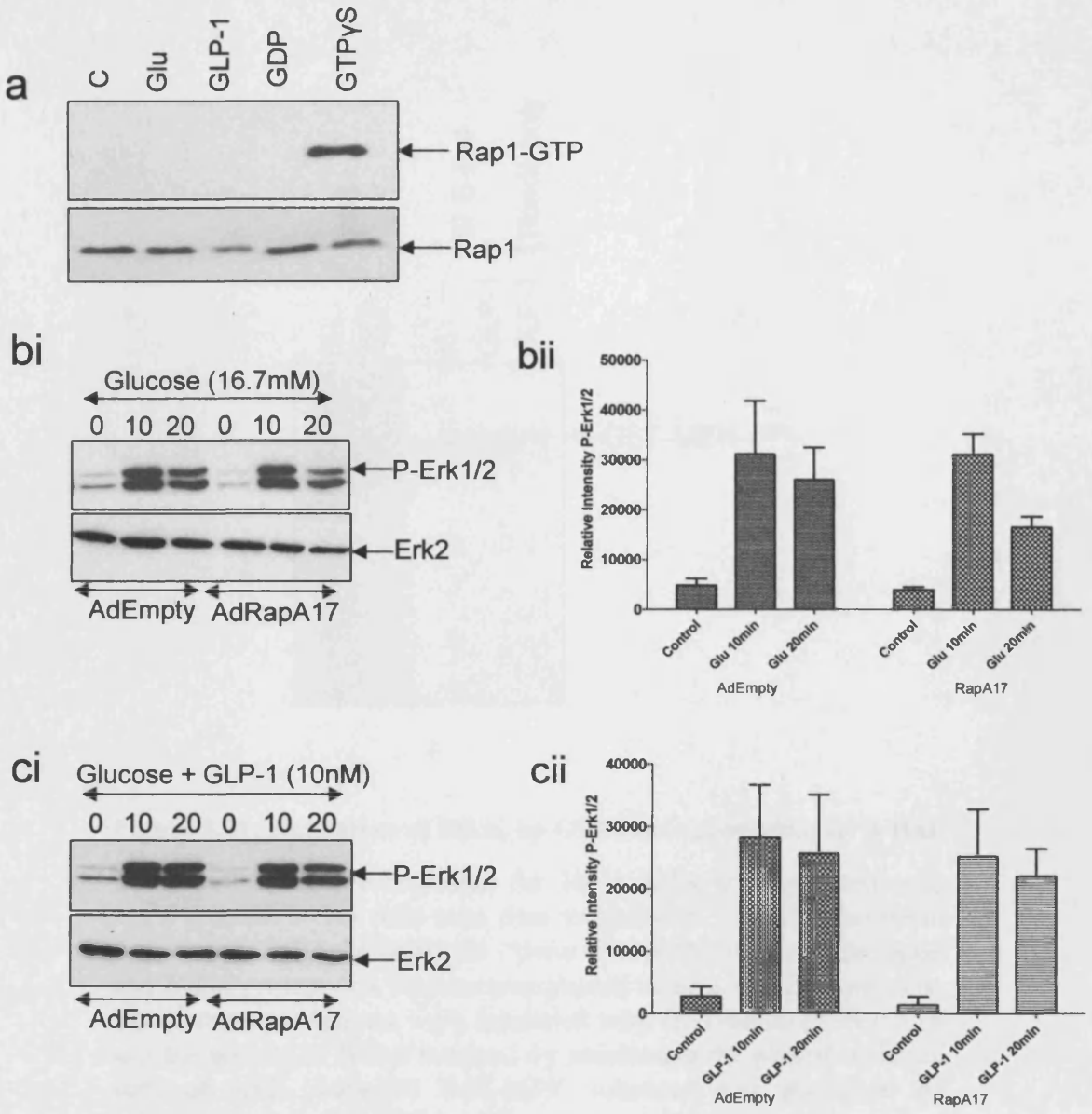


Figure 3.10: Role of Rap in Glucose and GLP-1 Stimulated Erk1/2 Phosphorylation

a) MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were then treated with either glucose (16.7mM) in the presence or absence of GLP-1 (10nM) for 10min. GTP-bound Rap1 was affinity-purified by GST pull down assay by using GST-RalGDS and detected with anti-Rap1 antibody. In addition, the two right hand lanes show negative and positive controls using 100μM of GDP and GTPγS, respectively. b,c) MIN6 cells were infected with Ad-Empty (control) and Ad-RapA17 for 48h. Following infection, the cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated for 10 or 20min in KRB containing bi) 16.7mM glucose ci) 16.7mM glucose and GLP-1 (10nM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204), total myc and total Erk2 as a loading control. bii,cii) Quantified data from phospho-ERK1/2 blots in *bi,ci* shown as means ± SEM; $n = 3$.

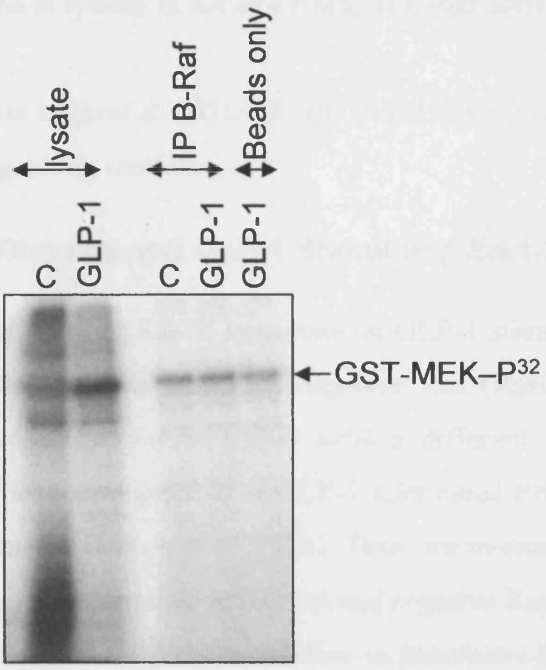


Figure 3.11: Activation of MEK by GLP1 is Independent of B-Raf.

MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. MIN6 cells were then treated with 16.7mM glucose in the presence of 10nM GLP1 for 10min. Protein lysates were prepared and B-Raf protein was immunoprecipitated using a specific antibody. The immunoprecipitates were incubated with GST-MEK [γ-^{32}P] ATP and the activity of B-Raf assessed by monitoring the phosphorylation state of MEK. Labelled GST-MEK substrate was visualized by autoradiography after SDS-PAGE. A representative autoradiogram of MEK phosphorylation detected after exposure to autoradiography film for 24-48h at room temperature is shown.

immunprecipitated B-Raf could not stimulate an increase in MEK phosphorylation following GLP-1 stimulation (**Figure 3.11**). Therefore, the GLP-1 stimulated MEK phosphorylation observed in lysates is not as a result of B-Raf activation.

Collectively, these results suggest that GLP-1 stimulated Erk1/2 activation is independent of the Rap and B-Raf signalling module.

3.2.5 Role of Ras in Glucose and GLP-1 Stimulated Erk1/2 Phosphorylation

The published results on whether Ras is important in GLP-1 stimulated Erk1/2 activation are inconsistent. In INS-1 cells, a dominant negative Ras (RasG15A) blocked glucose stimulated Erk1/2 activation whilst in MIN6 cells a different Ras dominant negative mutant RasN17, had no effect on glucose or GLP-1 stimulated Erk1/2 activation (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). Thus, we re-examined whether blocking Ras by adenoviral-mediated expression of a dominant negative Ras (RasN17) would affect glucose or GLP-1 induced Erk-1/2 phosphorylation in pancreatic β-cells. MIN6 cells were infected with either an empty virus (AdEmpty.EGFP) or AdRasN17. 48h post-infection, the cells were treated for 10 or 20min with 16.7mM glucose alone or 16.7mM glucose plus GLP-1 or EGF for 5min and the activation status of Erk1/2 determined. The over-expression of dominant negative Ras had no detectable inhibitory effect on the ability of GLP-1 to stimulate Erk1/2 activation but effectively inhibited EGF-stimulated Erk1/2 activation (**Figure 3.12b**). The over-expression of RasN17 had a small statistically insignificant effect on glucose stimulated Erk1/2 activation at 20min (**Figure 3.12a**). Therefore, glucose and GLP-1-stimulated Erk1/2 phosphorylation appears to be independent of Ras.

3.2.6 Determination of MEK Kinase Activity in MIN6 Cells

I have shown that GLP-1 stimulates the activation of Erk1/2 via a mechanism independent of both Ras and Rap. Therefore, the identity of the MEK kinase that responds to the increase in calcium and initiates the downstream signalling cascade leading to Erk1/2 activation is unknown. In order to identify the MEK kinase, I aimed to isolate a MEK kinase activity from lysates of cells stimulated with GLP-1 using recombinant MEK as a substrate in an *in vitro* kinase assay (**Figure 3.13**). To establish if GLP-1 and other secretagogues, glucose and depolarising concentrations of potassium, [K^{+50}], can induce an increase in MEK activity, MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. The cells were then incubated in 1mM or 16.7mM glucose, 16.7mM

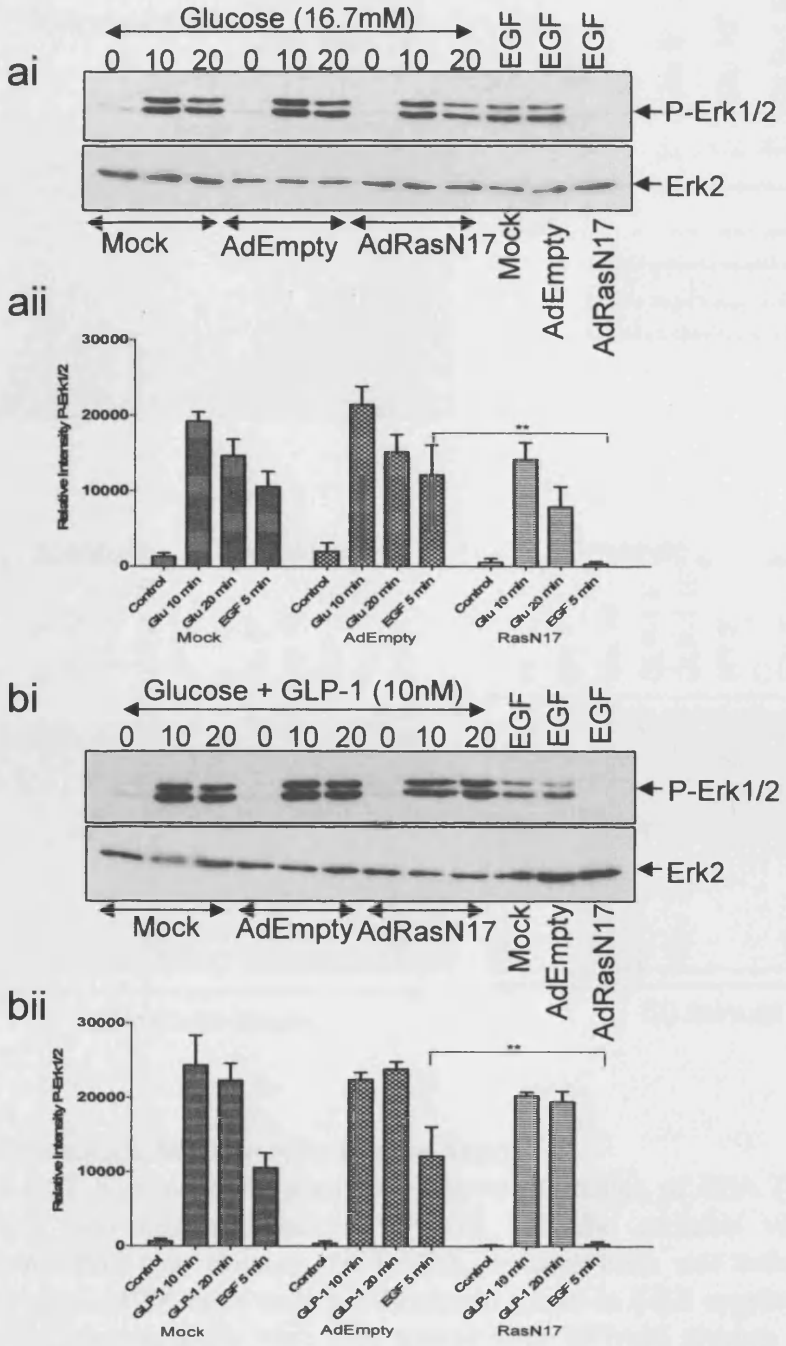


Figure 3.12: Role of Ras in Glucose and GLP-1 Stimulated Erk1/2 Phosphorylation

MIN6 cells were infected with Ad-Empty (control) or Ad-RasN17 for 48h. Following infection, the cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated for 10 or 20min in KRB containing ai) 16.7mM glucose or 20ng/ml EGF (positive control) and bi) 16.7mM glucose in the presence of GLP-1 (10nM) or 20ng/ml EGF. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control. aii,bii) Quantified data from phospho-Erk1/2 blots in *ai,bi* shown as means ± SEM; $n = 3$, ** < 0.01 P value obtained using a Two-way ANOVA.

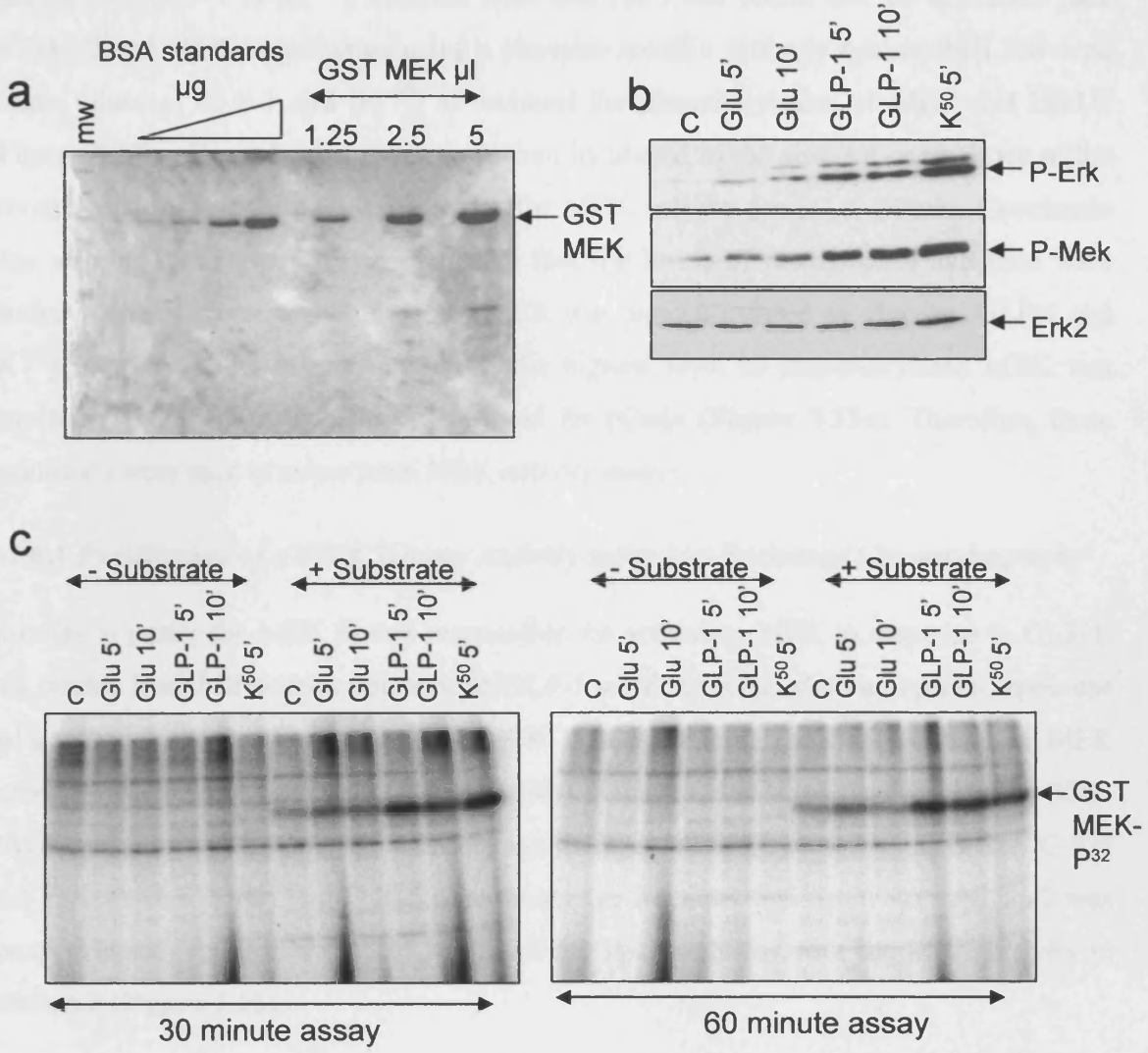


Figure 3.13: MEK *in vitro* Kinase Assay

a) GST purified MEK alongside known quantities of BSA (1, 1.25, 2.5, 5µg) was separated on SDS-PAGE and the proteins visualised by Coomassie blue staining. GST-MEK concentration was estimated to be ~2µg/µl. MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were then treated with 16.7mM glucose (Glu) in the presence or absence GLP-1 (10nM) and K+50 for 5 or 10min. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204), phospho-MEK or total Erk2 as a loading control. c) The same cell lysates as in (b) were used to carry out *in vitro* MEK kinase assays for 30 or 60min in the presence or absence of GST-MEK as a substrate as described in materials and methods. Labelled GST-MEK substrate was visualized by autoradiography after SDS-PAGE. Representative autoradiograms of MEK phosphorylation detected after exposure to autoradiography film for 24-48h at room temperature.

glucose plus GLP-1 or [K^{+50}]. Samples were taken at 5 and 10min and the activation state of Erk1/2 and MEK investigated using a phospho-specific antibody against their activated forms. Glucose, GLP-1 and [K^{+50}] all induced the phosphorylation of MEK and Erk1/2 (**Figure 3.13b**). Treated cell lysates were then incubated in the absence or presence of the recombinant MEK substrate and assayed for MEK activity for 30 or 60min. Coomassie blue staining of SDS-PAGE gels revealed that the levels of recombinant substrate were similar between kinase assay reactions. MEK was phosphorylated by glucose, GLP-1 and [K^{+50}] at both 30 and 60min. However, the highest level of phosphorylated MEK was detected at 10min GLP-1 treatment assayed for 60min (**Figure 3.13c**). Therefore, these conditions were used in subsequent MEK activity assays.

3.2.6.1 Purification of a MEK Kinase Activity using Ion Exchange Chromatography

In order to purify the MEK kinase responsible for activating MEK in response to GLP-1, cell lysates from MIN6 cells treated with GLP-1 were fractionated on a heparin-Sepharose column and each fraction assayed for MEK kinase activity. The highest level of MEK activity was eluted in fraction 3 (**Figure 3.14b**). Each fraction was also separated by SDS-PAGE and immunoblotted with anti-sera against four MEK kinases, A-Raf, B-Raf, C-Raf and Tpl2. A-Raf, B-Raf and C-Raf were present in fractions 4, 5 and 6 whereas Tpl2 was found primarily in fractions 1 and 3. Therefore, Tpl2 co-elutes with the MEK activity in fraction 3 (**Figure 3.15**).

3.2.7 Role of Tpl2 in GLP-1 Regulated Erk1/2 Phosphorylation in MIN6 Cells

Tpl2 is a member of the MAPKKK family of serine/threonine kinases. Tpl2 is activated by pro-inflammatory mediators and plays a key role in regulating the MAPK pathway in macrophages. The results presented above indicate that Tpl2 might be the activator of MEK in response to GLP-1 in pancreatic β-cells. To test whether Tpl2 is activated by GLP-1, I performed a Tpl2 *in vitro* kinase assay on MIN6 cells treated with GLP-1 or, as a positive control, on RAW264.7 cells (macrophages) stimulated with LPS (1μg/ml). The Tpl2 immunoprecipitates were incubated with GST-MEK and GST-Erk1/2 for 30min in the presence of 1mM unlabelled ATP. Fractions of the kinase reactions were then incubated with MBP and [γ-^{32}P]ATP and the activity of Tpl2 assessed by monitoring the phosphorylation state of MBP. *In vitro* kinase assays on Tpl2 immunprecipitated from LPS-stimulated RAW264.7 cells showed that the activity of the Tpl2 kinase is low prior to stimulation and

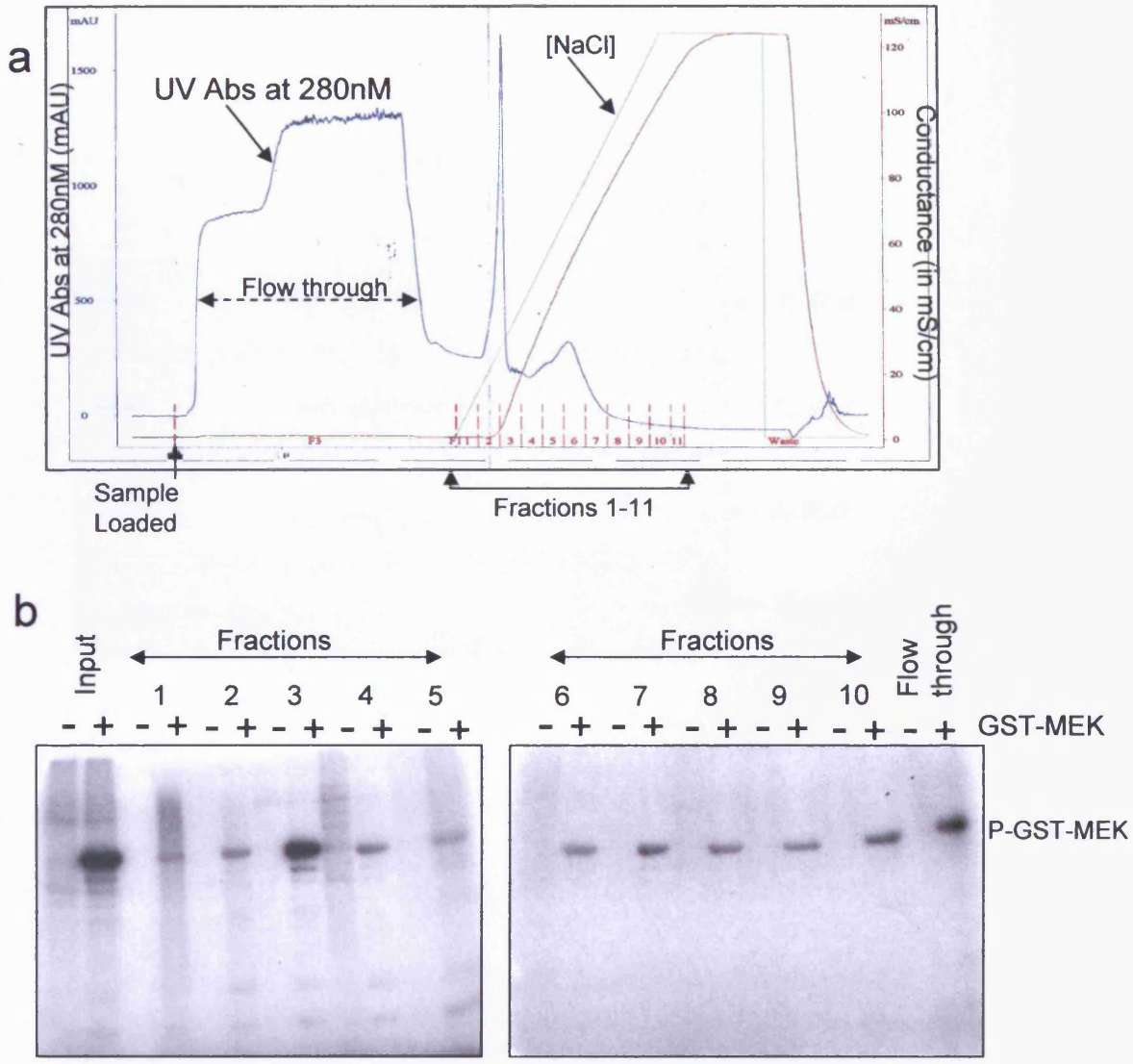


Figure 3.14: Partial Purification of a MEK1 Activator from GLP-1 Stimulated MIN6 Cells

MIN6 cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated with 16.7mM glucose in the presence of GLP-1 (10nM) for 10min. GLP-1 treated lysate was purified as described under materials and methods on a Heparin sepharose column. Fractions were collected over an increasing salt gradient 0-2M NaCl (see materials and methods) (a) The graph represents an elution profile measuring Abs at 280 nm. (b) An aliquot of each fraction was used in an *in vitro* kinase reaction in the absence (–) or presence (+) GST-MEK (4µg). Labelled GST-MEK substrate was visualized by autoradiography after SDS-PAGE. Representative autoradiograms of MEK phosphorylation detected after exposure to autoradiography film after 24-48h at room temperature.

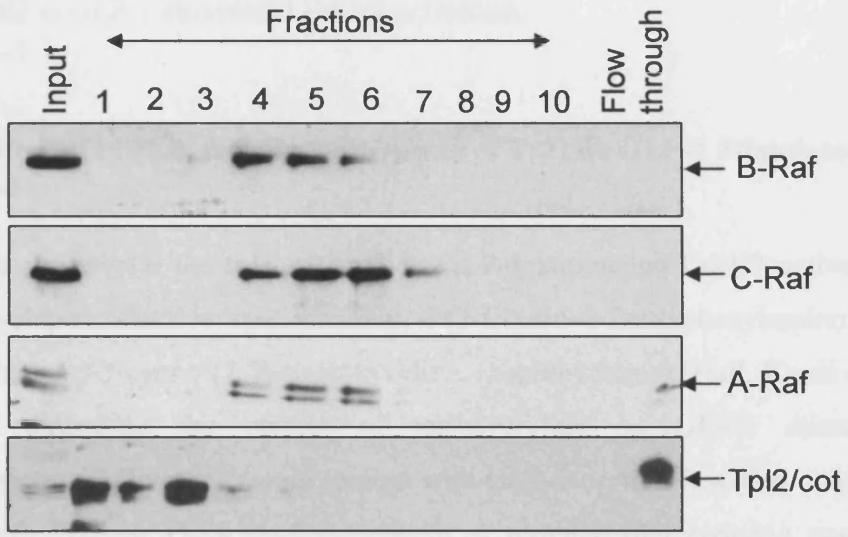


Figure 3.15: GLP-1 Stimulated MEK Kinase co-elutes with Tpl2

MIN6 cells were pre-incubated in KRB supplemented with 1mM glucose for 1h. Cells were then treated with 16.7mM glucose in the presence of GLP-1 (10nM) for 10min. GLP-1 treated lysate was then fractionated into 10 fractions as described under materials and methods on a Heparin sepharose column. Each fraction was run on a SDS-PAGE gel and Western blotted using anti-B Raf, C-Raf, A Raf or Tpl2/cot antibodies.

increases rapidly following LPS treatment (**Figure 3.16a**). *In vitro* kinase assays on Tpl2 immunprecipitated from GLP-1 stimulated MIN6 cells showed an increase in activity following GLP-1 stimulation (**Figure 3.16b**). However, I have been unable to consistently reproduce this finding. Therefore, further studies will need to be performed to confirm the role of Tpl2 in GLP-1 stimulated Erk1/2 activation.

3.2.7.1 Effect of Pharmacological Inhibition of Tpl2 on GLP-1 Stimulated Erk1/2 Activation

To further investigate the role of Tpl2 in GLP-1 stimulated Erk1/2 activation, I used a newly developed selective Tpl2 inhibitor, 4-(3-Chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-[1,7]-naphthyridine, (naphthyridine) (Hall, Kurdi et al. 2007). In order to determine the effects of naphthyridine on GLP-1 stimulated Erk1/2 phosphorylation, MIN6 cells were treated with GLP-1 or, as a negative control, cells were treated with EGF or PMA in the presence or absence of increasing concentrations of naphthyridine. The activation state of Erk1/2 was assessed using a phospho-specific antibody. GLP-1 stimulated Erk1/2 phosphorylation was dose dependently inhibited by naphthyridine (**Figure 3.17a**). However, naphthyridine also inhibited PMA and EGF stimulated Erk1/2 activation (**Figure 3.17b/c**). To further examine the efficacy of naphthyridine, I performed *in vitro* MEK kinase assays in the presence or absence of naphthyridine at concentrations found to be selective for Tpl2 (Hall, Kurdi et al. 2007). MIN6 cells were treated with GLP-1 or EGF or as a control RAW264.7 macrophages were treated with LPS. The treated lysates were then incubated with naphthyridine followed by an *in vitro* MEK kinase assay. At all concentrations of naphthyridine tested, GLP-1, EGF or LPS induced MEK phosphorylation was unaffected (**Figure 3.17d**). Taken altogether, these results call into question the specificity and effectiveness of naphthyridine as a specific and selective inhibitor of Tpl2. Therefore, its use in determining the role of Tpl2 in GLP-1 stimulated Erk1/2 activation is questionable.

3.2.7.2 Mechanisms of GLP-1 Stimulated Tpl-2 Activation in MIN6 cells

Based on data showing that GLP-1 stimulated Erk1/2 activation was dependent on Tpl2, I examined the possible signalling pathway by which GLP-1 may activate Tpl2 in pancreatic β-cells. In macrophages, Ikappa B Kinase (IKK) is an essential upstream activator of Tpl2. Inhibition of IKKß by 15dPGJ2 in macrophages blocks LPS stimulated Tpl2 activity (Waterfield, Jin et al. 2004). To investigate if GLP-1 stimulated Erk1/2 signalling requires

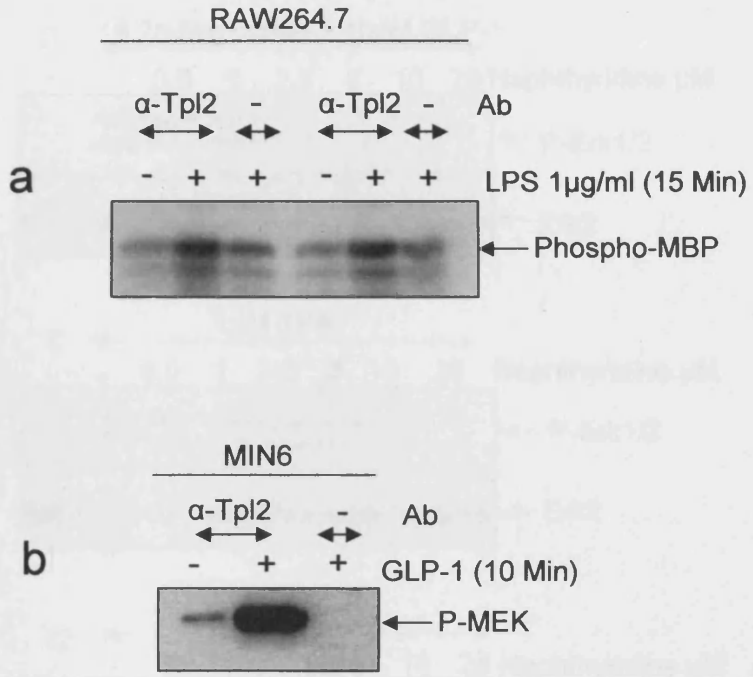


Figure 3.16: Role of Tpl2 in GLP-1 Stimulated Erk1/2 Activation

a) RAW264.7 cells were seeded 18h prior to treatment. Cells were stimulated with LPS (1μg/ml) for 15min or left untreated. Tpl2 was immunoprecipitated from RAW264.7 cell lysates with an anti-Tpl2 (70mer) antibody, and its MEK kinase activity was determined by coupled MEK/Erk kinase assay as described in the materials and methods. Labelled MBP substrate was visualized by autoradiography after SDS-PAGE. b) MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose. Cells were left untreated (-) or then treated for 10min with glucose (16.7mM) in the presence of GLP-1 (10nM) (+). Tpl2 was immunoprecipitated from MIN6 cell lysates with anti-Tpl2 (70mer) antibody, and its MEK kinase activity was determined by a cold MEK kinase assay as described in the materials and methods. Kinase reactions were separated by SDS PAGE analysis followed by immunblot analysis using a phospho-MEK antibody.

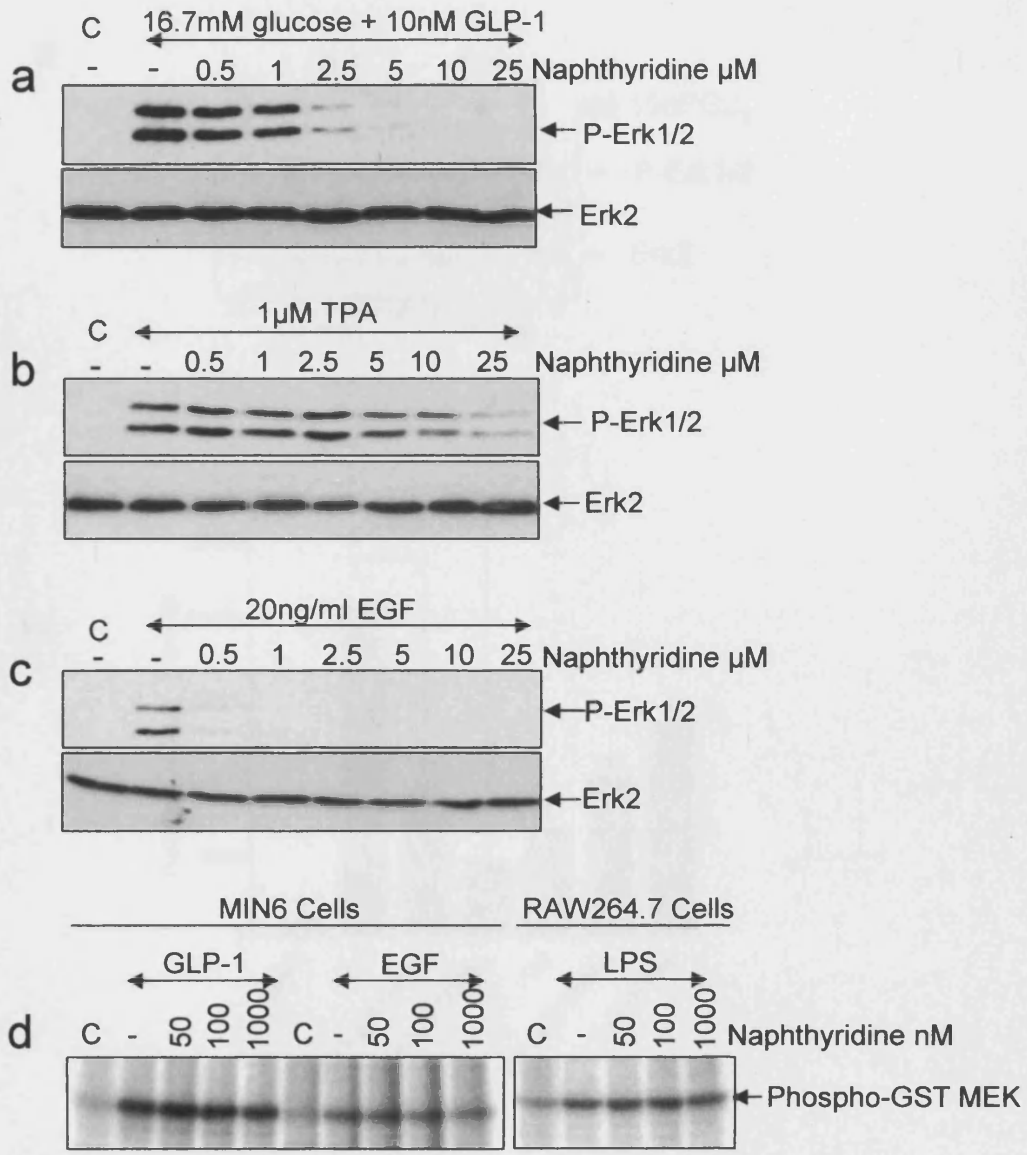


Figure 3.17: Characterisation of a Tpl2 Inhibitor in MIN6 Cells

MIN6 cells were pre-incubated at 1mM glucose in KRB for 1h (Control, (C)) in the presence of increasing concentrations of Tpl2 inhibitor (naphthyridine) as indicated in the figure. Cells were then treated with (a) 10nM GLP-1 in the presence of 16.7mM glucose for 10min (b) TPA (1µM) for 15min or (c) EGF (20ng/ml) for 5min. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control. d) *In vitro* kinase assay to test the selectivity of the Tpl2 inhibitor. Equal amounts of cell lysate from GLP-1 or EGF treated MIN6 cells or LPS stimulated RAW264.7 were incubated with increasing concentrations of Tpl2 inhibitor (naphthyridine) in the presence of recombinant MEK and [γ-^{32}P]-ATP for 1h. Labelled MEK substrate was visualized by autoradiography after SDS-PAGE. Representative autoradiogram of MEK phosphorylation detected after exposure to autoradiography film for 24-48h at room temperature.

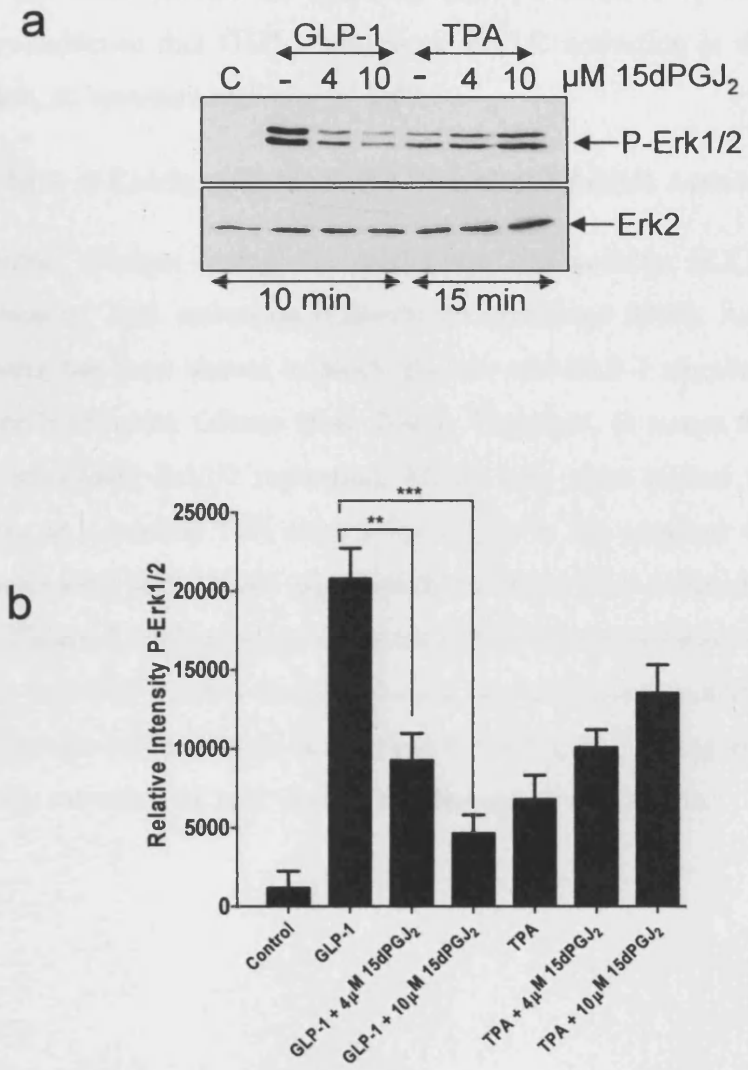


Figure 3.18: Role of Tpl-2 in GLP-1 Stimulated Erk1/2 Phosphorylation

a) MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose (Control, (C)). Cells were then treated with 16.7mM glucose plus 10nM GLP-1 for 10min or, as a control, TPA (1μM) for 15min in the presence or absence of 4μM or 10μM $15dPGJ_2$ (an IKKβ inhibitor). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control. b) Quantified data from phospho-ERK1/2 blots in a) shown as means ± SEM; $n = 3$. ** $P < 0.01$, *** $P < 0.001$, P value obtained using a One-way ANOVA.

IKK activation, MIN6 cells were treated with GLP-1 (10nM) or as a control TPA (1μM) in the presence or absence of 15dPGJ2. 15dPGJ2 significantly blocked GLP-1 stimulated Erk1/2 activation but had no effect on Erk1/2 stimulated by PMA (**Figure 3.18**). This provides evidence that GLP-1 stimulated Erk1/2 activation is dependent upon IKK for activation, an upstream activator of Tpl2.

3.2.7.3 Role of Calcineurin in GLP-1 Stimulated Erk1/2 Activation

In neurons, calcium acting via calcineurin can activate IKK, which is an essential component of Tpl2 activation (Lilienbaum and Israel 2003). Additionally, inhibition of calcineurin has been shown to block glucose and GLP-1 stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). Therefore, to assess the role of calcineurin in GLP-1 stimulated Erk1/2 activation, MIN6 cells were treated with GLP-1 (10nM) for 10min or as a control TPA (1μM) for 15min in the presence or absence of FK506 (a calcineurin inhibitor). FK506 significantly inhibited GLP-1 stimulated Erk1/2 activation at 10μM (**Figure 3.19**) but had no effect on Erk1/2 phosphorylation stimulated by PMA. This suggests that GLP-1 activates Erk1/2 via a mechanism dependent upon calcineurin. These results provide evidence that, in response to GLP-1 Ca^{2+} acting via calcineurin may play a role in the activation of Tpl2 via an IKK dependent mechanism.

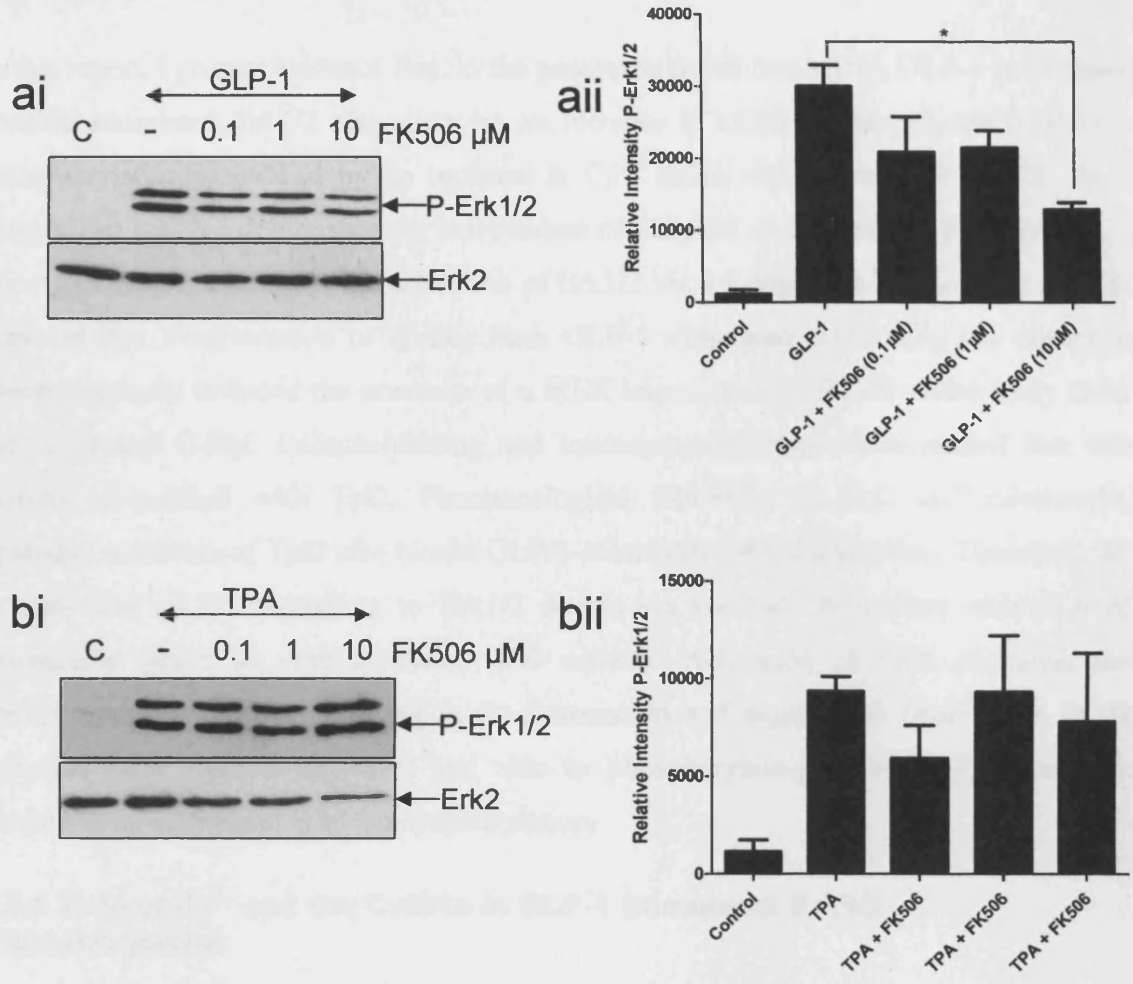


Figure 3.19: Role of Tpl-2 in GLP-1 Stimulated Erk1/2 Phosphorylation

MIN6 cells were pre-incubated for 1h in KRB supplemented with 1mM glucose (control (C)). Cells were then treated with ai) 16.7mM glucose plus 10nM GLP-1 for 10min or bi) as a control TPA (1µM) for 15min in the presence or absence of FK506 (a calcineurin inhibitor) at the concentrations indicated in the figure. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-Erk1/2 (Thr202, Tyr204) or total Erk2 as a loading control. aii,bii) Quantified data from phospho-Erk1/2 blots in *ai,bi* shown as means ± SEM; $n = 3$, * $P < 0.05$. P value obtained using a One-way ANOVA.

3.3 Discussion

In this report, I present evidence that, in the pancreatic β-cell line MIN6, GLP-1 potentiates glucose stimulated Erk1/2 activation via an increase in cAMP. This increase in Erk1/2 phosphorylation is evoked by an increase in Ca^{2+} influx which activates Erk1/2 via a mechanism which I demonstrate is independent of CaMKII and CaMKIV. Furthermore, I show that GLP-1 stimulates the activation of Erk1/2 via a mechanism independent of both Rap and Ras. Fractionation of lysates from GLP-1 stimulated cells using ion exchange chromatography revealed the presence of a MEK kinase activity which eluted away from the A, B and C-Raf. Immunoblotting and immunoprecipitation demonstrated that this activity co-purified with Tpl2. Pharmacological inhibition of IKK and calcineurin, upstream activators of Tpl2 also blocks GLP-1 stimulated Erk1/2 activation. Therefore, we propose that GLP-1 signalling to Erk1/2 occurs via the Ca^{2+} dependent activation of calcineurin which in turn increases IKK activity. Activation of IKK promotes the phosphorylation of p105 resulting in its dissociation and degradation from Tpl2. In its unbound state, Tpl2 is activated and able to phosphorylate/activate MEK, ultimately leading to an increase in Erk1/2 phosphorylation.

3.3.1 Role of Ca^{2+} and the CaMKs in GLP-1 Stimulated Erk1/2 Phosphorylation

In pancreatic β-cells, the activation of Erk1/2 has been shown to be dependent on a rise in intracellular free Ca^{2+} levels through Ca^{2+} efflux from the ER (Arnette, Gibson et al. 2003) and Ca^{2+} entry through L-type voltage-gated calcium channels (L-type VGCC) (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). In neurons, the mode of Ca^{2+} entry is also critical in determining which signalling pathway is activated and is thought to be mediated through the local increase in Ca^{2+} concentration around the mouth of the L-type VGCC resulting in the binding of Ca^{2+}/calmodulin to the L-type VGCC and the subsequent activation of Erk1/2 (Dolmetsch, Pajvani et al. 2001). Therefore, given the important role of Ca^{2+} in GLP-1 stimulated Erk1/2 activation, it is possible that the increased influx of calcium via elevations in cAMP is the mechanism by which GLP-1 potentiates glucose-dependent Erk1/2 activation. The Ca^{2+} dependency of glucose and GLP-1 stimulated Erk1/2 activation appeared to be dependent on calmodulin, as W7 (calmodulin inhibitor) inhibits glucose and GLP-1 stimulated Erk1/2 activation in MIN6 and INS-1 cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). Calmodulin is also required for the activation of CaMKs and pharmacological inhibition of CaMKII using KN62 and KN93

inhibits GLP-1 stimulated Erk1/2 activation in MIN6 cells and INS-1 cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). Therefore, CaMKII may play an important role in GLP-1 stimulated Erk1/2 activation (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). However, my data demonstrates that adenoviral mediated expression of dominant negative CaMKII has no inhibitory effect on glucose or GLP-1 stimulated Erk1/2 activation in the pancreatic β-cell line, MIN6 (**Figure 3.5**) but surprisingly lead to an increase in K^{+50} stimulated Erk1/2 activation. A possible explanation for this is that the activation of CaMKII can lead to the inhibition of certain isoforms of adenylyl cyclase (Dyer, Liu et al. 2005). Four isoforms of AC are regulated directly by Ca^{2+}: AC1 and AC8 are stimulated by Ca^{2+}-CaM, whereas AC5 and AC6 are inhibited by Ca^{2+}. In addition, certain isoforms are regulated by enzymes that are themselves Ca^{2+}-regulated: AC9 is inhibited by Ca^{2+}-calcineurin, and AC1 and AC3 are inhibited by Ca^{2+}-calmodulin-dependent protein kinases IV and II, respectively (Dyer, Liu et al. 2005). It has been demonstrated in rat islets that multiple isoforms of AC including 2-7 are expressed (Leech, Castonguay et al. 1999). Therefore, it is possible that the inhibition of CaMKII by the over-expression of the dominant negative form of CaMKII may lead to an increase in adenylyl cyclase (AC3) activity which elevates cAMP levels that augments glucose-stimulated Ca^{2+} entry through L-type VGCC leading to an increase in Erk1/2 activation.

In PC12 cells and cortical neurons, over-expression of CaMKIV has been shown to drive MAPK activation (Enslen, Tokumitsu et al. 1996). The mechanism by which CaMKIV can activate Erk1/2 is reported to be via the phosphorylation of the small G Protein Rap1, which increases Erk1/2 activation via B-Raf (Sahyoun, McDonald et al. 1991). However, over-expression of constitutively active CaMKIV was unable to stimulate or potentiate Erk1/2 activation in response to glucose and GLP-1 in MIN6 cells (**Figure 3.7**).

In addition to CaMKII or CaMKIV, CaMKI has been shown as well to stimulate Erk1/2 activity (Schmitt, Guire et al. 2005). In addition to its calcium/calmodulin dependence, CaMKI also requires phosphorylation to become fully active. CaMKI is activated by Thr177 phosphorylation by CaMKK (Haribabu, Hook et al. 1995). Interestingly, inhibition of CaMKK with STO-609 blocks depolarization-induced Erk1/2 activation in NG108 neuroblastoma cell line (Schmitt, Wayman et al. 2004). Whether CaMKI is able to mediate glucose and GLP-1 stimulated Erk1/2 phosphorylation is unknown.

Erk1/2 can also be activated in a Ca^{2+} dependent manner by the modulation of small G-proteins via the activation of specific guanine nucleotide exchange factors for the G-protein Ras including Ras-GRP and Ras-GRF (Walker, Cullen et al. 2003). Ras-GRF is activated by the binding of Ca^{2+} and calmodulin (CaM). Once bound, Ras-GRF facilitates the GTP exchange and Ras activation. Therefore, an increase in Ca^{2+} following glucose metabolism may lead to the activation of Erk1/2 via the activation of Ras. However, GLP-1 stimulated Erk1/2 activation is likely to be independent of Ras (Gomez, Pritchard et al. 2002).

3.3.2 Role of cAMP in GLP-1 Stimulated Erk1/2 Activation

It has previously been shown that, in the presence of stimulatory concentrations of glucose, GLP-1 stimulates the activation of adenylyl cyclase and an increase in cAMP (Drucker, Philippe et al. 1987). We provide evidence that the ability of GLP-1 to signal to Erk1/2 is dependent on an increase in cAMP, since artificial activation of adenylyl cyclase with forskolin can mimic the effect of GLP-1 on Erk1/2 phosphorylation (**Figure 3.8**). In addition, glucose led to a dose dependent increase in Erk1/2 activation that was potentiated by GLP-1 at all glucose concentrations. However, the intracellular cAMP levels remained constant and did not parallel Erk1/2 phosphorylation. In INS-1 cells, a study by Briaud *et al.* demonstrated that glucose-induced activation of Erk1/2 is dependent on an increase in Ca^{2+} influx via a PKA dependent mechanism (Briaud, Lingohr et al. 2003). They hypothesized that glucose stimulated Erk1/2 activation is dependent upon an increase in Ca^{2+} influx which activates Ca^{2+}/CaM regulated adenylyl cyclase isoform (AC3), leading to an increase of cAMP levels that activate PKA. PKA then causes Raf-1 or MEK phosphorylation which mediates Erk1/2 phosphorylation (Briaud, Lingohr et al. 2003). However, based on our data we hypothesise that elevations in cAMP which has a tonic effect in potentiating glucose stimulated Erk1/2 phosphorylation likely occurs via an increase in Ca^{2+} influx. Indeed, elevations in cAMP leads to the subsequent activation of PKA and cAMP regulated exchange factor II (Epac), which augments glucose-stimulated Ca^{2+} entry through L-type VGCC by regulating a multitude of events, which include the alteration of ion channel activity and increases in intracellular calcium (Holst 2007). PKA has been shown to phosphorylate the α1.2 subunit of L-type VGCC in pancreatic β-cells (Leiser and Fleischer 1996). Phosphorylations of a Ser1928 of the α1.2 subunit and both Ser478/479 of the β_{2a}-subunit by PKA increase the activity of VGCC, thus increasing Ca^{2+} influx (Bunemann, Gerhardstein et al. 1999). Closure of K_{ATP} channels as a result of an increase in intracellular ATP, a product of glucose metabolism, may also be affected by

PKA. It has been demonstrated that the point mutation at position Ser1448A in the ADP-sensing region of the sulfonylurea receptor (SUR1) which forms part of the K_{ATP} channel, removed the modulatory effects of PKA. Therefore, PKA-mediated phosphorylation of Ser1448 of the SUR1 subunit leads to K_{ATP} channel closure via an ADP-dependent mechanism and an increase in Ca^{2+} influx (Light, Manning Fox et al. 2002). In rodent islets and MIN6 cells it has been reported that PKA is able to promote the release of Ca^{2+} via the IP_3 receptors to amplify the levels of intracellular Ca^{2+} (Tsuboi, da Silva Xavier et al. 2003; Dyachok and Gylfe 2004). Like PKA, Epac also increase intracellular Ca^{2+} levels. Epac interacts with the ryanodine receptor (RyR) an ER resident channel to increase Ca^{2+} release from the ER (Tsuboi, da Silva Xavier et al. 2003).

In many cell types, hormones that increase intracellular cAMP inhibit Erk1/2 signalling (Schmitt and Stork 2001). However, in other cell types, such as PC12 and neuronal cells, increased intracellular cAMP leads to an increase in Erk1/2 activation (Vossler, Yao et al. 1997). cAMP activation or inhibition of Erk1/2 is thought to occur through the activation of either PKA or Epac. However, we have demonstrated that activation of Epac using the Epac-specific cAMP analogue 8-pCPT-2Me-cAMP, which selectively activate Epac but not PKA, had no effect on GLP-1 or forskolin induced Erk1/2 phosphorylation (**Figure 3.9**). Our results are consistent with other studies that showed that the activation of Epac using 8-pCPT-2Me-cAMP, activates a distinct pool of Rap1 which does not lead to Erk1/2 activation (Wang, Dillon et al. 2006). However, relocalization of Epac to the plasma membrane by a membrane targeting motif enables cAMP to activate Erk1/2 via Rap1 and B-Raf, suggesting that the spatial localization of Epac is important in its signalling.

3.3.3 Role of Rap in GLP-1 Stimulated Erk1/2 Activation

In PC12 cells, the activation of Rap1 leads to the activation of B-Raf, which in turn activates Erk1/2 (York, Yao et al. 1998; Stork 2005). Rap is regulated by guanine nucleotide exchange factors (GEFs) such as Epac and C3G which are activated in a PKA independent or PKA dependent manner, respectively. It has been shown that although Epac can activate Rap, this does not lead to Erk1/2 activation (Wang, Dillon et al. 2006). However, it has been reported that Rap1 activated by C3G is able to promote Erk1/2 phosphorylation (Wang, Dillon et al. 2006). Therefore, the mode of Rap1 activation is an important determinant for the regulation of Erk1/2 by cAMP. In human islets, Rap1 was shown to be in its GTP bound state. Furthermore, Rap1 co-immunoprecipitated with B-Raf following glucose and GLP-1 stimulation (Trumper, Ross et al. 2005). I was unable to

detect Rap.GTP following GLP-1 stimulation in MIN6 cells (**Figure 3.10a**), suggesting that there is no Rap.GTP after GLP-1 stimulation in MIN6 cells. However, a recent study in the β-cell line MIN6 revealed that GLP-1 increases the levels of Rap.GTP and that the increases in Rap.GTP plays an important role in PKA-independent potentiation of insulin secretion (Shibasaki, Takahashi et al. 2007). An increase in Rap.GTP in response to GLP-1 as seen in human islets maybe important in insulin secretion but not Erk1/2 activation (Shibasaki, Takahashi et al. 2007). Indeed, in MIN6 cells a dominant negative Rap (Rap1N17) has no effect on GLP-1 stimulated Erk1/2 phosphorylation in MIN6 cells (Gomez, Pritchard et al. 2002). Additionally, my data demonstrates that over-expression of the dominant negative RapA17 had no inhibitory effect on GLP-1 stimulated Erk1/2 phosphorylation (**Figure 3.10b/c**). This strengthens my hypothesis that GLP-1 stimulated Erk1/2 phosphorylation is independent of Rap1.

Following GLP-1 stimulation, immunoprecipitation of B-Raf, a downstream target of Rap1 had no effect on MEK phosphorylation, which indicates that B-Raf is not involved in GLP-1 stimulated Erk1/2 activation in agreement with Gomez *et al* (Gomez, Pritchard et al. 2002) (**Figure 3.11**). However, another possible mechanism for GLP-1 stimulated Erk1/2 activation maybe via Rap2. Rap2 shares a 60% identity to Rap1 and it has been reported to share many of the same effector proteins as Rap1, except for the RPIP8. RPIP8 is expressed preferentially in the brain and pancreatic β-cells and binds to Rap2 in a GTP-dependent manner, however its function is unknown (Janoueix-Lerosey, Pasheva et al. 1998). Rap2 also shares the same GEFs and GAPs as Rap1 indicating that Rap2 may function in the same signalling cascade as Rap1. The major difference between the two isoforms is the low sensitivity of Rap2 to GAPs, which results in the long half life of Rap2.GTP (Ohba, Mochizuki et al. 2000). The RapA17 dominant negative used in this study contains a mutation within the region responsible for binding to the terminal phosphate of GTP. Therefore a mutation at this position S17A results in a loss of affinity for GTP. In addition, over-expression of Rap1A17 also acts by titrating GEFs and hence interfere with the activation of endogenous Rap1 (Dupuy, L'Hoste et al. 2005). Given that Rap2 shares many of the same GEFs and GAPs as Rap1 the over-expression of Rap1A17 is likely to inhibit the activation of endogenous Rap2 by titrating GEFs required for its activation.

One possible mechanism by which Erk1/2 could be activated by cAMP, in a Rap-independent mechanism, is through the activation of Src which has been shown to activate

Erk1/2 in many cell types (Klinger, Kudlacek et al. 2002). Src has been reported to be activated via a number of mechanisms, including the direct activation by GPCR via the Gsα subunit (Ma, Huang et al. 2000). Alternatively, Src can be activated via the transactivation of tyrsosine kinase receptors (Ma, Huang et al. 2000). Erk1/2 has also been shown to be activated by Src following receptor endocytosis where β-arrestin functions as an adaptor protein for the recruitment of Src to form large signalling complexes (McDonald and Lefkowitz 2001). A study by Klinger *et al.* demonstrated that cAMP can activate Erk1/2 in a PKA dependent manner via the activation of Src (Klinger, Kudlacek et al. 2002). It has been shown that the phosphorylation of Ser17 on Src by PKA promotes its release from the plasma membrane which allows Src to be directed towards a specific set of targets (Schmitt and Stork 2002). Indeed, in INS-1 cells, inhibition of Src using PP2 caused a partial but significant reduction in Erk1/2 phosphorylation in response to glucose (Arnette, Gibson et al. 2003). Therefore, cAMP generated by GLP-1R activation may activate Src in a PKA dependent manner to mediate Erk1/2 activation.

3.3.4 Role of Ras in GLP-1 Stimulated Erk1/2 Activation

cAMP activation of Ras has been demonstrated in neuronal cells as well as endocrine cells (Ambrosini, Tininini et al. 2000; Busca, Abbe et al. 2000). In neuronal cells, it has been shown that depolarization induced Erk1/2 activation is dependent on Ca^{2+} influx and the activation of the small G-protein Ras (Obara, Horgan et al. 2007). This activation has been proposed to be via Ca^{2+} dependent activation of Ras specific GEFs, RasGRP and RasGRF (Norum, Dawood et al. 2007). Ras has also been demonstrated to be activated by cAMP by both PKA dependent and PKA independent mechanisms (Ambrosini, Tininini et al. 2000). The PKA independent activation of Ras by cAMP could be via the activation of a cAMP regulated GEF, cNRasGEF (cyclic nucleotide Ras GEF), following cAMP binding (Pham, Cheglakov et al. 2000). In PC12 cells, cAMP has been reported to activate Ras via the transactivation of the EGF or NGF receptors (Lee, Rajagopal et al. 2002). Therefore, it is possible that Ca^{2+} and/or cAMP activate Ras in pancreatic β-cells to activate Erk1/2.

Reports in the literature disagree as to the role of Ras in glucose and GLP-1 stimulated Erk1/2 activation in pancreatic β-cells. Adenoviral mediated expression of a dominant negative form of Ras (RasN17) had no effect on glucose stimulated Erk1/2 activation in INS-1 cells (Briaud, Lingohr et al. 2003) or glucose and GLP-1 stimulated Erk1/2 activation in MIN6 cells but effectively blocked EGF stimulated Erk1/2 activation (Gomez, Pritchard et al. 2002). In contrast, expression of a different dominant negative

Ras (RasG15A) blocked glucose stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). However, in this last study there was no positive or negative control to show the effectiveness of the RasG15A adenovirus and adenovirus over-expression can inhibit glucose and GLP-1 stimulated Erk1/2 signalling. The differences in these results may be due to the mechanism by which these dominant negative mutants work. Ras(S17N) fails to bind effector proteins but instead binds tightly to guanine nucleotide exchange factors thereby titrating GEFs, which interferes with the activation of endogenous Ras. The Ras(G15A) mutant also belongs to this class of dominant negative mutant, although it is considered to be free of nucleotides and therefore even more efficient at sequestering Ras guanine nucleotide exchange factors (Fiordalisi, Holly et al. 2002). Another possibility for the conflicting data may be due to the different cell types used in each study. However, in MIN6 cells, adenoviral mediated over-expression of the dominant negative RasN17 had no effect on GLP-1 stimulated Erk1/2 activation but effectively blocks EGF stimulated Erk1/2 activation which is known to signal to Erk1/2 via Ras (**Figure 3.12**). In addition, RasN17 had no effect on GLP-1 stimulated Erk1/2 activation in mouse islets but effectively blocked EGF stimulated Erk1/2 activation in these cells (Gomez and Herbert unpublished results).

Previous studies are in agreement that glucose stimulated Erk1/2 activation is dependent on the upstream activation of MEK (Benes, Poitout et al. 1999; Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003; Briaud, Lingohr et al. 2003). However, the mechanism of MEK activation in pancreatic β-cells is unclear. In MIN6 cells, it has been shown that GLP-1 stimulated Erk1/2 activation is independent of Raf since none of the three isoforms were activated following GLP-1 stimulation as assessed by an *in vitro* kinase assay (Gomez, Pritchard et al. 2002). In contrast, the expression of a dominant negative form of Raf-1 (RafC4B) blocked glucose stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003). However, I have identified a GLP-1 stimulated MEK kinase which co-elutes with a Tpl2 and not with the Rafs (**Figure 3.14,15**).

3.3.5 Role of Tpl2 in GLP-1 Stimulated Erk1/2 Phosphorylation

I have identified that a GLP-1 stimulated MEK kinase co-elutes with Tpl2. The role of Tpl2 in pancreatic β-cells has not been examined before. Activation of Tpl2 is dependent on several steps. In contrast to Raf, Tpl2 is stabilized by association with p105 of the nuclear factor kappa B (NF-kB) pathway (Waterfield, Zhang et al. 2003) which inhibits its kinase activity (Beinke, Deka et al. 2003). Stimulation with LPS or TNFα promotes its

release from p105. Tpl2 activation is dependent on IκB kinase (IKKβ) which functions by phosphorylating p105 and triggering its proteolysis (Waterfield, Jin et al. 2004). In this unbound state, Tpl2 is active but unstable and is subject to rapid degradation by the proteasome. Tpl2 also undergoes phosphorylation at Thr290 by IKKβ in response to LPS, which parallels an increase in its activity (Cho, Melnick et al. 2005).

I have demonstrated that GLP-1 stimulated Erk1/2 activation is blocked in the presence of the IKK inhibitor, 15dPGJ2 (**Figure 3.18**). This data suggests that IKK is required for the activation of Tpl2, and hence specifically mediates the activation of the MEK/Erk1/2 pathway in pancreatic β-cells. Since the influx of Ca^{2+} through L-type VGCC alone has been shown to be sufficient for the activation of Erk1/2 (Gomez, Pritchard et al. 2002), and GLP-1 augments glucose-stimulated Ca^{2+} influx through L-type VGCC, it is likely that the increased influx of calcium is the predominant mechanism by which GLP-1 induces glucose-dependent Erk1/2 activation.

Although our data provides evidence for the requirement of IKK in GLP-1 stimulated Erk1/2 activation, the role of IKK in mediating the calcium signal following GLP-1 stimulation is unknown. In neurons, it has been shown that the IKK complex may act as an integrator receiving signals triggered by Ca^{2+} and membrane receptors (Lilienbaum and Israel 2003). These authors demonstrated that a rise in intracellular Ca^{2+} through the opening of L-type VGCC at the plasma membrane and the indirect release of Ca^{2+} from intracellular stores is able to enhance basal NFκB activity, a target of IKK (Lilienbaum and Israel 2003). Subsequent work demonstrated that these effects were dependent on the Ca^{2+} sensor, calmodulin. Calmodulin triggers the activity of calcineurin, a phosphatase which also increases basal NFκB activity (Lilienbaum and Israel 2003). Interestingly, inhibition of calcineurin with FK506 attenuated GLP-1 stimulated Erk1/2 activation in MIN6 cells (**Figure 3.19**) and glucose and GLP-1 stimulated Erk1/2 activation in INS-1 cells (Arnette, Gibson et al. 2003).

Therefore, in pancreatic β-cells, glucose stimulation results in an increase in Ca^{2+} influx specifically via L-type VGCC, which is enhanced by GLP-1. GLP-1 acts to potentiate Ca^{2+} influx by modulating ion channel activity in a PKA dependent manner. I hypothesise that the increase in Ca^{2+} around the mouth of the pore of the channel leads to increased binding of calmodulin which leads to the activation of calcineurin which in turn activates IKK. An increase in IKK promotes the phosphorylation and targeted degradation of p105, resulting

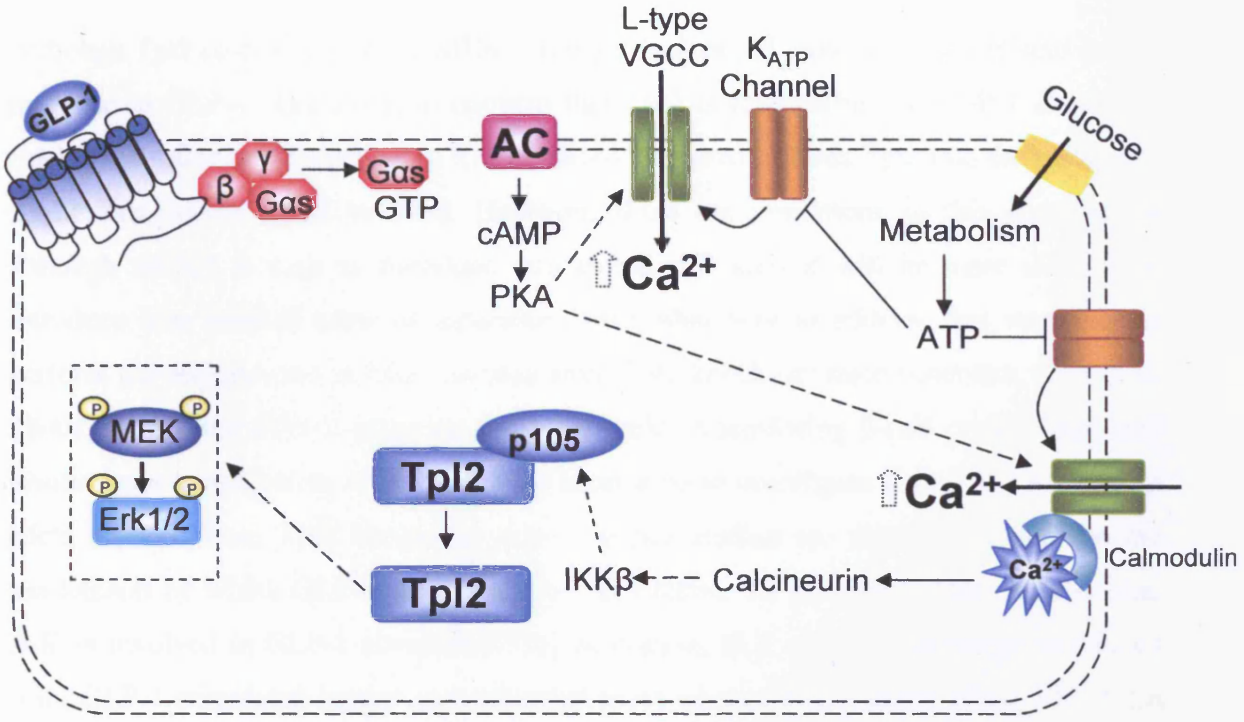


Figure 3.20: Proposed Model for GLP-1 Stimulated Erk1/2 Activation. In pancreatic β-cells, glucose metabolism leads to an increase in the ATP/ADP ratio, the closure of ATP-sensitive K+ channels, the depolarization of the cell membrane, the opening of L-type VGCC, and the subsequent influx of extracellular calcium into the cell. This influx of calcium leads to the activation of calmodulin. In the presence of stimulatory concentrations of glucose, binding of GLP1 to the GLP-R leads to the activation of adenylyl cyclase (AC), an increase in cAMP, and the activation of PKA. These events are known to augment glucose-stimulated Ca^{2+} entry through L-type VGCC. The entry of calcium through L-type VGCC together with the activation of calmodulin leads to the activation of calcineurin and the activation of IKKβ. IKKβ phosphorylates p105 which promotes Tpl2 activation and subsequent activation of MEK and Erk1/2.

in the activation of Tpl2 which specifically mediates MEK and Erk1/2 activation (**Figure 3.20**).

3.3.6 Future Directions

Although Tpl2 co-elutes with the MEK activity this does not prove that Tpl2 is activated in response to GLP-1. Therefore, to confirm that Tpl2 is responsible for GLP-1 stimulated Erk1/2 activation small interfering RNA (siRNA) targeted towards Tpl2 into the pancreatic β-cell line, MIN6 could be used. However, there are limitations to this approach as although siRNA is easy to introduce into clonal cell lines it will be more difficult to introduce into isolated islets of langerhan. One other way to address this would be to perform our experiments in islets isolated from Tpl2 knock-out mice (Dumitru, Ceci et al. 2000). Given that Erk1/2 plays an important role in regulating β-cell proliferation and insulin gene transcription, it would also be interesting to investigate if these are affected in islets isolated from Tpl2 knock-out mice. Further studies are required to confirm the mechanism by which GLP-1 stimulates Tpl2 activation. To provide further evidence that IKK is involved in GLP-1 stimulated Tpl2 activation, IKK could be immunoprecipitated from GLP-1 stimulated lysates and subjected to an *in vitro* kinase assay using GST-IκBα as a substrate, in the presence or absence of $15dPGJ_2$ (IKK inhibitor). A further series of studies should explore the role of calcineurin in GLP-1 stimulated Tpl2 activation and an alternative approach to pharmacological inhibition would be to use an adenovirus expressing a constitutively active form of calcineurin (De Windt, Lim et al. 2000; Kim, Moon et al. 2004). I would predict that the over-expression of constitutively active calcineurin will potentiate GLP-1 stimulated Erk1/2 activation in β-cells.

Chapter 4: cAMP Dependent Activation of mTOR and the Phosphorylation of Ribosomal Protein S6 by Glucagon Like Peptide-1 in Pancreatic β-cells

4.1 Introduction

4.1.1 Mammalian Target of Rapamycin (mTOR)

The target of rapamycin (TOR) was first identified in yeast as a protein that was sensitive to the immunosuppressive agent rapamycin (Kunz, Henriquez et al. 1993). The mammalian counterpart, referred to as mTOR, was identified shortly after (Sabatini, Erdjument-Bromage et al. 1994). mTOR is a multidomain protein which plays a key role in regulating many aspects of cell growth and proliferation. mTOR contains a serine/threonine protein kinase domain, belonging to the phosphatidylinositol kinase related kinase (PIKK) family (Inoki, Ouyang et al. 2005). mTOR also contains a FKBP12/rapamycin binding domain which is next to the kinase domain and therefore may inhibit mTOR activity by preventing substrate binding (Edinger, Linardic et al. 2003) or inhibiting the autophosphorylation (Peterson, Beal et al. 2000). mTOR also possess a series of alpha helices that form tandem HEAT repeats and serve as surfaces for protein-protein interactions. Signalling via mTOR is increased by nutrients and growth factors and is decreased during nutrient deprivation. Deregulation of the mTOR pathway is associated with many diseases such as diabetes and cancer (Dann, Selvaraj et al. 2007).

4.1.1.1 mTOR Forms Two Multiprotein Complexes

mTOR forms two functional protein complexes, termed mTORC1 and mTORC2. mTORC1 consists of Raptor (regulated associated protein of mTOR) (Kim, Sarbassov et al. 2002; Kim, Sarbassov et al. 2003; Kim and Sabatini 2004), mammalian LST8 (mLST8) (Kim, Sarbassov et al. 2003) and PRAS40 (proline rich Akt substrate) (Vander Haar, Lee et al. 2007). It is this complex which is acutely sensitive to rapamycin (Sarbassov, Ali et al. 2004). Raptor is required for mTORC1 to phosphorylate its two key downstream targets, ribosomal S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein (4E-BP) (Kim, Sarbassov et al. 2002). Knockdown of Raptor using siRNA not only results in a reduction in S6K1 phosphorylation but also a decrease in cell size (Kim, Sarbassov et al. 2002). In addition, over-expression of Raptor promotes an increase in mTOR activity *in*

vitro (Kim, Sarbassov et al. 2002). There is also evidence that raptor maintains mTOR stability since knockdown of Raptor expression reduces mTOR levels in the cell (Kim, Sarbassov et al. 2002).

Several studies have shown that the knockdown of mLST8 results in a decrease in S6K phosphorylation as well as a decrease in cell size, a similar phenotype to that of Raptor knockdown (Kim, Sarbassov et al. 2003). However, in mLST8 knock-out MEFs, S6K1 and 4EBP-1 phosphorylation are unaffected and the association between raptor and mTOR remains intact. Therefore, the role mLST8 plays in mTORC1 is not clear. Although deletion of mLST8 had no effect on mTORC1, the loss of mLST8 completely abrogates the interaction between rictor and mTORC2, preventing the phosphorylation of PKB on Ser473 (Guertin, Stevens et al. 2006). This suggests that mLST8 plays an important role in maintaining a stable interaction of the mTORC2 complex.

PRAS40 is an mTOR binding partner that mediates PKB signalling to mTOR. Vander Haar *et al* demonstrated that PRAS40 binds the mTOR kinase domain and its interaction with mTOR is induced during nutrient or serum deprivation, conditions that inhibit mTOR signalling (Vander Haar, Lee et al. 2007). It has been demonstrated that PKB can phosphorylate PRAS40 on Thr246, which appears to release the inhibitory effect of PRAS40 on mTORC1 (Kovacina, Park et al. 2003).

mTORC2 contains mTOR, mLST8, rictor (rapamycin independent companion of mTOR) and mSin1 (mitogen-activated protein kinase-associated protein 1) (Sarbassov, Ali et al. 2004). In contrast to mTORC1, mTORC2 is insensitive to acute rapamycin treatment or nutrient withdrawal (Sarbassov, Ali et al. 2004). Knockdown of Rictor has no effect on the phosphorylation of S6K1 or 4EBP1 but it does regulate the phosphorylation of PKB on Ser473. In addition, Rictor$^{-/-}$ knock-out MEFs show diminished PKB Ser473 phosphorylation (Sarbassov, Guertin et al. 2005). Another potential substrate of mTORC2 is PKCα, as reduction of rictor expression by siRNA led to a decrease in the phosphorylation of PKCα at Ser657 in Hela cells (Sarbassov, Ali et al. 2004).

4.1.2 Upstream Regulators of mTOR

4.1.2.1 TSC1/2

Tuberous sclerosis complex (TSC) is a genetic syndrome characterized by the formation of benign tumours (Kwiatkowski and Manning 2005). TSC is due to mutations in either the TSC1 or TSC2 genes. One interesting feature of these tumours is that they contain very large cells suggesting that TSC1/TSC2 plays an important role in cell growth. The TSC1 and TSC2 gene products encode hamartin (130KDa) and tuberin (198KDa) respectively. TSC1 and TSC2 form a heterodimer that inhibits cell growth and proliferation. Therefore, loss of function of either TSC1 or TSC2 leads to unregulated cell growth and tumourigenesis (Kwiatkowski and Manning 2005). Over-expression of TSC1 and TSC2 suppresses the phosphorylation of the mTOR targets S6K1 and 4E-BP1 (Inoki, Li et al. 2002), whereas knock-down of TSC2 by siRNA enhances S6K1 phosphorylation (Fingar, Richardson et al. 2004). TSC1 and TSC2 do not inhibit the function of mTOR directly; instead, their effects on mTOR are mediated via the GTP binding protein Rheb (Ras homologue enriched in brain), which activates mTOR in its GTP bound form (Inoki, Li et al. 2003; Tee, Manning et al. 2003). In unstimulated conditions, the TSC1/TSC2 complex has strong and highly specific GAP activity towards Rheb, whereas upon growth factor stimulation, TSC2 phosphorylation inhibits its ability to act as a Rheb-GAP. At least three signalling pathways have been reported to regulate the GAP activity of TSC2 via direct phosphorylation: these include the PI3K/PKB pathway (Tee, Anjum et al. 2003), the Erk1/2-RSK pathway (Roux, Ballif et al. 2004) and the LKB1-AMPK pathway (Inoki, Zhu et al. 2003). The sites phosphorylated on TSC2 by PKB, Erk1/2, p90RSK and AMPK are shown in **Figure 4.1**.

Regulation of TSC1/2 by the PI3K/PKB pathway: The activation of a receptor such as the insulin receptor by insulin stimulation is followed by its autophosphorylation on tyrosine residues. Adaptor proteins which include insulin receptor substrate molecules (IRS) bind via their SH2 domains to the intracellular portion of the receptor which leads to the recruitment of PI3K. PI3K phosphorylates membrane PtdIns(4,5)P_2 at position 3 to generate PtdIns(3,4,5)P_3. This lipid leads to the recruitment of PKB to the membrane where it is phosphorylated by PDK1 on Thr308 (Vanhaesebroeck and Alessi 2000) and mTORC2 on Ser473 (Sarbassov, Guertin et al. 2005) (**Figure 4.2**). PKB phosphorylates several proteins including the forkhead transcription factor 1 (FOXO1) isoform, BCL2-antagonist of cell death (BAD), glycogen synthase kinase 3 (GSK3) and TSC2. Several PKB phosphorylation sites have been identified on TSC2 and include Ser939, Ser1130, Ser1132 and Thr1462 (Inoki, Li et al. 2002). Phosphorylation at these sites inhibits TSC2 GAP activity towards Rheb. It has been reported that the PI3K/PKB pathway can also

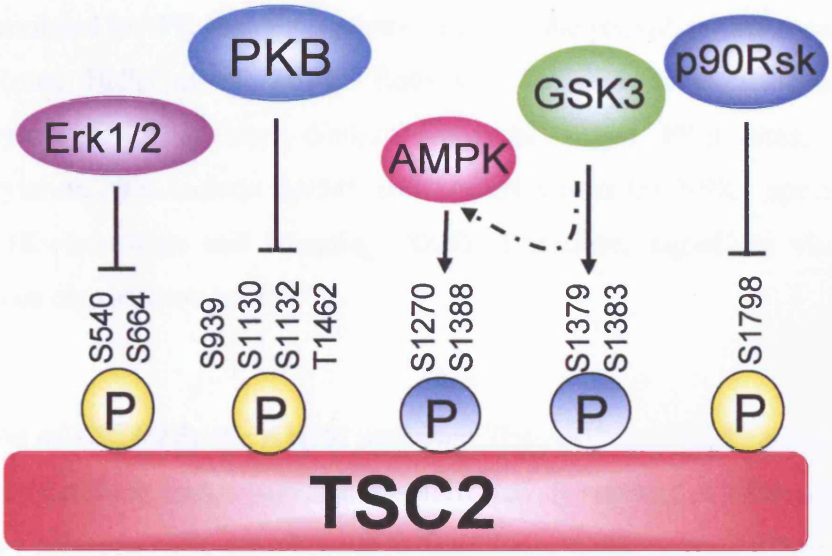


Figure 4.1: Phosphorylation Sites in TSC2

Schematic representation of the main phosphorylation sites in TSC2 (tuberin). Yellow circles represent phosphorylations which inhibit TSC2 activity and increase mTORC1 activity. Blue circles represent phosphorylations which activate TSC2 activity and thereby inhibit mTORC1 activity.

regulate TSC2 activity independently of the direct phosphorylation of TSC2, via PKB phosphorylation of the transcription factor FOXO1 (Cao, Kamioka et al. 2006). Phosphorylation of FOXO1 results in its cytosolic retention where it binds to TSC2 and dissociates the TSC1/TSC2 complex. This inhibits the GAP activity towards Rheb, leading to the activation of mTOR and its downstream targets (Cao, Kamioka et al. 2006).

Regulation of TSC1/2 by the MAPK pathway: It has been demonstrated that the growth factor-stimulated MAPK (Erk1/2) pathway leads to the phosphorylation and inactivation of TSC2 (Roux, Ballif et al. 2004). Both ERK1/2 and its downstream target RSK1 phosphorylate TSC2 at sites distinct from the major PKB sites; Erk1/2 specific phosphorylation sites include Ser540 and Ser664 whilst the RSK1 specific site includes Ser1798 (Kwiatkowski and Manning 2005). Therefore, signalling via ERK1/2-RSK1 pathway can also activate mTORC1.

Regulation of TSC1/2 by the AMPK pathway: The AMP activated protein kinase (AMPK) is a sensor of cellular energy status and is activated by elevated AMP levels (Hardie 2005). AMPK is a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits. AMPK is activated by phosphorylation of its α-subunit on Thr172 by LKB1 (derived as a code name for Peutz-Jeghers syndrome causative gene) (Reviewed by Hardie 2005). Once activated, AMPK phosphorylates and influences many downstream targets. Among these, AMPK phosphorylates TSC2 at Ser1270 and Ser1388, two sites distinct from the PKB sites (Inoki, Zhu et al. 2003). In contrast to the phosphorylation of TSC2 by PKB or Erk1/2, AMPK-mediated phosphorylation enhances the ability of the TSC1/TSC2 complex to act as a Rheb-GAP, and therefore blocks Rheb-dependent mTOR activation under conditions of energy stress.

Regulation of TSC1/2 by the GSK3 pathway: Glycogen synthase kinase 3 (GSK-3) is responsible for phosphorylating and inactivating glycogen synthase and phosphorylation of GSK-3 by PKB inactivates the kinase. However, it has recently been shown that GSK-3 can also phosphorylate TSC2 on Ser1379 and Ser1383 (Inoki, Ouyang et al. 2006). This study demonstrated that phosphorylation of TSC2 by AMPK provides the priming phosphorylation sites for subsequent phosphorylation by GSK-3. Therefore, AMPK and

GSK-3 coordinately phosphorylate TSC2 and increase GAP activity towards Rheb, which represses mTOR signalling (Inoki, Ouyang et al. 2006).

4.1.2.2 Rheb

Rheb is a small GTP binding protein that is highly conserved from yeast to mammals and has homology with the Rap subfamily (Aspuria and Tamanoi 2004). Insulin stimulation increases the amount of GTP bound to Rheb and the amount of Rheb:GTP is higher in TSC2 null cells deprived of serum compared to wild type cells (Garami, Zwartkruis et al. 2003). Overexpression of TSC1/2 results in a decrease in Rheb:GTP which is restored when TSC2 is mutated in the GAP domain, providing evidence that Rheb is downstream of TSC1/2 (Garami, Zwartkruis et al. 2003). Additionally, over-expression of Rheb increases mTOR activity in the absence of growth factor stimulation, as measured by S6K1 and 4E-BP1 phosphorylation (Long, Lin et al. 2005). The mechanism by which Rheb could regulate mTOR is poorly understood. Rheb can directly bind to the catalytic domain of mTOR, independently of mLST8, which also binds near to this catalytic domain. Rheb also shows a weak interaction with raptor. However, the recent discovery of FKBP38 (a member of the FK506-binding protein (FKBP) family) as an endogenous inhibitor of mTOR, revealed that Rheb in its GTP bound state may activate mTOR by interacting with FKBP38 and preventing its binding to mTOR (Bai, Ma et al. 2007).

4.1.3 Activation of mTOR

4.1.3.1 Nutrients

The mTOR pathway is activated by nutrient rich conditions, particularly by high levels of amino acids (Proud 2002). Leucine has been identified as the most effective stimulator of mTOR (Xu, Kwon et al. 2001). Several reports, describe the identification of proteins that modulate amino acid signalling through mTOR (Gulati and Thomas 2007). For example, amino acid withdrawal causes TSC2 phosphorylation, thereby inhibiting mTOR function (Inoki, Li et al. 2002), whereas over-expressing TSC1 and TSC2 inhibits amino acid induced S6K and 4E-BP1 phosphorylations (Inoki, Li et al. 2002). In cells that lack TSC2, amino acid starvation still causes inactivation of mTOR signalling (Smith, Finn et al. 2005). Therefore, the role of TSC2 on amino acid regulation of mTOR is the subject of debate. Rheb over-expression overcomes amino acid insufficiency via a rapamycin-sensitive mechanism, suggesting that Rheb participates in nutrient signalling through mTOR (Tee, Manning et al. 2003). However, it has been proposed that the decrease in the

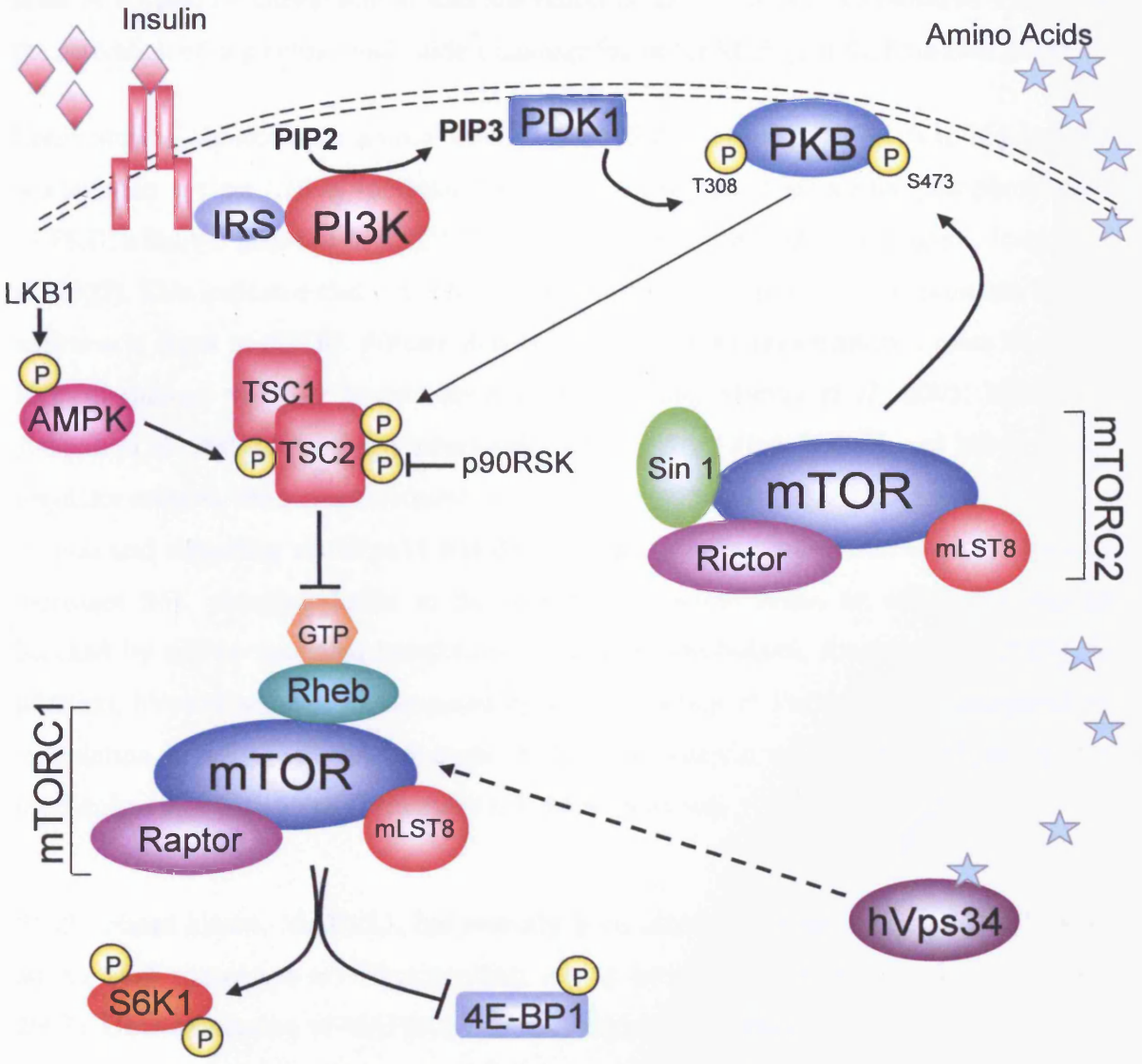


Figure 4.2: mTOR Signalling in Mammalian Cells

mTORC1 is the rapamycin sensitive complex, consisting of mTOR, Raptor and mLST8. Growth factor signals are represented by insulin. Activation of receptor tyrosine kinase activates Class 1 PI3K through direct binding to the receptor or via IRS proteins, which bind and activate PI3K. PI3K phosphorylates PIP2 to form PIP3 leading to the recruitment of PDK-1 and PKB to the membrane. PDK-1 phosphorylates Thr308 on PKB whilst mTORC2 phosphorylates Ser473 resulting in full activation of PKB. PKB phosphorylates TSC2, leading to mTORC1 activation, the phosphorylation and inhibition of 4E-BP1 (negative regulator of translation) leading to its release from eIF4E. mTORC1 also phosphorylates and activates S6K1/2. mTORC1 can also be negatively regulated by decreases in energy status and the activation of AMPK which phosphorylates and activates TSC2. Amino acids have been reported to regulate mTORC1 via the activation of hVps34.

level of Rheb.GTP during amino acid starvation is not due to the activation of TSC2 but the activation of a guanine nucleotide exchange factor for Rheb (Smith, Finn et al. 2005).

Early studies showed that amino acid induced S6K phosphorylation was blocked by wortmannin, a class 1 PI3K inhibitor. Yet amino acids did not induce the phosphorylation of PKB, a known inhibitor of TSC1/2 (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005). This indicated that a novel wortmannin sensitive pathway is responsible for the amino acid input to mTOR. Further studies identified this component as a class III PI3K, hVps34 (human vacuolar protein sorting 34) (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005). hVps34 is structurally distinct from class 1 PI3K and has a unique regulator subunit, the serine/threonine protein kinase p150 (Nobukuni, Kozma et al. 2007). Amino acid signalling via hVps34 was demonstrated by over-expression of hVps34 which increases S6K phosphorylation in the presence of amino acids, an effect that can be blocked by siRNA mediated knockdown of hVps34 (Nobukuni, Joaquin et al. 2005). In addition, hVps34 activity, as measured by the production of PtdIns(3)P_1, is increased by stimulation of cells with amino acids (Nobukuni, Joaquin et al. 2005). However, the mechanism by which hVps34 activates mTOR is unknown.

Ste20-related kinase, MAP4K3, has recently been identified as an important regulator of amino acid stimulated mTOR signalling (for a detailed review see Cook and Morley 2007). Over-expression of MAP4K3 increases the phosphorylation of S6K1 at Thr389 and ribosomal protein S6 (rpS6) at Ser240/244 in a rapamycin sensitive manner (Findlay, Yan et al. 2007). Furthermore, amino acids stimulate an increase in MAP4K3 activity that is insensitive to rapamycin, indicating that MAP4K3 is activated upstream of mTOR. Knockdown of MAP4K3 using siRNA results in a reduction in S6K1 and rpS6 phosphorylation in response to amino acids, independently of TSC1/2. Additionally, knock-down of MAP4K3 also results in a decrease in HeLa cell size to similar levels observed during rapamycin treatment in serum stimulated cells (Findlay, Yan et al. 2007).

4.1.3.2 Regulation of mTOR by Energy

mTORC1 activity is also regulated by the energy status of the cell. mTORC1 signalling has been shown to be inhibited by a reduction in cellular ATP (Dennis, Jaeschke et al. 2001), since depletion of ATP levels with the glucose analogue, 2-deoxyglucose inhibits S6K1 and 4E-BP1 phosphorylation in an mTOR dependent manner (Dennis, Jaeschke et al. 2001). Subsequent studies have shown that energy depletion inhibits mTOR via the

activation of AMP-activated protein kinase (AMPK) (see 4.1.2). The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status and is activated by elevated AMP levels (Reviewed by Hardie 2005). AMPK is a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits. Under conditions of hypoxia, exercise, ischaemia, heat shock and low glucose, AMPK is activated allosterically by rising cellular AMP and through the phosphorylation of the catalytic subunit. Phosphorylation of Thr172 on the α-subunit is required for kinase activity and is catalysed by a number of kinases, including LKB1 (Hardie 2005). Once activated, AMPK phosphorylates multiple downstream substrates with the aim of conserving ATP levels. Conditions which activate AMPK, promote the dephosphorylation of the mTOR substrates S6K and 4E-BP1. Artificial activation of AMPK using AICAR (aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) reduces the phosphorylation of S6K1, whereas over-expression of a rapamycin resistant S6K1 is insensitive to AICAR (Kimura, Tokunaga et al. 2003). This data supports a link between mTOR and AMPK. AMPK inhibits mTOR signalling by phosphorylating TSC2 which enhances the ability of the TSC1/2 complex to act as a Rheb-GAP, and therefore blocks Rheb-dependent mTOR activation under conditions of energy stress.

Hypoxia and glucose deprivation, likely through a decrease in energy, can also decrease mTOR signalling through the rapid induction of REDD1 (regulated in development and DNA damage responses) expression. REDD1 inhibits mTOR activity in a TSC1/TSC2-dependent manner (Brugarolas, Lei et al. 2004; Sofer, Lei et al. 2005) by competing with 14-3-3 for binding to TSC2. Dissociation of 14-3-3 leads to an increase in TSC2 activity which in turn inhibits mTORC1 activity (DeYoung, Horak et al. 2008).

4.1.3.3 Growth Factors and Hormones

Many growth factors and hormones including insulin are thought to activate mTOR via the activation of PI3K or the Erk1/2 signalling pathway, as described in 4.1.2. Briefly, the activation of PKB subsequently induces the phosphorylation of TSC2 within the TSC1/2 complex. Phosphorylation of TSC2 inactivates the complex, allowing the levels of Rheb.GTP to accumulate leading to the activation of mTORC1 (**Figure 4.2**). Agents, that result in the activation of the MAPK pathway such as phenylephrine or phorbol esters also increases mTORC1 activity via site specific phosphorylation of TSC2. This phosphorylation event inhibits the TSC2 GAP activity towards Rheb thereby increasing mTORC1 activity (Wang and Proud 2002).

4.1.4 Signalling Downstream of mTOR

4.1.4.1 The Ribosomal Protein S6 Kinase (S6K1/2)

Mammalian cells express two forms of S6 kinase, S6K1 and S6K2, each encoded by a separate gene (Shima, Pende et al. 1998). Each exists as two splice variants, S6K1 contains a short p70 isoform which is cytosolic and a p85 isoform which is nuclear. S6K2 contains a p54 and p56 isoforms that are both primarily nuclear. Activation of S6K1 is associated with increased hierarchical phosphorylation of at least eight residues by independently regulated protein kinases (Weng, Kozlowski et al. 1998). Phosphorylation of the autoinhibitory domain at four closely clustered phosphorylation sites Ser411, Ser418, Thr421 and Ser424 is required to inhibit the interaction between the N-terminus domain and the autoinhibitory domain (Weng, Kozlowski et al. 1998). This in turn allows the phosphorylation of Thr389 and Thr229 which propagate full kinase activity. The phosphorylation of S6K by mTOR occurs at Thr389 and Ser371, which are essential for S6K activation (Burnett, Barrow et al. 1998). Phosphorylation at Thr389 acts as a docking site for PDK1. PDK1 then phosphorylates Thr229 in the activation loop of the catalytic domain which allows complete activation of S6K1 (Pullen, Dennis et al. 1998). The rapamycin sensitivity of S6K reveals that mTOR is an essential input into the regulation of S6K (**Figure 4.3a**).

Studies in *Drosophilia* and mice models in which S6K1 is deleted have demonstrated S6K1 is an important positive regulator of cell growth (Shima, Pende et al. 1998) (Montagne, Stewart et al. 1999). In *Drosophilia,* deletion of the S6K gene (dS6K), leads to embryonic lethality and the few flies that survive show a reduction in body mass as a result of smaller cell size rather than reduced cell number (Montagne, Stewart et al. 1999). In mice, deletion of the S6K1 gene ($S6K1^{-/-}$) is not lethal but the mice are significantly smaller than their wild type counterparts (Pende, Kozma et al. 2000). These mice exhibit smaller cell size rather than decreased cell number. Myoblasts and pancreatic β-cells are particularly affected. A decrease in β-cell mass co-insides with a reduction in pancreatic insulin and mild glucose intolerance (Pende, Kozma et al. 2000). A possible explanation for this increased insulin sensitivity in the $S6K1^{-/-}$ mice is that S6K1 exerts a negative feedback loop by phosphorylating IRS1 and inhibiting downstream signalling (Harrington, Findlay et al. 2004). In contrast, mice in which the S6K2 gene is deleted have a normal body size compared to their wild type littermates (Pende, Um et al. 2004). Deletion of both

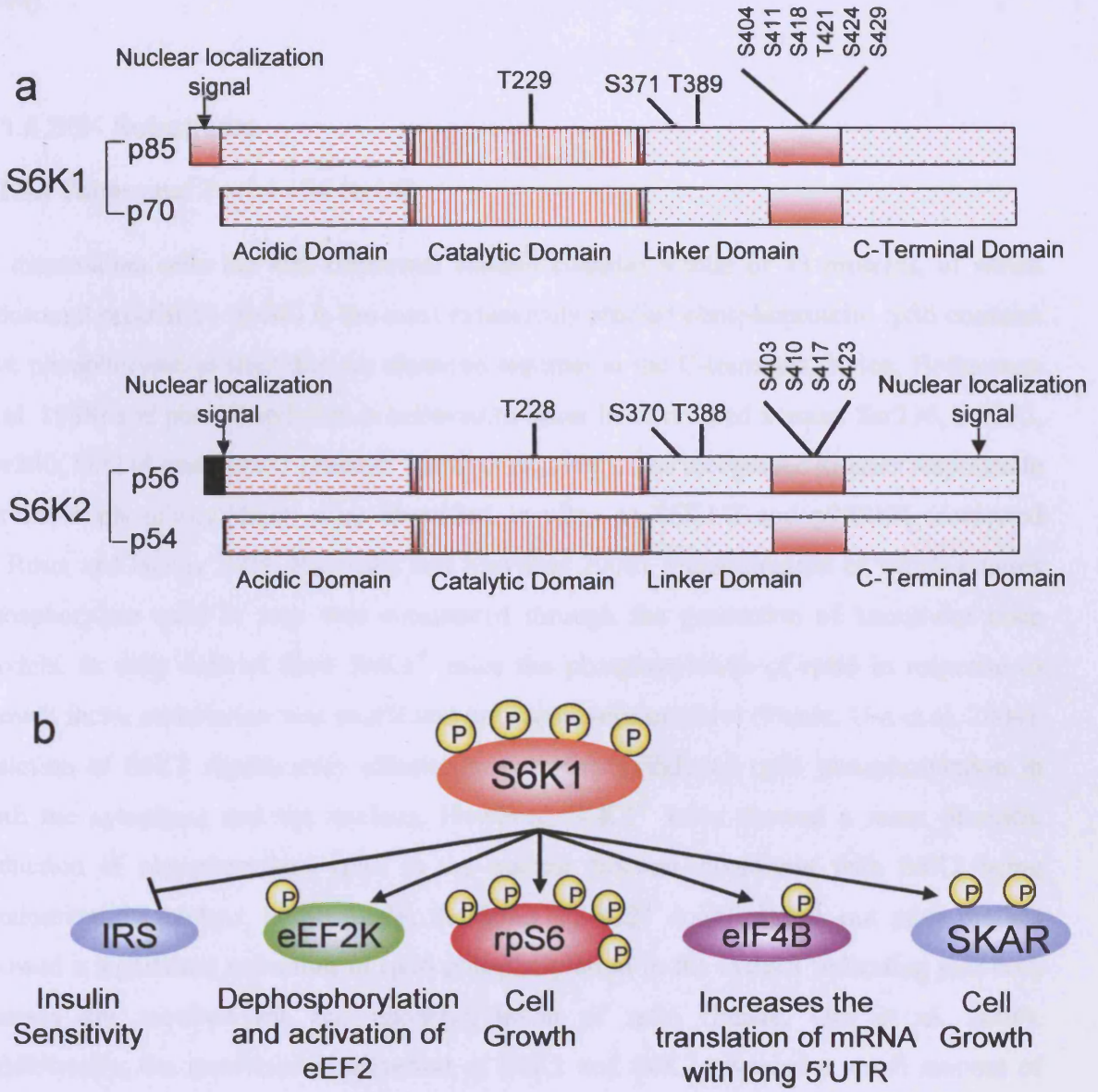


Figure 4.3: Structure and Function of Ribosomal Protein S6 Kinase

a) Schematic representation of the phosphorylation sites in ribosomal protein S6 kinase1 and 2. b) Activation of Ribosomal protein S6 kinase (S6K) via mTORC1 leads to the phosphorylation of multiple downstream effectors including rpS6, eEF2 kinase, eIF4B, SKAR and IRS proteins. See text for details.

S6K1 and S6K2 ($S6K1^{-/-}$/$S6K2^{-/-}$) show a significant decrease in viability (Pende, Um et al. 2004).

4.1.5 S6K Substrates

4.1.5.1 Ribosomal Protein S6 (rpS6)

In mammalian cells the 40S ribosomal subunit contains a total of 33 proteins, of which ribosomal protein S6 (rpS6) is the most extensively studied phosphoprotein. rpS6 contains five phosphorylation sites that are clustered together at the C-terminus (Krieg, Hofsteenge et al. 1988) and phosphorylation is believed to occur in an ordered manner Ser236, Ser235, Ser240, Ser244 and Ser247 (Ferrari, Bandi et al. 1991). The recognised kinases responsible for these phosphorylations were identified *in vitro* as S6K1/2 and p90RSK (reviewed in Roux and Blenis 2004; Ruvinsky and Meyuhas 2006). Determination of which kinases phosphorylate rpS6 *in vivo* was accelerated through the generation of knock-out mice models. In cells derived from $S6K1^{-/-}$ mice the phosphorylation of rpS6 in response to growth factor stimulation was unaffected but rapamycin sensitive (Pende, Um et al. 2004). Deletion of S6K2 significantly affected growth factor induced rpS6 phosphorylation in both the cytoplasm and the nucleus. However, $S6K2^{-/-}$ mice showed a more dramatic reduction of phosphorylated rpS6 in the nuclear fraction, consistent with S6K2 being predominantly nuclear. Interestingly, the $S6K1^{-/-}$ $S6K2^{-/-}$ double knock-out mice models showed a significant reduction in rpS6 phosphorylation in the cytosol indicating that both kinases are required for full phosphorylation of rpS6 (Pende, Um et al. 2004). Additionally, the combined inactivation of S6K1 and S6K2 revealed a small amount of phosphorylated rpS6 on Ser235/236 that was rapamycin insensitive, indicating the presence of another S6K. Inhibition of MEK, an upstream activator of p90RSK, with the MEK inhibitor (U0126) revealed that inhibition of the MAPK pathway inhibited rpS6 Ser235/236 phosphorylation in the $S6K1^{-/-}$ $S6K2^{-/-}$ double knock-out MEFs. This data provides evidence that p90RSK can phosphorylate rpS6 on Ser235/236 in the absence of either S6K1 or S6K2.

Although rpS6 was discovered many years ago, the function of rpS6 phosphorylation remains unknown. It was thought to play an important role in the up-regulation of 5'TOP (oligopyrimidine tract at their transcriptional start) mRNAs as the phosphorylation of rpS6 was shown to be important in recruiting 5'TOP mRNAs onto polysomes. However,

translation of 5'TOP mRNAs is regulated normally in the $rpS6^{P-/-}$ knock-in mouse in which all five phosphorylatable serine residues are substituted to alanines ($rpS6^{P-/-}$) (Ruvinsky, Sharon et al. 2005). In $rpS6^{P-/-}$ mouse embryonic fibroblasts (MEFs) the association and engagement of phosphorylated rpS6 with polysomes is similar between $rpS6^{P-/-}$ and wild type MEFs (Ruvinsky, Sharon et al. 2005). Further work on this mouse model revealed that rpS6 plays an important role in regulating cell size (Ruvinsky, Sharon et al. 2005). $rpS6^{P-/-}$ MEFs were significantly smaller compared to the $rpS6^{P+/+}$ MEFs. The size of the $rpS6^{P-/-}$ MEFs was further reduced in the presence of rapamycin, indicating that rpS6 is a key effector of mTOR signalling regulating cell size (Ruvinsky, Sharon et al. 2005). $rpS6^{P-/-}$ mice had smaller pancreatic β-cells and interleukin 7-dependent cells derived from feotal livers (Ruvinsky, Sharon et al. 2005). Another phenotype of the $rpS6^{P-/-}$ mice is a reduction in circulating insulin levels, a similar phenotype to that observed in $S6K1^{-/-}$ mice (Pende, Kozma et al. 2000). In addition, the $rpS6^{P-/-}$ mice show increased insulin sensitivity which is also observed in S6K1 deficient mice (Pende, Kozma et al. 2000). Therefore, many of the phenotypes seen in $S6K1^{-/-}$ mice are also observed in $rpS6^{P-/-}$ mice.

A recent study by the Blenis group showed a functional role for rpS6 phosphorylation, in that it facilitated protein synthesis in HEK293 cells by increasing cap dependent translation by assisting in the formation of the pre-initiation complex (Roux, Shahbazian et al. 2007). This mechanism was dependent on Erk1/2 signalling acting via RSK that promotes site specific phosphorylation of rpS6 on Ser235/236 in an mTOR independent fashion. Phosphorylation of rpS6 on Ser235/236 was shown to promote the recruitment to the 7-methylguanosine cap complex (Roux, Shahbazian et al. 2007).

4.1.5.2 Eukaryotic Elongation Factor 2 (eEF2)

Eukaryotic elongation factor kinase 2 (eEF2K) plays a role in negatively regulating translation elongation by phosphorylating eukaryotic elongation factor 2 (eEF2) which mediates the translocation step of elongation. eEF2 is inactivated by phosphorylation at Thr56 and thus eEF2K inhibits eEF2 function. Previous studies have shown that, in cardiomyocytes, insulin stimulates the activation of PI3K and S6K, resulting in a decrease in eEF2K activity and the subsequent dephosphorylation of eEF2 (Wang, Wang et al. 2000). These effects were blocked in the presence of rapamycin, indicating that mTOR acting through S6K1 negatively regulates eEF2 (Wang, Wang et al. 2000). Later studies reported that IGF-1 treatment stimulates the phosphorylation eEF2K on Ser366 by S6K1

which inhibits its activity (Wang, Li et al. 2001). The MAPK pathway can also activates eEF2 as p90RSK also phosphorylates eEF2K on Ser366 (Wang, Li et al. 2001).

4.1.5.3 Eukaryotic Translation Initiation Factor 4B (eIF4B)

eIF4B acts as a co-factor for the RNA helicase eIF4A and increases the rate of translation of mRNAs with long and structured 5' UTRs (Raught, Peiretti et al. 2004). Studies have shown that eIF4B is phosphorylated on Ser422 in response to serum in a rapamycin sensitive manner. Further work showed that this is dependent on S6K1 as the expression of a rapamycin resistant S6K inhibits the phosphorylation of Ser422 of eIF4B in response to serum stimulation (Raught, Peiretti et al. 2004).

4.1.5.4 (S6K1 Aly/REF-like target) SKAR

Another potentially interesting S6K1 target is the protein known as SKAR. SKAR is a RNA binding protein which couples transcription, pre-mRNA splicing, and nucleo cytoplasmic mRNA transport (Richardson, Broenstrup et al. 2004). SKAR has been shown to bind specifically to S6K1 and, following insulin stimulation, SKAR is phosphorylated by S6K1 at Ser383 and Ser385 *in vitro*, in a rapamycin sensitive manner (Richardson, Broenstrup et al. 2004). Interestingly, in HEK293 cells, reduction of SKAR expression by siRNA results in a decrease in cell size, indicating that SKAR plays an important role in regulating cell growth (Richardson, Broenstrup et al. 2004).

4.1.5.5 Insulin Receptor Substrate Proteins (IRS Proteins)

The IRS proteins play an important role in transducing many of the cell's responses to insulin to downstream effectors. The ability of S6K to negatively regulate PKB signalling and hence mTORC1 activation was first discovered in *Drosophilia* carrying deletions in the *d*TSC1 and *d*TSC2 genes. *d*TSC1/2 deletion leads to constitutive dS6K activation and inhibition of dPKB. This inhibition was relieved by reducing dS6K signalling (Radimerski, Montagne et al. 2002). It was later determined that S6K directly phosphorylated IRS1 at Ser302 in $TSC2^{-/-}$MEFs, which may prevent its association with the insulin receptor (Harrington, Findlay et al. 2004). In pancreatic β-cells, chronic exposure to elevated glucose decreases IRS2 expression as a result of mTOR activation (Briaud, Dickson et al. 2005). Prolonged mTOR signalling resulted in an increase in Ser/Thr phosphorylation of IRS2, leading to its proteasomal degradation. These effects were alleviated by the expression of a kinase-dead mTOR which reduced Ser/Thr phosphorylation of IRS2 and

maintained IRS2 protein levels (Briaud, Dickson et al. 2005). Both IRS2 degradation and phosphorylation at Ser/Thr residues leads to decreased PKB signalling and therefore inhibits mTORC1 activity. In addition to modulating PKB activity, S6K1 has also been shown to directly phosphorylate mTOR on Thr2446 and Ser2448 (Holz and Blenis 2005). The significance of these phosphorylations is unclear since substitution of these residues to alanine does not affect mTOR activity (Sekulic, Hudson et al. 2000).

4.1.6 Eukaryotic Initiation Factor 4E Binding Proteins (4E-BPs)

Eukaryotic initiation factor 4E binding proteins (4E-BPs) are repressor proteins which play an important role in translation initiation. There are 3 members to the 4E-BP family, namely 4E-BP1, 4E-BP2 and 4E-BP3, each encoded by a separate gene (Hay and Sonenberg 2004). Of the three 4E-BPs, 4E-BP1 is the most extensively studied in mammals. When 4E-BP1 is hypophosphorylated, it competes with eIF4G for binding to eIF4E and can therefore inhibit the assembly of the eIF4F complex and hence the assembly of the pre-initiation complex (Haghighat, Mader et al. 1995) (**Figure 4.4b**). 4E-BP1 is phosphorylated at seven phosphorylation sites which been mapped in 4E-BP1, Thr37, Thr46, Ser65, Thr70, Ser83, Ser101 and Ser112, phosphorylation of these sites occur in an ordered manner (**Figure 4.4a**) (Beugnet, Wang et al. 2003). Phosphorylation of certain residues is required for subsequent modification of other residues. For example, prior phosphorylation at both Thr37 and Thr46 (Gingras, Gygi et al. 1999) is required for the subsequent phosphorylation of Thr70 and Ser65 in 4E-BP1. Phosphorylation at Thr70 is essential for allowing phosphorylation at Ser65 and both residues play an important role in regulating the binding of 4E-BP1 to eIF4E, with Thr70 promoting release and Ser65 preventing re-binding (Fadden, Haystead et al. 1997; Gingras, Gygi et al. 1999). In addition, the phosphorylation of Thr70 and Ser65 also depends upon a further site, Ser101 (Beugnet, Wang et al. 2003). The phosphorylation of Thr70, Ser65, Thr37 and Thr46 is increased in response to insulin and is sensitive to rapamycin, demonstrating an important role of mTOR in the phosphorylation of 4E-BP1 on these sites (Gingras, Gygi et al. 1999). The phosphorylation of Ser112 has been reported to also be required for the release of 4E-BP1 from eIF4E (Reviewed by Proud 2002).

4.1.7 Other Potential mTORC1 Targets

While S6K and 4E-BP1 are the best characterized downstream targets of mTOR, there is evidence that mTOR phosphorylates other proteins. Other potential substrates phosphorylated by mTOR include the novel PKC isoforms, nPKCδ and nPKCε. PKCδ is

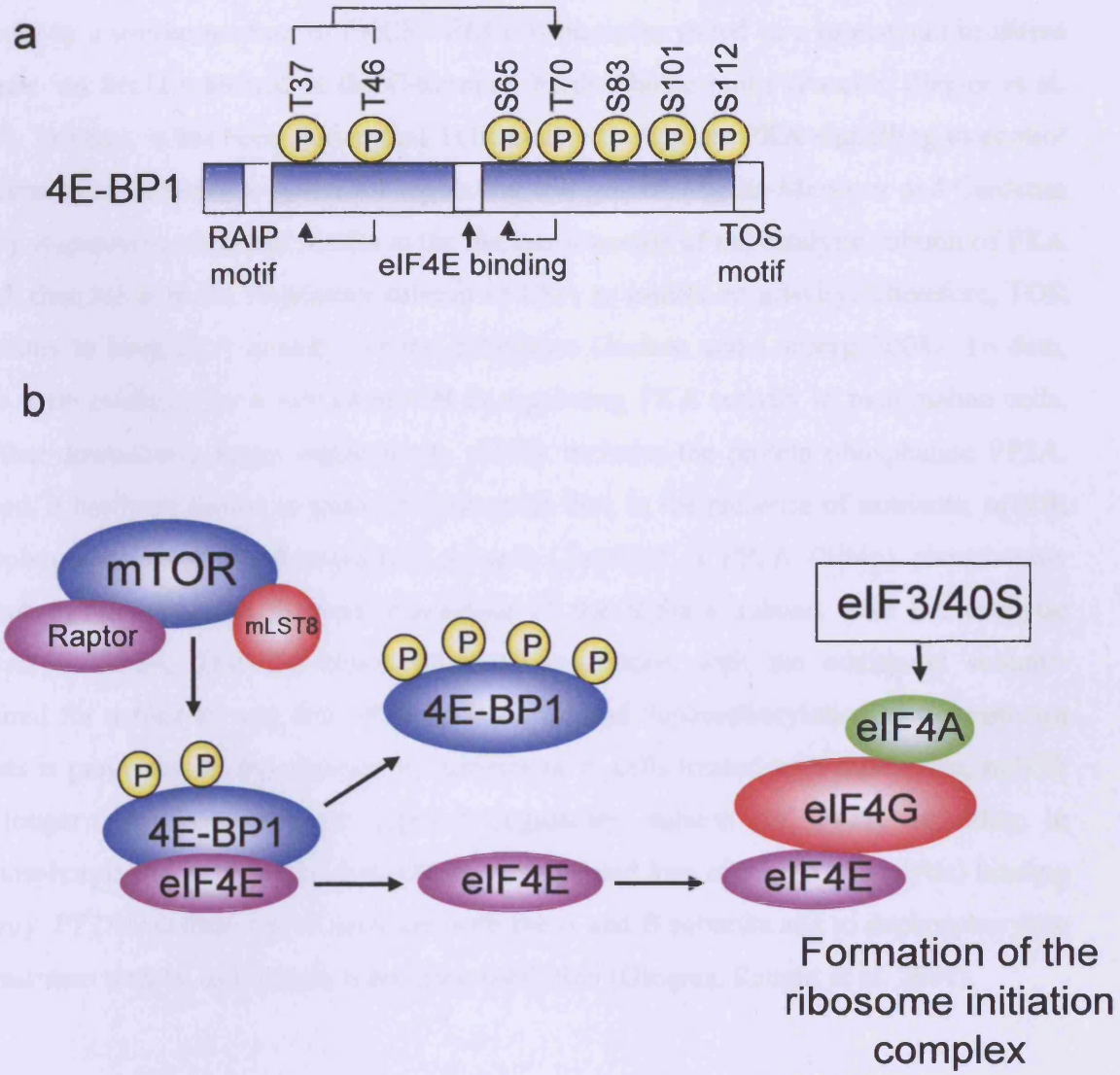


Figure 4.4: Structure and Function of 4E-BP1

a) Schematic representation of the phosphorylation sites in 4E-BP1.

b) Phosphorylation of 4E-BP1 results in its release from eIF4E enabling it to bind to eIF4G, which allows the assembly of the ribosome initiation complex.

phosphorylated on Ser622 which is located in the hydrophobic motif, in a rapamycin sensitive manner. Phosphorylation at Ser622 can be alleviated by over-expressing a rapamycin resistant mTOR (Parekh, Ziegler et al. 1999). It was also established that PKCε behaves in a similar manner to PKCδ. PKCε is phosphorylated in a rapamycin sensitive manner on Ser729, located at the C-terminal hyrdrophobic motif (Parekh, Ziegler et al. 1999). In yeast, it has been shown that TOR is able to regulate PKA signalling to control the expression of genes required for regulating cell growth (Zurita-Martinez and Cardenas 2005). Rapamycin treatment results in the nuclear retention of the catalytic subunit of PKA which then binds to the regulatory subunit of PKA to inhibit its activity. Therefore, TOR functions to keep PKA activity in the cytoplasm (Jacinto and Lorberg 2008). To date, there is no evidence for a role of mTOR in regulating PKA activity in mammalian cells. Another downstream target regulated by mTOR includes the protein phosphatase PP2A. Indeed, it has been shown in yeast and mammals that, in the presence of nutrients, mTOR phosphorylates the alpha-4 regulatory subunit (Tap42p) of PP2A (Sit4p) phosphatase. Phosphorylation induces a direct interaction of the alpha-4 subunit with the catalytic subunit of PP2A. This interaction prevents association with the additional subunits required for activation and thus PP2A is inhibited and dephosphorylation of downstream targets is prevented. In the absence of nutrients or in cells treated with rapamycin, mTOR no longer phosphorylates the alpha-4 regulatory subunit of PP2A resulting in dephosphorylation of the alpha-4 regulatory subunit and loss of PP2AC (catalytic) binding activity. PP2AC is then free to associate with the A and B subunits and to dephosphorylate downstream targets, resulting in translation inhibition (Gingras, Raught et al. 2001).

4.1.8 mTOR Signalling in Pancreatic β-cells

4.1.8.1 Importance of mTOR Signalling in Pancreatic β-cells

There is a large amount of evidence that the control of β-cell growth is greatly influenced by mTOR and its downstream effectors. β-cell specific PDK1 knock-out mice which have defective mTOR signalling have smaller pancreatic β-cells compared to their wild-type counterparts and suffer from diminished levels of pancreatic insulin (Hashimoto, Kido et al. 2006). In addition, β-cell specific over-expression of a constitutively active form of PKB, a downstream target of PDK-1 and a key regulator of mTOR, results in increases in both β-cell size and mass resulting in hyperinsulinemia and hypoglycaemia (Bernal-Mizrachi, Wen et al. 2001; Tuttle, Gill et al. 2001). Conversely, mice which have a pancreatic-specific deletion of TSC2 ($\beta TSC2^{-/-}$), the negative regulator of mTOR

signalling, exhibit an increase in the size of the β-cells plus β-cell mass (Shigeyama, Kobayashi et al. 2008). These mice also had hypoglycaemia and hyperinsulinemia which, at 40 weeks, resulted in a decrease in β-cell mass due to chronic mTORC1 signalling resulting in constitutive S6K1 activation and a decrease in IRS/PKB signalling (Shigeyama, Kobayashi et al. 2008). Indeed, S6K1 directly phosphorylated IRS1 at Ser302 in $TSC2^{-/-}$MEFs, which may prevent its association with the insulin receptor (Radimerski, Montagne et al. 2002). Similarly, in INS-1 cells, chronic exposure to elevated glucose resulted in an increase in Ser/Thr phosphorylation of IRS2 leading to its proteasomal degradation. These effects were alleviated by the expression of a kinase-dead mTOR which reduced Ser/Thr phosphorylation of IRS2 and maintained IRS2 protein levels (Briaud, Dickson et al. 2005).

Recent studies have demonstrated that S6K1/2 and the phosphorylation of ribosomal protein S6 (rpS6) play a particularly important role in regulating pancreatic β-cell size (Ruvinsky, Sharon et al. 2005; Ruvinsky and Meyuhas 2006). The generation of the $rpS6^{P-/-}$ knock-in mice in which all five phosphorylatable serines were substituted to alanines demonstrated the importance of rpS6 phosphorylation in β-cell physiology. The $rpS6^{P-/-}$ mice display a smaller pancreatic β-cell size than their wild-type counterparts and suffer from diminished levels of pancreatic insulin, hypoinsulinemia and impaired glucose tolerance (Ruvinsky, Sharon et al. 2005). A recent study in INS-1 cells demonstrated the importance of mTOR in β-cell survival since the over-expression of either a rapamycin resistant S6K1 or increased expression of eIF4E increased β-cell survival in the presence of low glucose concentrations (conditions which activate AMPK) and this effect was markedly enhanced when the two constructs were co-expressed together (Cai, Wang et al. 2008). In addition, the activation of AMPK by low glucose or AICAR resulted in a decrease in β-cell survival which could be restored to similar levels as cells cultured in high glucose by over-expressing a rapamycin resistant S6K1 mutant (Cai, Wang et al. 2008).

4.1.8.2 Regulation of mTOR by Nutrients in Pancreatic β-cells

Nutrients, in particular glucose, have been recognized as a important signalling mediator of pancreatic β-cell function, playing a key role in modulating insulin secretion. In addition to its role in regulating insulin secretion, glucose has also been identified as an important signalling mediator of β-cell proliferation and growth (Hugl, White et al. 1998). In RINm5F cells, a pancreatic β-cell line, glucose stimulates the phosphorylation of the

downstream target of mTOR, S6K1, in a rapamycin sensitive manner (Xu, Kwon et al. 1998). Glucose also stimulates the rapid phosphorylation of 4E-BP1, another downstream target of mTOR in a time and concentration dependent manner in rat isolated islets (Xu, Marshall et al. 1998). Inhibition of insulin signalling using wortmannin, a PI3K inhibitor, or incubation of islets at 24°C, which blocks insulin secretion, prevents glucose-stimulated phosphorylation of 4E-BP1 (Xu, Marshall et al. 1998). Furthermore, treatment of rat islets with exogenous insulin mimics the effects of elevated glucose (Xu, Marshall et al. 1998). Therefore, glucose stimulates the phosphorylation of 4E-BP1 via the autocrine effect of insulin (Xu, Marshall et al. 1998). Amino acids are required for glucose or exogenous insulin to stimulate the phosphorylation of 4E-BP1 in βTC3 cell line (Xu, Marshall et al. 1998) and amino acids alone can also induce the rapamycin sensitive phosphorylation of 4E-BP1 in RINm5F cells. These results demonstrate that amino acids are both necessary and sufficient to mediate the phosphorylation of 4E-BP1 (Xu, Marshall et al. 1998). The branched chain amino acids leucine, valine and isoleucine are particularly effective in stimulating the phosphorylation of 4E-BP1, of which leucine is the most potent (Xu, Kwon et al. 1998). In β-cells, a combination of leucine and glutamine stimulates the phosphorylation of S6K1 by serving as a metabolic fuel for the mitochondria and an allosteric activator of glutamate dehydrogenase (Xu, Kwon et al. 1998). Therefore, in β-cells, both glucose and amino acids stimulate signalling through mTOR. However, until recently it remained unknown whether AMPK modulates the mTOR signalling pathway in β-cells. In isolated rat islets or MIN6 cells, artificial activation of AMPK using AICAR, phenformin or oligomycin blocks glucose stimulated S6K1 activation and the phosphorylation of its downstream target rpS6 (Gleason, Lu et al. 2007). Furthermore, leucine or its non metabolizable analog, BCH, inhibits AMPK activity to the same extent as high glucose in MIN6 cells (Gleason, Lu et al. 2007). These authors hypothesise that glucose and amino acids inhibit AMPK by elevating cellular ATP via enhanced flux through the TCA cycle (Gleason, Lu et al. 2007). Therefore glucose and amino acids inhibit AMPK phosphorylation and activity in pancreatic β-cells.

4.1.8.3 Regulation of mTOR by cAMP in Pancreatic β-cells

Forskolin, an artificial activator of adenylyl cyclase, or exenatide, a GLP-1R agonist, increases S6K1 phosphorylation in rat islets in a rapamycin sensitive manner (Kwon, Marshall et al. 2004). Diazoxide, an activator of K^{ATP} channels which inhibits insulin secretion, had no effect on forskolin or exenatide stimulated S6K1 phosphorylation.

Therefore, cAMP mediated S6K1 activation is independent of insulin secretion. cAMP has also been shown to mobilize Ca^{2+} from intracellular stores in β-cells (Tsuboi, da Silva Xavier et al. 2003) and treatment of cells with the intracellular Ca^{2+} chelator BAPTA resulted in inhibition of exenatide stimulated S6K1 phosphorylation in rat islets (Kwon, Marshall et al. 2004). Tsuboi *et al* showed that, in MIN6 cells, GLP-1 increases Ca^{2+} release from intracellular Ca^{2+} stores, leading to an increase in mitochondrial Ca^{2+} and an increase in ATP production (Tsuboi, da Silva Xavier et al. 2003). Therefore, Kwon *et al* hypothesized that cAMP dependent mobilization of intracellular calcium increases ATP production via the up regulation of mitochondrial dehydrogenase, with the increase in ATP acting as a fuel for mTOR (Kwon, Marshall et al. 2004).

In INS-1 cells, a recent study by Hurley *et al* demonstrated, that glucose dependent insulinotropic polypeptide (GIP) induced elevations in cAMP can inhibit AMPK activity (Hurley, Barre et al. 2006). Furthermore, other agents that elevate cAMP including forskolin and IBMX were also shown to inhibit AMPK activity and reduce the phosphorylation of Thr172 in the activation loop. The ability of cAMP to inhibit AMPK occurs via the inhibition of the upstream AMPK kinases, CaMKKα and CaMKKβ and not LKB1 via a PKA dependent mechanism (Hurley, Barre et al. 2006). Furthermore, activation of PKA by forskolin also modulates AMPK via its phosphorylation on Ser485/491. However, phosphorylation of AMPK at Ser485/491 is necessary but not sufficient to inhibit AMPK activity. The authors hypothesized that Ser485/491 is an autophosphorylation site which is required to enable the inhibition of AMPK in response to energy depletion (Hurley, Barre et al. 2006). Therefore, cAMP may activate mTOR via AMPK inhibition.

4.1.9 Aims

Ribosomal protein S6 kinases 1 and 2 (S6K) are critical downstream effectors of mTOR, a protein kinase that is able to integrate signals from hormones and nutrients to co-ordinate changes in cell growth and proliferation. Recent studies have demonstrated that S6K1/2 and the phosphorylation of ribosomal protein S6 (rpS6) play a particularly important role in regulating pancreatic β-cell size. Furthermore, glucose and GLP-1, potent stimulators of β-cell growth have been reported to activate mTOR. However, the molecular mechanisms by which glucose and GLP-1 stimulate the phosphorylation of rpS6 in pancreatic β-cells are not fully understood. Therefore, the aim of this study was to identify the transduction

pathways by which glucose and GLP-1 regulates rpS6 phosphorylation in pancreatic β-cells.

4.2 Results

4.2.1 Glucose and GLP-1 Stimulate rpS6 Phosphorylation in Pancreatic β-cells

To characterise the effects of glucose and GLP-1 on rpS6 phosphorylation in β-cells, MIN6 cells and islets of langerhans were incubated in KRB supplemented with amino acids for 1h. Cells were then incubated for a further 1h in the same buffer containing 20mM glucose in the presence of absence of GLP-1 (10nM) and the phosphorylation state of rpS6 was determined using phospho-specific antibodies directed towards Ser235/236 and Ser240/244. Incubation of both MIN6 cells and rat islets at 20mM glucose resulted in an increase in the phosphorylation of rpS6 at Ser235/236 and Ser240/244, which was significantly potentiated by GLP-1 (**Figure 4.5**).

In order to characterise the signalling pathways activated by GLP-1 that may be responsible for rpS6 phosphorylation, MIN6 cells were pre-incubated for 1h in KRB supplemented with amino acids prior to incubation for up to 60min in 20mM glucose in the presence or absence of GLP-1. At specific times post-stimulation, the phosphorylation state of rpS6 was determined using phospho-specific antibodies directed towards Ser235/236 and Ser240/244. Incubation of cells in KRB supplemented with 20mM glucose led to the phosphorylation of rpS6 at both Ser235/236 and Ser240/244 within 20min and was sustained up to 60min (**Figure 4.6**). The addition of GLP-1 in the presence of 20mM glucose led to a robust, rapid, and sustained increase in rpS6 phosphorylation, which was first detected at 5min and was sustained up to 60min. Glucose or GLP-1 induced changes in rpS6 phosphorylation paralleled increases in the phosphorylation of S6K, as assessed by mobility shift in polyacrylamide gel electrophoresis and using phospho-specific antibodies to Thr389 and Thr229. 4E-BP1, another downstream target of mTOR, was also phoshorylated in response to either glucose or GLP-1, as assessed by mobility shift assays. GLP-1 and glucose also stimulated the phosphorylation of potential activators of the mTOR pathway, Erk1/2 and PKB, as assessed using phospho-specific antibodies directed towards the phosphorylated forms of these proteins. Erk1/2 activation by glucose was transient and peaked at 10min whereas GLP-1 stimulated Erk1/2 activation peaked at 10min which was sustained for up to 60min. In contrast, PKB phosphorylation in response to glucose or GLP-1 paralleled changes in the phosphorylation of S6K1 and 4E-BP1. It is therefore possible that glucose and GLP-1 stimulates rpS6 phosphorylation via a PKB/S6K dependent mechanism.

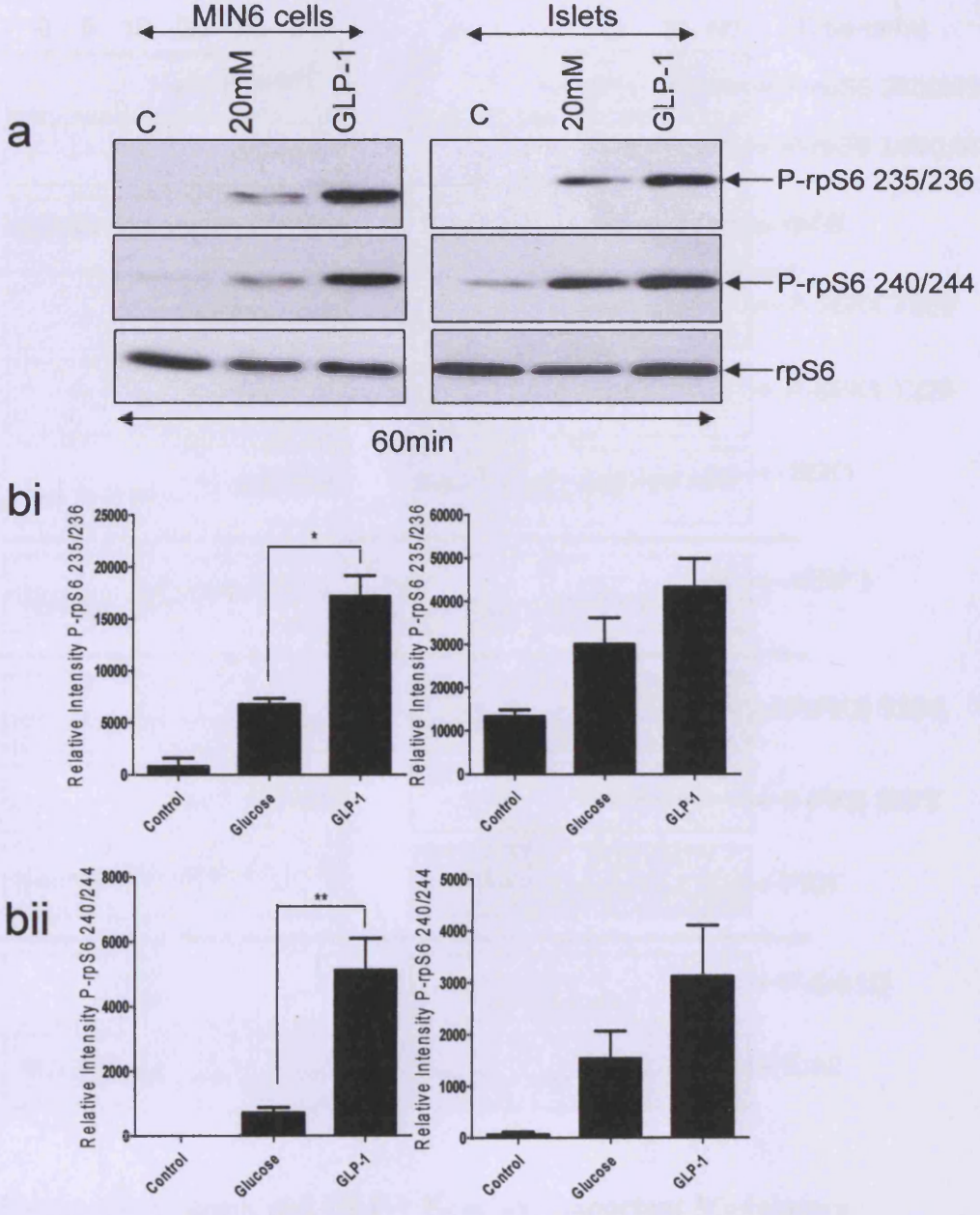


Figure 4.5: Glucose and GLP-1 Stimulate the Phosphorylation of rpS6 in Pancreatic β-Cells

MIN6 cells and rat islets of langerhans were pre-incubated in KRB supplemented with amino acid (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing 20mM glucose or 20mM glucose plus GLP-1 (10nM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244 and total rps6 as a loading control. bi,ii) Quantified data from phospho-rpS6 235/236 and 240/244 blots in *a* shown as means ± SEM; $n = 3$. $^{*}P < 0.05$, P value obtained using a One-Way ANOVA.

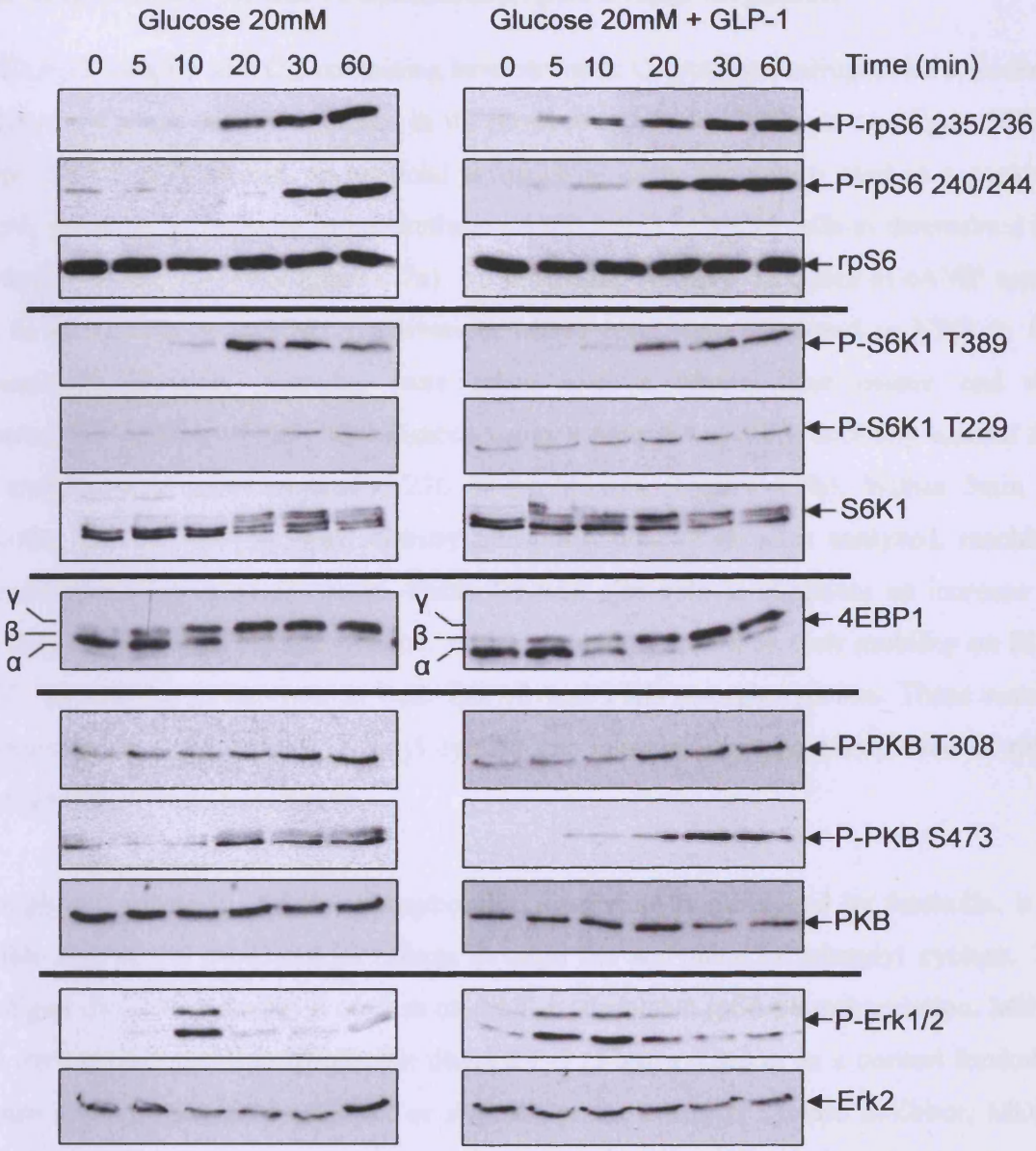


Figure 4.6: Glucose and GLP-1 Regulate Important Modulators of the mTOR Signalling Pathway

MIN6 cells were pre-incubated in KRB supplemented with amino acid (0.5X) for 1h. Cells were then treated in KRB containing 20mM glucose or 20mM glucose plus GLP-1 (10nM) for the times indicated in the figure. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-S6K1 (Thr389 and Thr229), phospho-PKB (Thr308 and Ser473), phospho-Erk1/2 (Thr202/Tyr204), total PKB, Erk2, S6K1, 4E-BP1 and total rpS6 as a loading control. All results are representative of 3 independent experiments.

4.2.2 Role of cAMP in GLP-1 Stimulated rpS6 Phosphorylation

The GLP-1R couples to a $G_{\alpha s}$-containing heterotrimeric G-proteins leading to the activation of adenylyl cyclase and the increase in the production of cAMP (Doyle and Egan 2007). Indeed, GLP-1 or forskolin, an artificial activator of adenylyl cyclase used as a positive control, stimulate an increase in intracellular cAMP levels in MIN6 cells as determined by radioimmunoassay (RIA) (**Figure 4.7a**). To determine whether increases in cAMP could lead to an increase in mTORC1 activation, MIN6 cells were incubated in KRB in the presence of forskolin. Samples were taken over a 60min time course and the phosphorylation state of rpS6 investigated using a phospho-specific antibody against the activated forms of rpS6 on Ser235/236 or Ser240/244 (**Figure 4.7b**). Within 5min of forskolin stimulation, rpS6 was robustly phosphorylated at all sites analyzed, reaching maximal phosphorylation at 60min. Forskolin was also able to stimulate an increase in both S6K1 and 4E-PB1 phoshorylation, as determined by a shift in their mobility on SDS PAGE, as well as an increase in both Erk1/2 and PKB phosphorylation. These results indicate that the activation of adenylyl cyclase can stimulate the phosphorylation of rpS6 and downstream targets of mTOR.

Since glucose-dependent GLP-1 phosphorylation of rpS6 is mimicked by forskolin, it is possible that GLP-1 mediates its effects through the activation of adenylyl cyclase. To investigate the role of adenylyl cyclase on GLP-1 stimulated rpS6 phosphorylation. MIN6 cells were treated with 20mM glucose plus GLP-1 (**Figure 4.8a**) or as a control forskolin (**Figure 4.8b**) in either the presence or absence of the adenylyl cyclase inhibitor, MDL-12330A. MDL-12330A dose-dependently inhibited GLP-1 and forskolin-stimulated rpS6 phosphorylation.

4.2.2.1 The Role of Cyclic Nucleotide Phosphodiesterases in GLP-1 Stimulated rpS6 Phosphorylation

Phosphodiesterases are proteins which targets the hydrolysis of cAMP. Pancreatic β-cells express several PDE isoforms including PDE1C, PDE3B and PDE4. PDE3B has been shown to play an important role in regulating insulin secretion, and more recently has been shown to be localized within insulin granules (Walz, Wierup et al. 2007). To provide further evidence that cAMP can activate mTOR and downstream signalling to rpS6, we prevented cAMP breakdown by inhibiting known PDE isoforms. MIN6 cells were pre-

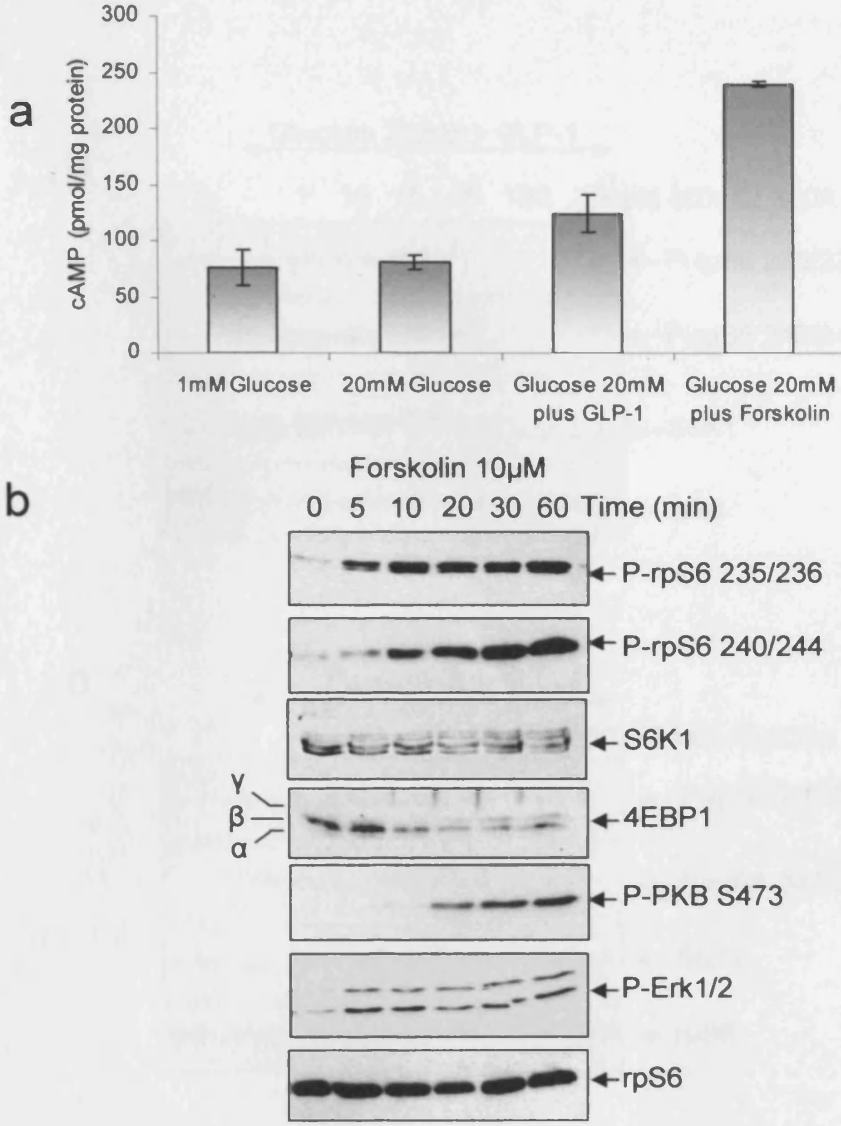


Figure 4.7: GLP-1 and Forskolin Stimulate an Increase in cAMP and Increases in cAMP can Stimulate the Phosphorylation of rpS6

(a) MIN6 cells were pre-incubated in KRB (plus amino acids 0.5X) supplemented with 1mM IBMX to prevent cAMP breakdown for 1h. Cells were then treated in KRB containing 20mM glucose, 20mM glucose plus GLP-1 (10nM) or forskolin (10μM), an activator of adenylyl cyclase. The intracellular cAMP content in MIN6 cells was determined as described in the materials and methods. (b) MIN6 cells were pre-incubated in KRB (plus amino acids) for 1h. Cells were then treated in KRB containing forskolin (10μM) for the times indicated in the figure. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Ser473), phospho-Erk1/2 (Thr202/Tyr204), S6K1, 4E-BP1 and total rpS6 as a loading control. All results are representative of 3 independent experiments

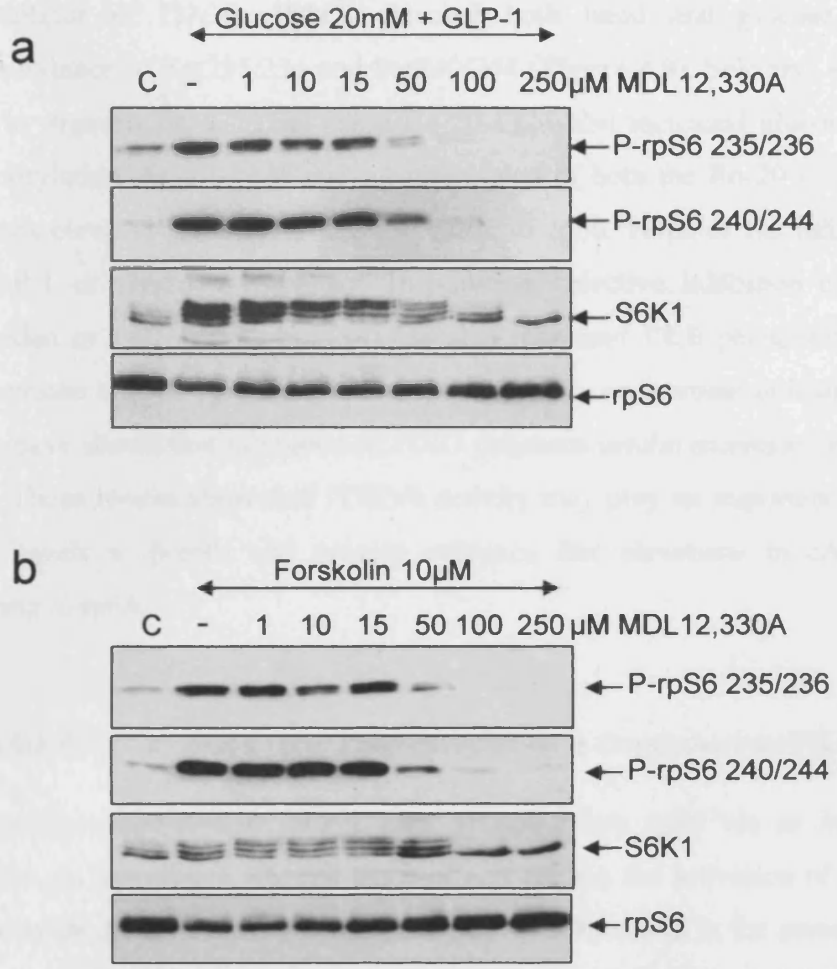


Figure 4.8: Role of cAMP in rpS6 Phosphorylation

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing (a) 20mM glucose plus GLP-1 (10nM) or (b) forskolin (10μM), in the absence or presence of increasing concentrations of MDL12,330A (an irreversible inhibitor of adenylyl cyclase). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, S6K1 and total rpS6 as a loading control. All results are representative of 3 independent experiments.

incubated for 1h in KRB supplemented with amino acids. The cells were then incubated in 20mM glucose, 20mM glucose plus GLP-1 or forskolin alone in the presence or absence of IBMX (inhibitor of all PDE isoforms), siguazodan (an inhibitor of PDE3), and Ro-20-1724 (an inhibitor of PDE4). IBMX elevated both basal and glucose stimulated rpS6 phosphorylation at Ser235/236 and Ser240/244 (**Figure 4.9**). Selective inhibition of either PDE3 by siguazodan or PDE4 using Ro-20-1724 also increased glucose stimulated rpS6 phosphorylation. Ro-20-1724 and a combination of both the Ro-20-1724 and siguazodan inhibitors elevated the basal phosphorylation of rpS6. None of the inhibitors potentiated the GLP-1 or forskolin responses. In addition, selective inhibition of either PDE3 by siguazodan or PDE4 using Ro-20-1724 also increased PKB phosphorylation at Ser473. This increase in PKB phosphorylation may be due to an increase in insulin secretion since studies have shown that inhibition of PDE3 enhances insulin secretion (Walz, Wierup et al. 2007). These results show that PDE3/4 activity may play an important role in regulating cAMP levels in β-cells and provide evidence that elevations in cAMP can increase signalling to rpS6.

4.2.2.2 GLP-1 Stimulated rpS6 Phosphorylation is Dependent on PKA but not Epac

We provide evidence that GLP-1 may phosphorylate rpS6 via an increase in cAMP. Therefore, to investigate whether these effects are via the activation of PKA, MIN6 cells were incubated with 20mM glucose plus GLP-1 or forskolin in the presence or absence of H89, a selective PKA inhibitor. At 5μM, H89 completely blocked GLP-1 and forskolin stimulated rpS6 phosphorylation at all 4 sites (**Figure 4.10ai,ii**). These results suggest that GLP-1 and forskolin stimulate the phosphorylation of rpS6 via PKA. In addition, GLP-1 stimulated PKB phosphorylation on Ser473 was unaffected by H89.

cAMP does not only mediate its actions via PKA but also via the cAMP regulated exchange factors Epac1 and 2. Epac 2 has been shown to be expressed in pancreatic islets of Langerhans (Kang, Chepurny et al. 2001) and has been shown to increase S6K1 activity in an mTOR dependent manner (Bos 2003). To determine whether Epac activation could increase the phosphorylation of rpS6, MIN6 cells were treated with 8-CPT-2-Me-cAMP (CPT-cAMP) an Epac-selective cAMP analogue. CPT-cAMP was unable to stimulate rpS6 phosphorylation at Ser235/236 or Ser240/244 (**Figure 4.10bi**). In Chinese hamster ovary cells (CHO-K1), it has been shown that glucose exerts a permissive effect on the regulation of the downstream targets of mTOR by insulin (Patel, Wang et al. 2001). Therefore, it is

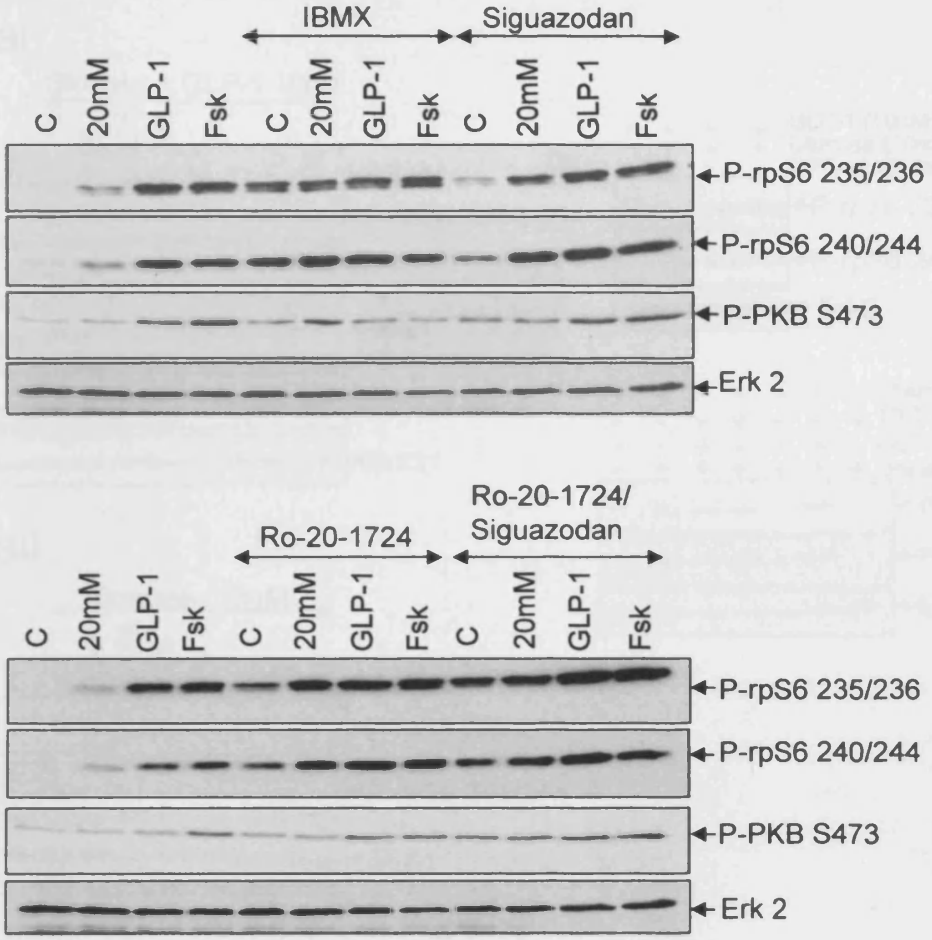


Figure 4.9: Role of cAMP in rpS6 Phosphorylation

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing 20mM glucose, 20mM glucose plus 10nM GLP-1 or 10μM forskolin (Fsk) alone in the presence or absence of 1mM IBMX (to block all PDE isoforms), 10μM siguazodan (an inhibitor of PDE3), 100μM Ro-20-1724, (an inhibitor of PDE4) or siguazodan and Ro-20-1724 together (to block both PDE3/4). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Ser473) and total Erk2 as a loading control. All results are representative of 2 independent experiments

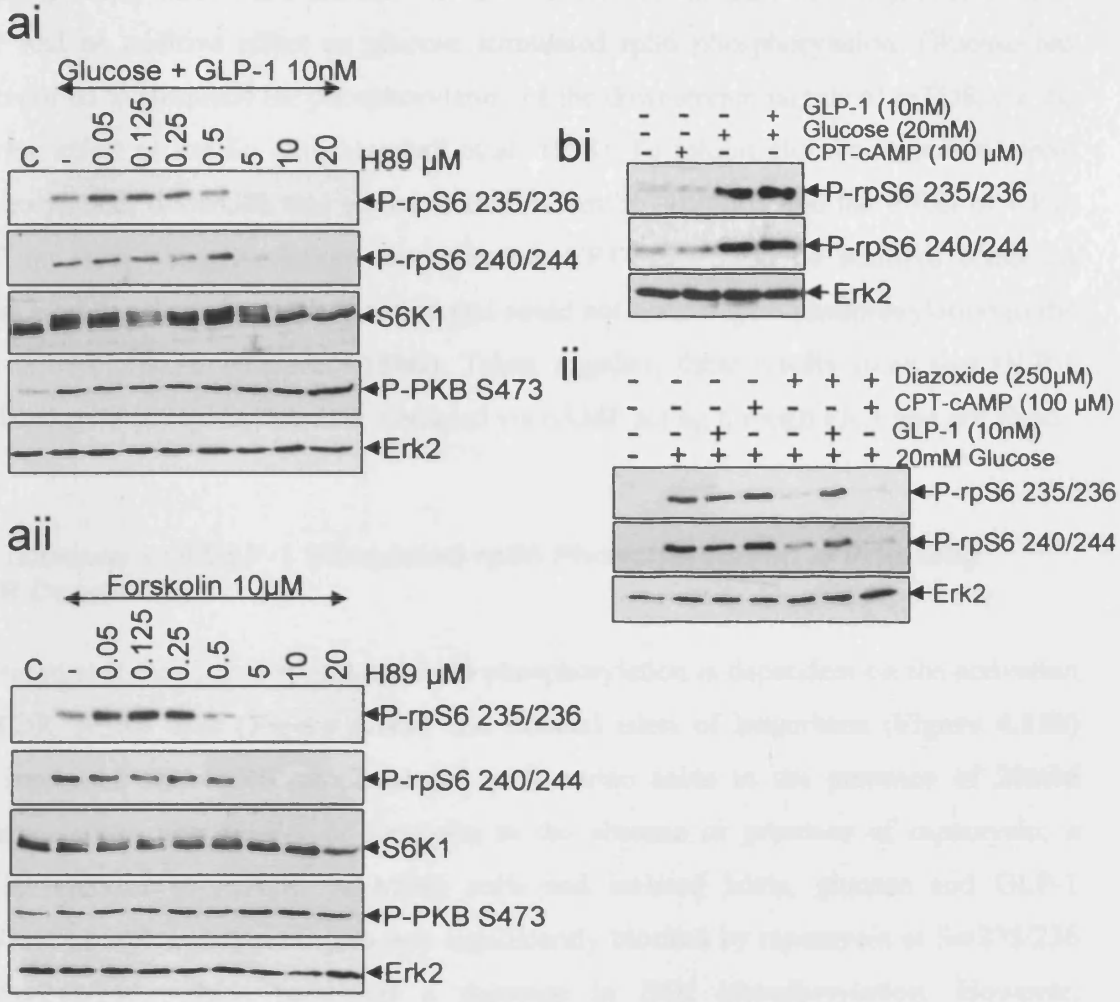


Figure 4.10: GLP-1 Stimulated rpS6 Phosphorylation is Dependent on PKA but not Epac

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing ai) 20mM glucose plus GLP-1 (10nM) or aii) forskolin (10μM) in the presence or absence of increasing concentrations of H89, a PKA inhibitor. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Ser473), S6K1 and total Erk2 as a loading control. bi) MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB at either 20mM glucose or 20mM glucose plus GLP-1 (10nM) and 8-CPT-2-Me-cAMP (100μM) (CPT-cAMP) bii) or in the presence or absence of diazoxide (250μM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244 and total Erk2 as a loading control. All results are representative of 3 independent experiments.

possible that glucose may be required for CPT-cAMP to activate rpS6 phosphorylation. Therefore, MIN6 cells were treated with CPT-cAMP in the presence of glucose. CPT-cAMP had no additive effect on glucose stimulated rpS6 phosphorylation. Glucose has been reported to stimulate the phosphorylation of the downstream targets of mTOR via the autocrine effect of insulin (Xu, Marshall et al. 1998). To inhibit glucose stimulated rpS6 phosphorylation, diazoxide was added to inhibit insulin secretion and the effect of CPT-cAMP on rpS6 phosphorylation was assessed. CPT-cAMP had no additive effect on glucose stimulated rpS6 phosphorylation and could not restore rpS6 phosphorylation in the presence of diazoxide (**Figure 4.10bii**). Taken together, these results show that GLP-1 stimulated rpS6 phosphorylation is mediated via cAMP acting through PKA and not Epac.

4.2.3 Glucose and GLP-1 Stimulated rpS6 Phosphorylation is Primarily mTOR Dependent

To determine if the GLP-1 stimulated rpS6 phosphorylation is dependent on the activation of mTOR, MIN6 cells (**Figure 4.11a**) and isolated islets of langerhans (**Figure 4.11b**) were incubated with KRB supplemented with amino acids in the presence of 20mM glucose, glucose plus GLP-1 or forskolin in the absence or presence of rapamycin, a specific inhibitor of mTOR. In MIN6 cells and isolated islets, glucose and GLP-1 stimulated phosphorylation of rpS6 was significantly blocked by rapamycin at Ser235/236 and Ser240/244, which paralleled a decrease in S6K phosphorylation. However, Ser235/236 remained partly phosphorylated in cells treated with rapamycin following forskolin stimulation, indicating that Ser235/236 can be phosphorylated via an mTOR independent pathway. As expected, the phosphorylation of PKB at Ser473 in response to glucose, GLP-1 and forskolin was unaffected by rapamycin. Together, these results show that glucose, GLP-1 and forskolin stimulated rpS6 phosphorylation is dependent upon the activation of mTOR.

4.2.4 GLP-1 Stimulates the Phosphorylation of rpS6 via a Mechanism which is Independent on the Autocrine Effect of Insulin

Glucose metabolism in pancreatic β-cells leads to an increase in intracellular calcium through an influx of calcium through L-type VGCC, which is augmented by hormones such as GLP-1. This rise in intracellular Ca^{2+} stimulates a number of important pancreatic β-cell functions including insulin secretion. It has previously been reported that the autocrine effect of insulin is responsible for glucose stimulated activation of mTOR

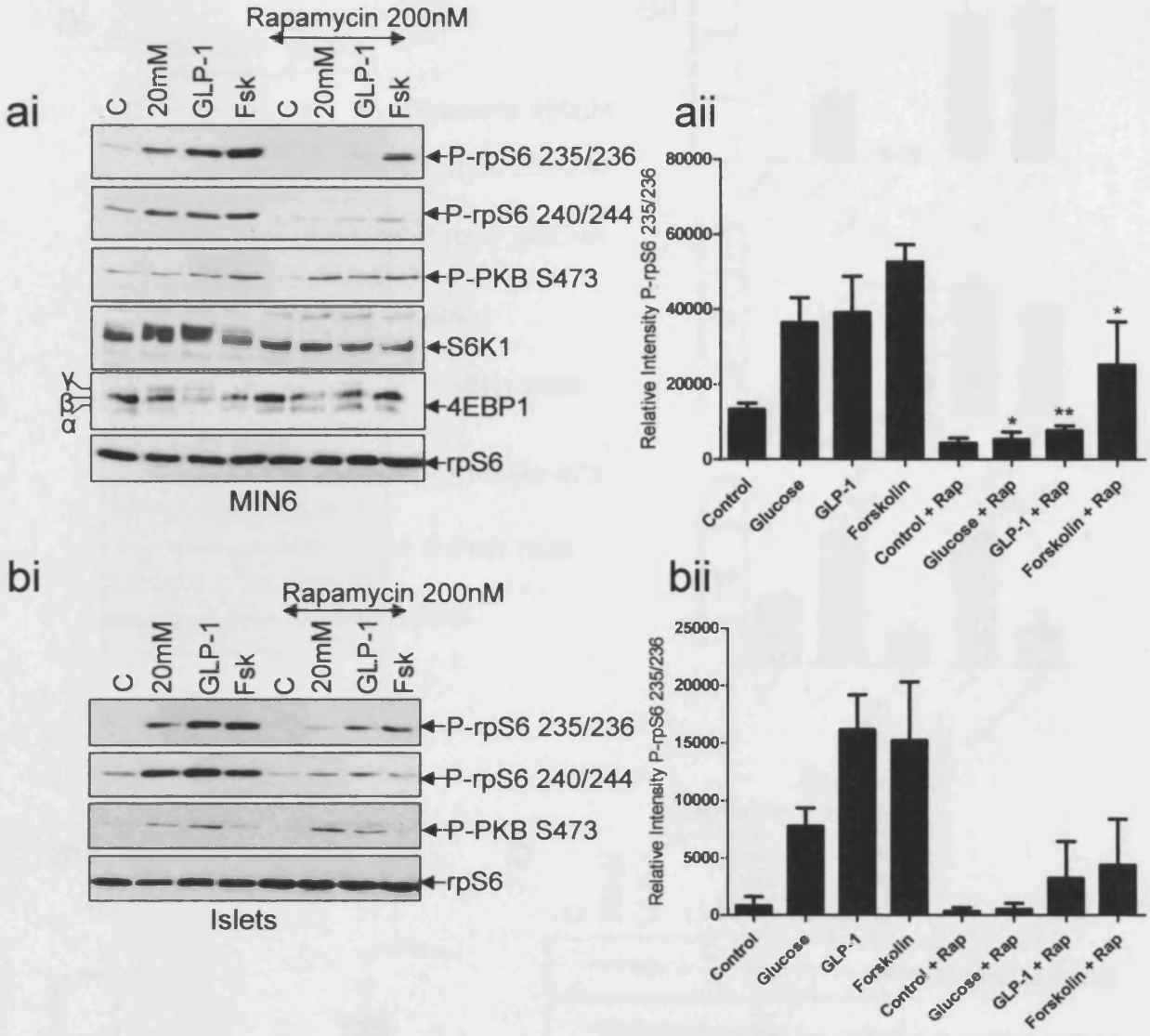


Figure 4.11: Glucose and GLP-1 Dependent rpS6 Phosphorylation is Primarily mTOR Dependent.

(ai) MIN6 cells and (bi) islets of langerhans were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing glucose (20mM), 20mM glucose plus 10nM GLP-1 or 10μM forskolin (Fsk) in the presence or absence of 200nM rapamycin. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Ser473), S6K1 and total rpS6 as a loading control. aii) Quantified data from phospho-rpS6 235/236 blots in *ai* shown as means ± SEM; $n = 3$. $*P < 0.05$, $**P < 0.01$. P value obtained using a Two-way ANOVA. bii) Quantified data from phospho-rpS6 235/236 blots in *bi* shown as means ± SEM; $n = 2$.

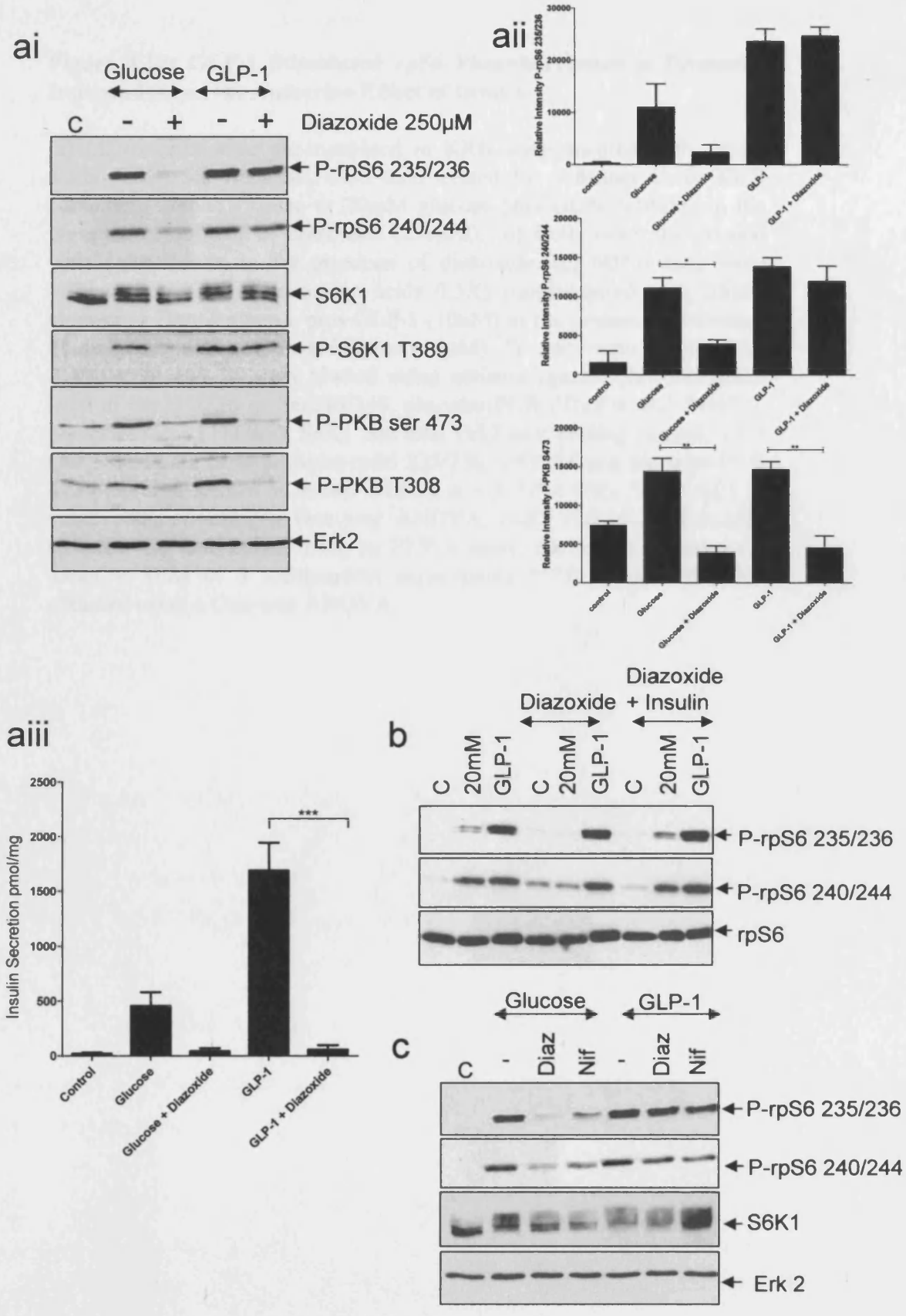

ai
Glucose
GLP-1
C
-
+
-
+
Diazoxide 250μM
P-rpS6 235/236
P-rpS6 240/244
S6K1
P-S6K1 T389
P-PKB ser 473
P-PKB T308
Erk2
aii
Relative Intensity P-rpS6 235/236
Relative Intensity P-rpS6 240/244
Relative Intensity P-PKB S473
control
Glucose
Glucose + Diazoxide
GLP-1
GLP-1 + Diazoxide
aiii
Insulin Secretion pmol/mg
Control
Glucose
Glucose + Diazoxide
GLP-1
GLP-1 + Diazoxide
b
Diazoxide
Diazoxide + Insulin
C
20mM
GLP-1
P-rpS6 235/236
P-rpS6 240/244
rpS6
c
Glucose
GLP-1
C
-
Diaz
Nif
P-rpS6 235/236
P-rpS6 240/244
S6K1
Erk 2

Figure 4.12

Figure 4.12: GLP-1 Stimulated rpS6 Phosphorylation is Primarily Independent on the Autocrine Effect of Insulin

(a) MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB containing 20mM glucose or 20mM glucose plus GLP-1 (10nM) in the presence or absence of diazoxide (250μM). (b) Cells were also treated with 1μM insulin in the presence of diazoxide. (c) MIN6 cells were incubated in KRB (plus amino acids 0.5X) supplemented with 20mM glucose or 20mM glucose plus GLP-1 (10nM) in the presence or absence of diazoxide (250μM) or nifidipine (10μM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Thr308 and Ser473), phospho-S6K1 (Thr389), S6K1 and total Erk2 as a loading control. (aii) Quantified data from phospho-rpS6 235/236, 240/244 and phospho-PKB 473 blots in *ai* shown as means ± SEM; $n = 3$, $*P < 0.05$, $**P < 0.01$. P value obtained using a One-way ANOVA. (aiii) The level of insulin secreted was determined using an ELISA assay, the results shown are a mean ± SEM of 3 independent experiments $***P < 0.001$ P value obtained using a One-way ANOVA.

(McDaniel, Marshall et al. 2002). To determine whether insulin secretion is also an important determinant in GLP-1 stimulated rpS6 phosphorylation, MIN6 cells were incubated at 20mM glucose or at 20mM glucose plus GLP-1 in the presence or absence of diazoxide, an activator of K_{ATP} channel that inhibits insulin secretion, and its effects on rpS6 phosphorylation and insulin secretion determined. As expected, diazoxide blocked glucose stimulated insulin secretion and inhibited glucose stimulated rpS6 and S6K1 phosphorylation (**Figure 4.12ai**), which could be restored by the addition of insulin (**Figure 4.12b**). In contrast, diazoxide had no statistically significant effect on GLP-1 stimulated rpS6 on Ser235/236 but had a small, yet statistically insignificant, inhibitory effect on Ser240/244 phosphorylation (**Figure 4.12ai and ii**). In addition, GLP-1 stimulated S6K1 activation was unaffected by diazoxide, as assessed by the mobility shift of S6K1 and its phosphorylation on Thr389. Importantly, GLP-1 stimulated PKB activation was blocked by diazoxide. To provide additional evidence that GLP-1 stimulated rpS6 phosphorylation is independent of insulin secretion, MIN6 cells were treated with 20mM glucose or 20mM glucose plus GLP-1 in the presence or absence of another inhibitor of insulin secretion, nifedipine (a specific L-Type VGCC blocker). Like diazoxide, nifedipine also blocked glucose stimulated rpS6 phosphorylation (**Figure 4.12c**) yet neither inhibited GLP-1 stimulated rpS6 phosphorylation at Ser235/236 but had a small effect on Ser240/244 (**Figure 4.12c**).

Collectively, our data shows that glucose stimulated rpS6 phosphorylation is dependent on the autocrine effect of insulin likely acting through PKB. In contrast, GLP-1 stimulated rpS6 phosphorylation is unaffected by diazoxide or nifedipine whereas signalling to PKB is blocked, indicating that GLP-1 signalling to rpS6 is independent of insulin secretion and PKB activation.

4.2.5 GLP-1 Stimulated rpS6 Phosphorylation is Independent of PKB

My previous data indicated that glucose stimulated rpS6 phosphorylation is dependent on insulin secretion likely via a PKB dependent mechanism. In contrast, GLP-1 stimulated rpS6 phosphorylation was unaffected by inhibitors of insulin secretion which blocked PKB (**Figure 4.12a**). To provide further evidence that PKB activation is not required for GLP-1 stimulated rpS6 phosphorylation but is required for glucose stimulated rpS6 phosphorylation, we investigated the activation of rpS6 in response to glucose, GLP-1 and forskolin in MIN6 cells expressing a dominant negative form of PKB (DN-PKB) in which Thr308 and Ser473 are replaced by alanines (Kotani, Ogawa et al. 1999). Glucose

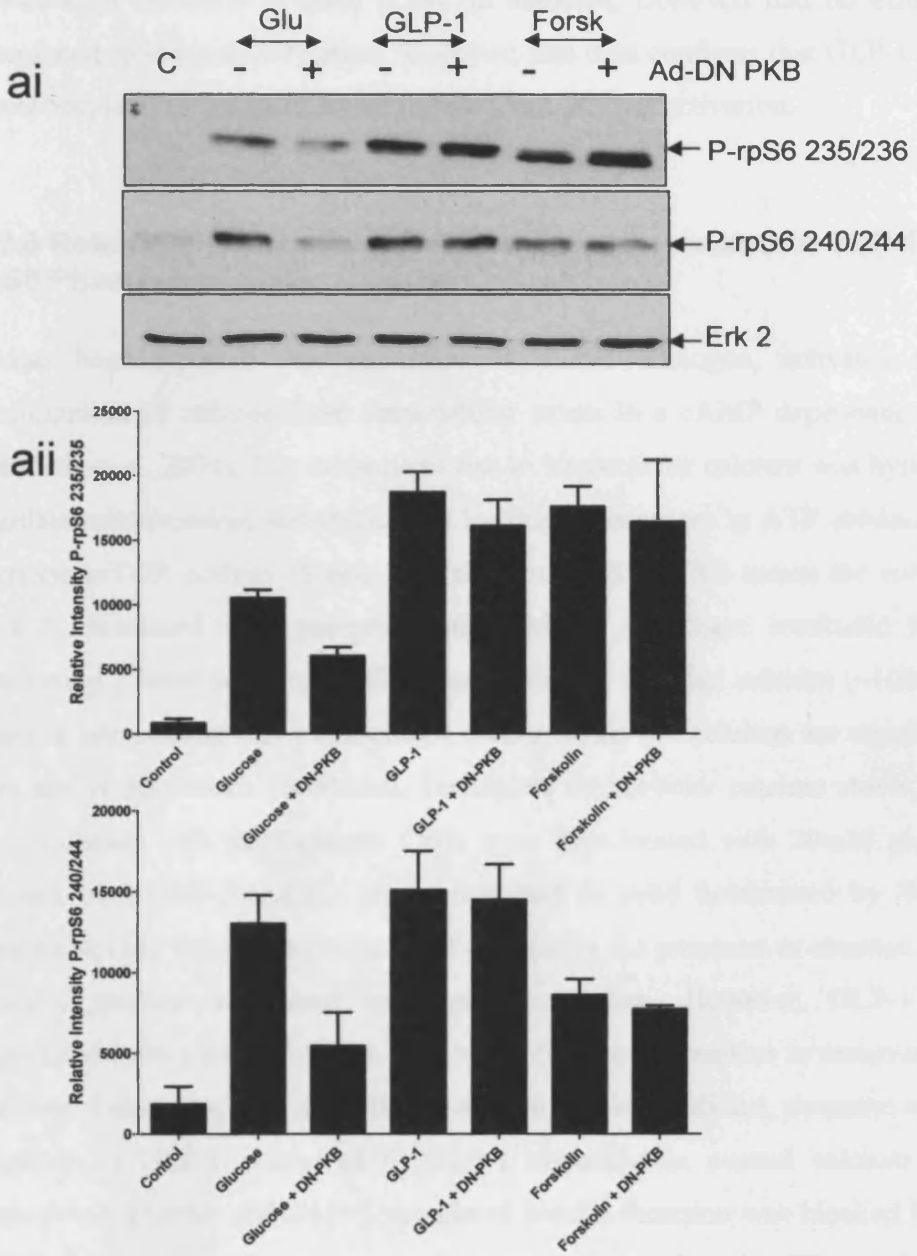


Figure 4.13: GLP-1 Stimulated rpS6 Phosphorylation is Independent of PKB

MIN6 cells were mock infected or infected with recombinant adenovirus expressing dominant negative PKB (DN-PKB) for 48h. Following infection, the cells were pre-incubated for 1h in KRB supplemented with amino acids (0.5X). Cells were then treated for a further 1h with 20mM glucose, glucose plus 10nM GLP-1 or 10μM forskolin. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244 and total Erk2 as a loading control. aii) Quantified data from phospho-rpS6 235/236 and 240/244 blots in *ai.* shown as means ± SEM; $n = 3$.

stimulated rpS6 phosphorylation was significantly reduced in cells over expressing DN-PKB, whereas the phosphorylation of rpS6 in response to GLP-1 was unaffected in the presence of DN-PKB (**Figure 4.13**). In addition, DN-PKB had no effect on forskolin stimulated rpS6 phosphorylation. Together, this data confirms that GLP-1 stimulates rpS6 phosphorylation via a mechanism independent of PKB activation.

4.2.6 Release of Intracellular Calcium is not Important for GLP-1 Stimulated rpS6 Phosphorylation

It has been reported that exenatide, a GLP-1 analogue, activates mTOR via the mobilization of calcium from intracellular stores in a cAMP dependent manner (Kwon, Marshall et al. 2004). The subsequent rise in intracellular calcium was hypothesized to up-regulate mitochondrial dehyrogenases leading to increases in ATP production which then increase mTOR activity (Kwon, Marshall et al. 2004). To assess the role of calcium in GLP-1 stimulated rpS6 phosphorylation, MIN6 cells were incubated in normal KRB containing 2.5mM calcium or KRB that contained nominal calcium (~100nM calcium) so that the intracellular and extracellular concentrations of calcium are equal and there is no flux across the plasma membrane. To deplete intracellular calcium stores, cells were also pre-incubated with thapsigargin. Cells were then treated with 20mM glucose or 20mM glucose plus GLP-1 and the phosphorylation of rpS6 determined by Western Blotting (**Figure 4.14a**). Incubation at nominal calcium in the presence or absence of thapsigargin, blocked glucose stimulated rpS6 phosphorylation. However, GLP-1 and forskolin stimulated rpS6 phosphorylation on Ser235/236 was insensitive to removal of intracellular calcium. I also observed a small, but statistically insignificant, decrease at Ser240/244 in response to GLP-1 compared to GLP-1 treatment in normal calcium conditions. As anticipated, glucose and GLP-1 stimulated insulin secretion was blocked by incubation in nominal calcium in the presence of absence of thapsigargin **(Figure 4.14b)**. This data suggests that GLP-1 stimulated rpS6 phosphorylation is not dependent upon an increase in intracellular calcium concentration.

4.2.7 Role of PI3K in rpS6 Phosphorylation in MIN6 cells

To investigate whether rpS6 phosphorylation by glucose or GLP-1 was dependent upon PI3K, MIN6 cells were incubated with 20mM glucose (**Figure 4.15a**), 20mM glucose plus GLP-1 (**Figure 4.15b**) or forskolin (**Figure 4.15c**) in the presence or absence of increasing concentrations of the PI3K inhibitors LY294002 and wortmannin. Both PI3K inhibitors

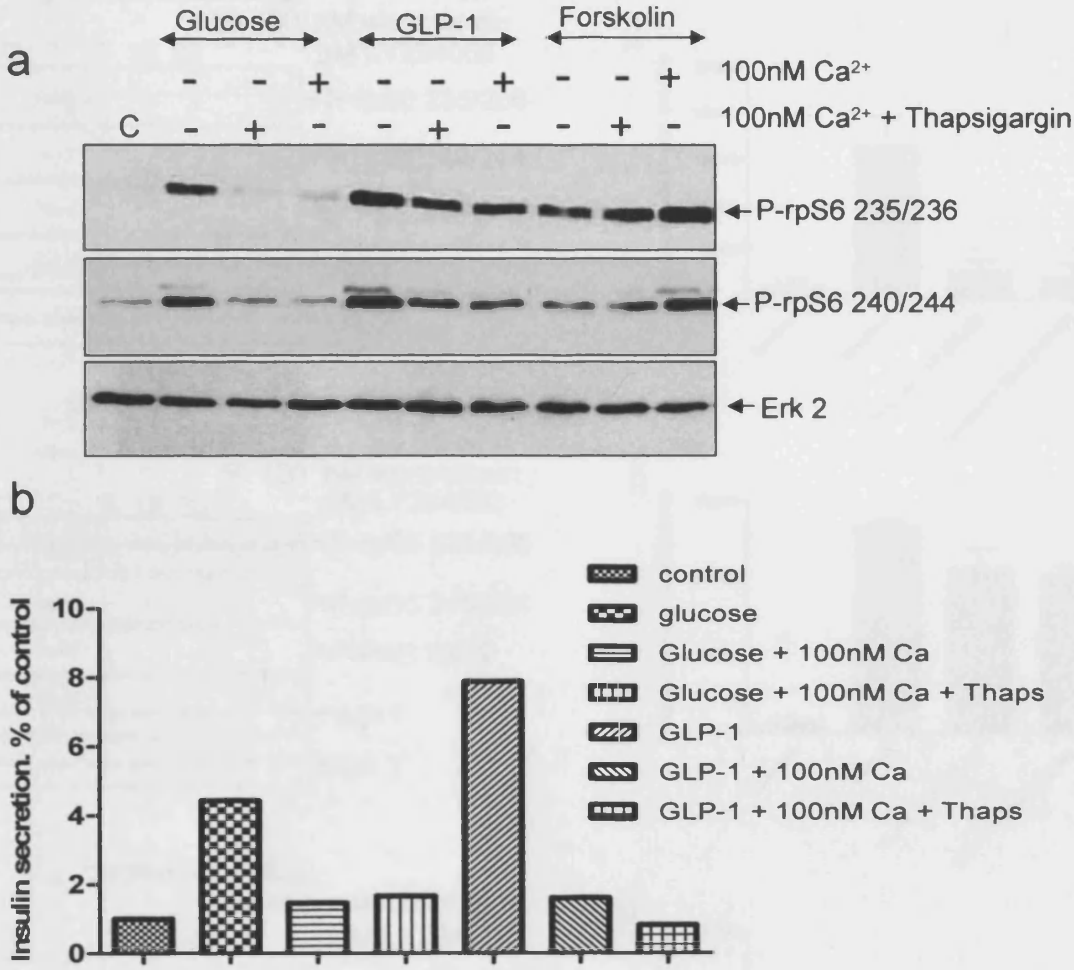


Figure 4.14: Release of Intracellular Calcium is not Important for GLP-1 Stimulated rpS6 Phosphorylation

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h with 20mM glucose, glucose plus 10nM GLP-1 or 10μM forskolin, in the presence or absence of modified KRB containing nominal calcium (100nM), which prevents calcium influx by balancing the extracellular and intracellular calcium levels or nominal calcium in the presence of 1μM thapsigargin. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244 and total Erk2 as a loading control. b) The level of insulin secreted was determined using an ELISA assay as described in the materials and methods.

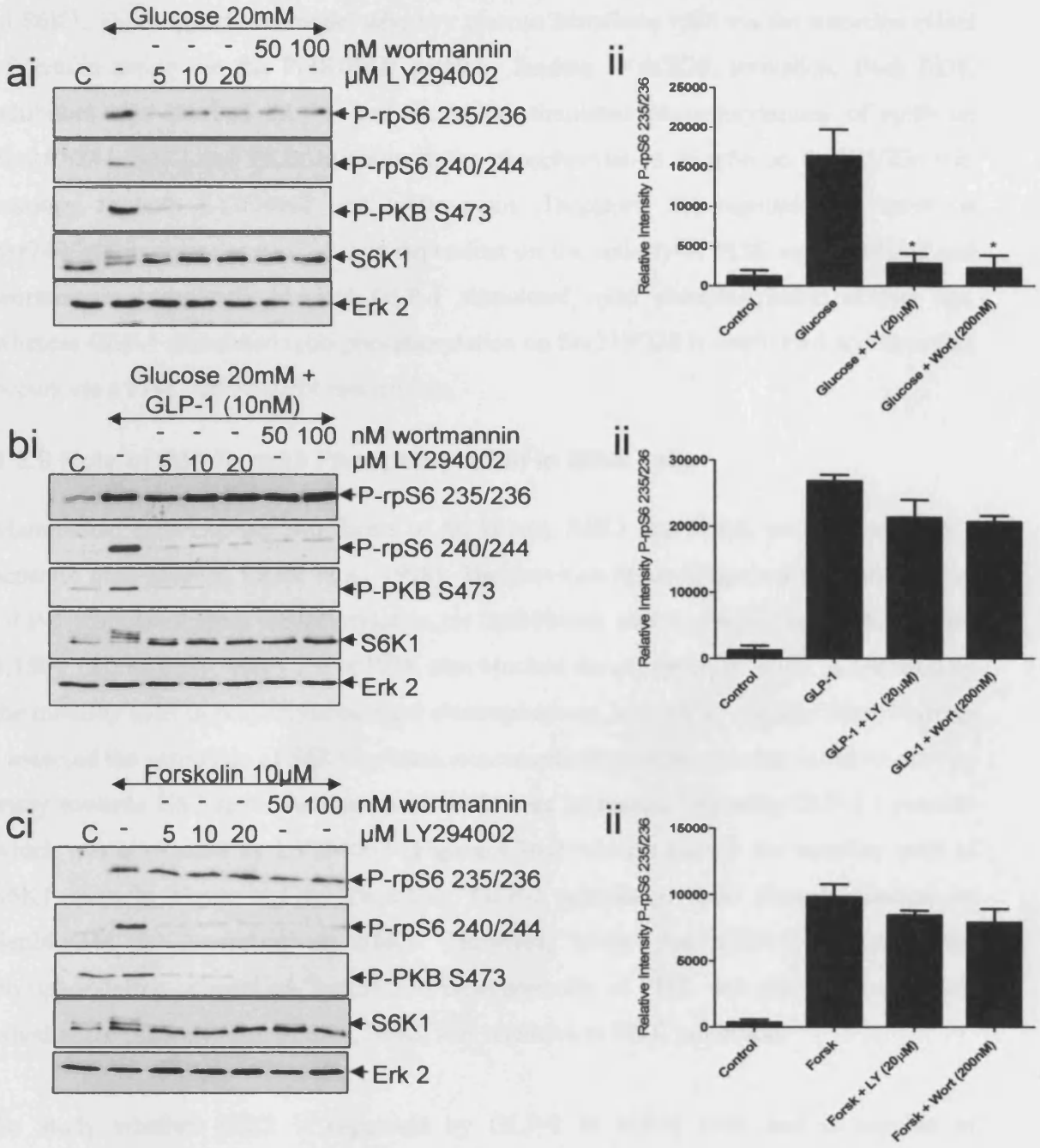


Figure 4.15: Role of PI3-K in rpS6 Phosphorylation in MIN6 Cells

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h with (a) 20mM glucose, (b) glucose plus 10nM GLP-1 or (c) 10μM forskolin in the presence or absence of increasing concentrations of LY294002 or wortmannin (two PI3-K inhibitors). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho-PKB (Ser473), S6K1 and total Erk2 as a loading control. (aii,bii,cii). Quantified data from phospho-rpS6 235/236 blots in *ai,bi,ci.* shown as means ± SEM; $n = 3$. $^*P < 0.05$. P value obtained using a Two-way ANOVA.

blocked glucose stimulated phosphorylations of rpS6 on Ser235/236 and Ser240/244 and of S6K1. This supports the model whereby glucose stimulates rpS6 via the autocrine effect of insulin acting via the PI3K/PKB pathway leading to mTOR activation. Both PI3K inhibitors also blocked GLP-1 and forskolin stimulated phosphorylations of rpS6 on Ser240/244, S6K1 and PKB. In contrast, the phosphorylation of rpS6 on Ser235/236 was resistant to both LY294002 and wortmannin. Therefore, the regulation of rpS6 on Ser240/244 in response to GLP-1 is dependent on the activity of PI3K as LY294002 and wortmannin completely blocked GLP-1 stimulated rpS6 phosphorylation at this site, whereas GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is unaffected and therefore occurs via a PI3K independent mechanism.

4.2.8 Role of S6K in rpS6 Phosphorylation in MIN6 cells

Mammalian cells express two forms of S6 kinase, S6K1 and S6K2, each encoded by a separate gene (Shima, Pende et al. 1998). The previous figure (**Figure 4.15**) showed that GLP-1 stimulated rpS6 phosphorylation on Ser240/244 was dependent on PI3K (**Figure 4.15b**). Interestingly, inhibition of PI3K also blocked the activation of S6K1 as assessed by the mobility shift in polyacrylamide gel electrophoresis. In order to confirm these findings I assessed the activation of S6K1 by immunoprecipitating the enzyme and using an activity assay towards GST-rpS6. Activation of S6K1 was increased following GLP-1 treatment which was attenuated by LY294002 (**Figure 4.16a**) which parallels the mobility shift of S6K1 seen in Figure 4.11b. Therefore, GLP-1 stimulated rpS6 phosphorylation on Ser240/244 is dependent on S6K1. However, given that GLP-1 stimulates the phosphorylation of rpS6 on Ser235/236 independently of PI3K and S6K1 I investigated whether the other isoform of S6K, S6K2 was sensitive to PI3K inhibition.

To study whether S6K2 is regulated by GLP-1 in MIN6 cells and is capable of phosphorylating rpS6 on Ser235/236 in the presence of LY294002, I treated MIN6 cells with glucose (20mM) plus GLP-1 for 60min. Activation of S6K2 was observed after GLP-1 treatment, as assessed by immunoprecipitating the enzyme and using an activity assay towards GST-rpS6. Furthermore, the activity of S6K2 was blocked in the presence of the PI3K inhibitor, LY294002 (**Figure 4.16b**). This data indicates that GLP-1 stimulated rpS6 phosphorylation on Ser240/244 is dependent on S6K activation, whereas the LY294002 resistant phosphorylation of rpS6 on Ser235/236 in response to GLP-1 is not dependent on either S6K1 or S6K2.

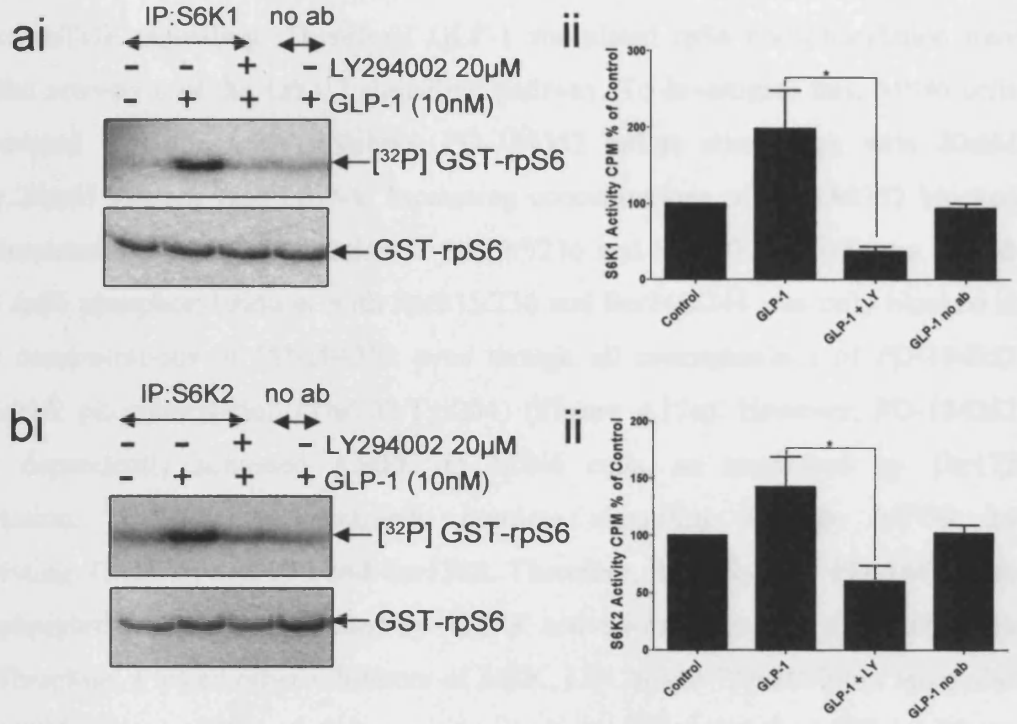


Figure 4.16: GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is independent of S6K1 and S6K2

MIN6 cells were pre-incubated for 1h in KRB supplemented with amino acids (0.5X). Cells were then treated for a further 1h with 20mM glucose plus 10nM GLP-1. Endogenous S6K1 (a) and S6K2 (b) were immunoprecipitated and assayed for their phosphotransferase activity against recombinant GST-rpS6 fusion protein *in vitro*. Labelled GST-rpS6 substrate was visualized by autoradiography after SDS-PAGE. Representative autoradiograms of rpS6 phosphorylation detected after exposure to autoradiography film 24-48h at room temperature. aii,bii) relative ^{32}P incorporation is displayed in the histogram above the autoradiogram. Activity is measured in CPM expressed relative to that of untreated control cells shown as means ± SEM; $n = 3$. $*P < 0.05$, P value obtained using a One-way ANOVA.

4.2.9 Role of the Erk1/2 Pathway in GLP-1 Stimulated rpS6 Phosphorylation

Our data has shown that the phosphorylation of rpS6 in response to GLP-1 is independent of insulin secretion and PKB activation. It has previously been shown that Erk1/2 and its downstream target RSK1 can phosphorylate and inactivate TSC2 on Ser1798, a negative regulator of mTOR signalling. Therefore, GLP-1 stimulated rpS6 phosphorylation may occur via the activation of the Erk1/2 signalling pathway. To investigate this, MIN6 cells were pre-treated with the MEK inhibitor PD-184352 before stimulation with 20mM glucose or 20mM glucose plus GLP-1. Increasing concentrations of PD-184352 blocked glucose stimulated rpS6 phosphorylation at Ser235/236 and Ser240/244, whereas GLP-1 stimulated rpS6 phosphorylation at both Ser235/236 and Ser240/244 was only blocked at the higher concentrations of PD-184352 even though all concentrations of PD-184352 blocked Erk1/2 phosphorylation (Thr202/Tyr204) (**Figure 4.17a**). However, PD-184352 also dose dependently activated AMPK in MIN6 cells, as monitored by Thr172 phosphorylation. AMPK can negatively regulate signalling through mTOR by phosphorylating TSC2 on Ser1270 and Ser1388. Therefore, the effects of PD-184352 on rpS6 phosphorylation could be mediated by AMPK activation rather than the inactivation of MEK. Therefore, I tested other inhibitors of MEK, U0126 and PD98059 but these also activated AMPK (data not shown). It has previously been shown that the MEK inhibitors, PD98059 and U0126 are strong activators of AMPK whilst PD-184352 was not affecting AMPK activation in HEK 293 cells (Dokladda, Green et al. 2005). In light of these findings, the role of Erk1/2 in rpS6 phosphorylation remains unclear. To establish whether strong activators of the Erk1/2 signalling cascade could stimulate rpS6 phosphorylation in pancreatic β-cells, islets of langerhans were treated with phorbol ester, Phorbol 12-myristate 13-acetate (PMA), epidermal growth factor (EGF) or depolarising concentrations of potassium (K^{+50}) (which have previously been shown to activate Erk1/2 in pancreatic β-cells (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003)), in the presence or absence of glucose. EGF and K^{+50} were unable to stimulate rpS6 phosphorylation on their own (**Figure 4.17b**), although PMA was able to stimulate a small but significant increase in rpS6 phosphorylation on Ser235/236 and Ser240/244.

To further investigate whether Erk1/2 activation contributes to GLP-1 stimulated rpS6 phosphorylation, MIN6 cells were infected with increasing titres of recombinant adenovirus encoding MKP3. MKP3 is a dual-specific phosphatase that dephosphorylates the activation loop of Erk1/2 with very high specificity (Rolfe, McLeod et al. 2005). 48h

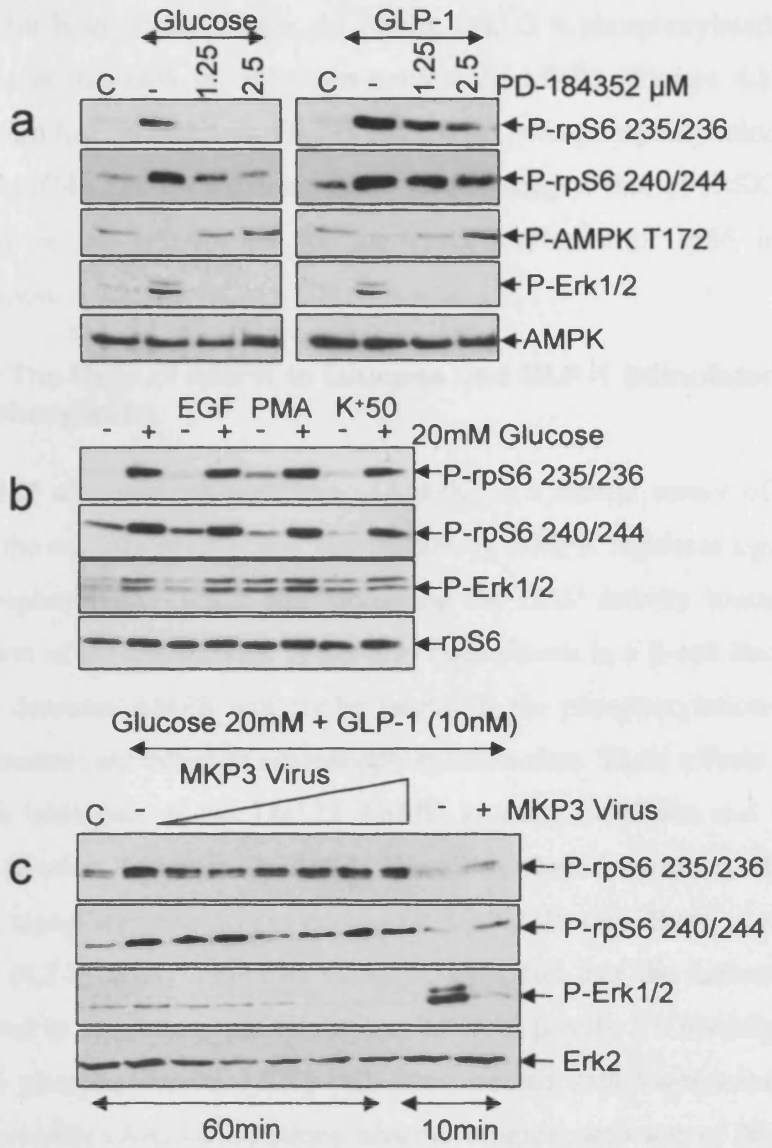


Figure 4.17: Role of the Erk1/2 Pathway in GLP-1 Stimulated rpS6 Phosphorylation

a) MIN6 cells were pre-incubated in KRB (plus amino acids 0.5X) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 20mM glucose or 20mM glucose plus GLP-1 (10nM) in the presence or absence of increasing concentrations of PD184352, as indicated in the figure. b) Islets of langerhans were treated with agents known to stimulate ERK activation, 20ng/mlEGF for 5min, 1µM PMA for 15min or 50mM potassium [K^{+50}] for 5min. c) MIN6 cells were mock infected or infected with increasing concentrations of a recombinant adenovirus over-expressing MKP3 for 48h. Following infection, MIN6 cells were pre-incubated in KRB (plus 0.5X amino acids) for 1h. Cells were then incubated for 10 or 60min in KRB supplemented with 20mM glucose plus 10nM GLP-1. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho Erk1/2 (Thr202/Tyr204), phospho AMPK (Thr172), total AMPK and total Erk2 as loading control.

post-infection, MIN6 cells were incubated in KRB supplemented with 20mM glucose plus GLP-1 for both 10 and 60min. At 10min, Erk1/2 is phosphorylated in response to GLP-1 and this is inhibited in cells over-expressing MKP3 (**Figure 4.17c**). However, MKP3 expression had no effect on GLP-1 stimulated rpS6 phosphorylation on either Ser235/236 or Ser240/244. Taken together, these results suggest that p90RSK or indeed the Erk1/2 pathway is not responsible for the phosphorylation of rpS6 in response to GLP-1 stimulation.

4.2.10 The Role of AMPK in Glucose and GLP-1 Stimulated rpS6 Phosphorylation

The AMP activated protein kinase (AMPK) is a central sensor of cellular energy status within the cell (for review see Hardie 2004)). AMPK regulates signalling through mTOR by phosphorylating TSC2 and increasing the GAP activity towards Rheb, resulting in inhibition of mTOR activity. It has also been shown in a β-cell line, INS-1, that cAMP is able to decrease AMPK activity by inhibiting the phosphorylations of Thr172 as well as other residues identified as autophosphorylation sites. These effects of cAMP are mediated through inhibition of the Thr172 AMPK kinases, CaMKKα and CaMKKβ and not via LKB1 (Hurley, Barre et al. 2006). Therefore, there is a cross talk between cAMP and AMPK signalling pathways in pancreatic β-cells (Hurley, Barre et al. 2006). Our previous results (4.2.8) using inhibitors of MEK indicated that the activation of AMPK maybe important in regulating rpS6 phosphorylation in β-cells. To investigate the role of AMPK in rpS6 phosphorylation, MIN6 cells were treated with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a strong pharmacological activator of AMPK before incubation for a further 1h in 20mM glucose or 20mM glucose plus GLP-1. As expected, treatment of MIN6 cells at high glucose and GLP-1 led to the phosphorylation of rpS6, which was paralleled by a decrease in the phosphorylation of AMPK on Thr172. Increasing concentrations of AICAR increases AMPK activity, as assessed by an increase in Thr172 phosphorylation, and blocked glucose stimulated rpS6 phosphorylation at Ser235/236 and Ser240/244 (**Figure 4.18a**). GLP-1 stimulated phosphorylation of Ser240/244 was also inhibited by AICAR. In contrast, GLP-1 stimulated rpS6 phosphorylation on Ser235/236 was unaffected by AICAR. To provide additional information on the role of AMPK on GLP-1 stimulated rpS6 phosphorylation, I used a mutant form of AMPK that acts as a constitutively active kinase ($\alpha 1^{312}$). MIN6 cells were infected with increasing titres of recombinant adenovirus encoding CA-AMPK along side a control GFP expressing virus

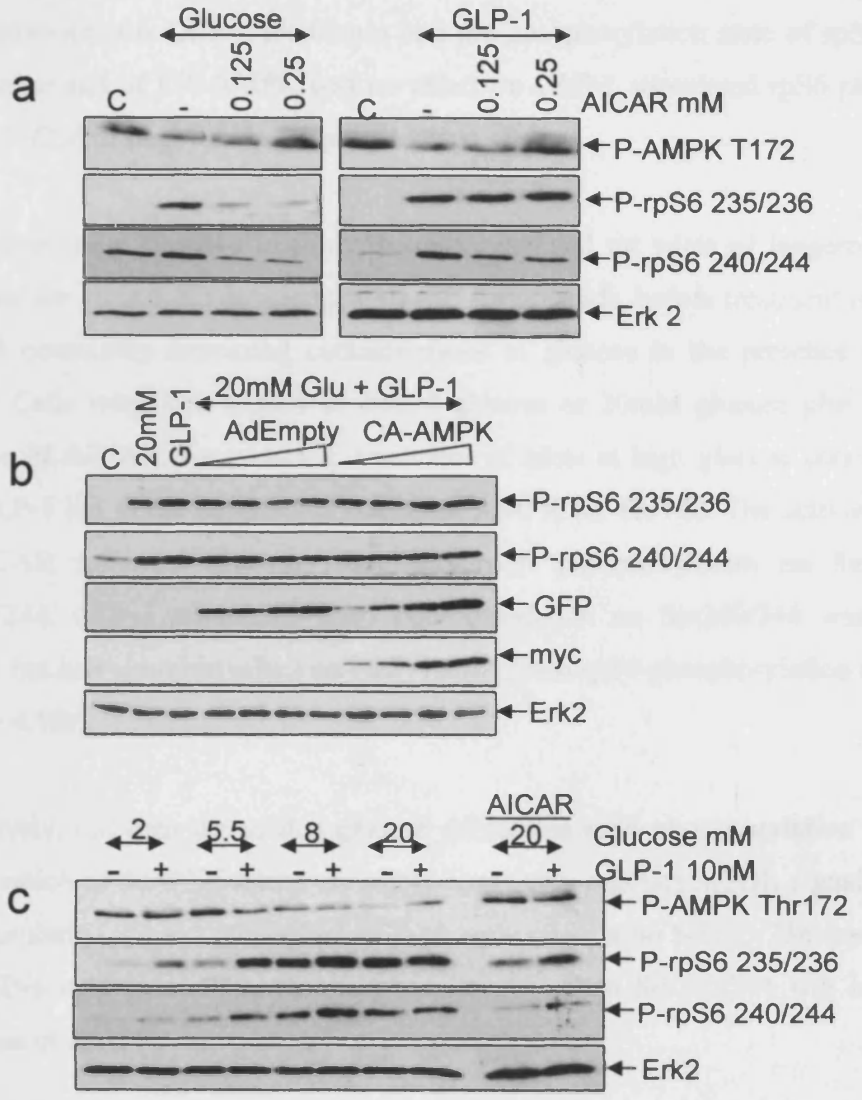


Figure 4.18: Role of AMPK in GLP-1 Stimulated rpS6 Phosphorylation

a) MIN6 cells were pre-incubated in KRB (plus 0.5X amino acids) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 20mM glucose or 20mM glucose plus 10nM GLP-1 in the presence or absence of increasing concentrations of AICAR, an artificial activator of AMPK. b) MIN6 cells were mock infected or infected with an empty adenovirus (AdEmpty) over-expressing GFP or a recombinant adenovirus over-expressing CA-AMPK for 48h. Following infection, MIN6 cells were pre-incubated in KRB (plus 0.5X amino acids) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 20mM glucose plus 10nM GLP-1. c) Islets of langerhans were pre-incubated in KRB (plus amino acids 0.5X) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 2, 5.5, 8 and 20mM glucose in the presence or absence of 10nM GLP-1. Cells were also treated at 20mM glucose or 20mM glucose plus GLP-1 in the presence or absence of 0.5mM AICAR. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, phospho AMPK (Thr172), total myc, total GFP and total Erk2 as a loading control.

(AdEmpty). 48h post-infection, MIN6 cells were incubated in KRB supplemented with 20mM glucose plus GLP-1 for 60min and the phosphorylation state of rpS6 determined. Over-expression of CA-AMPK had no effect on GLP-1 stimulated rpS6 phosphorylation on Ser235/236 or Ser240/244 (**Figure 4.18b**).

To confirm these findings in primary tissue, isolated rat islets of langerhans were pre-incubated for 1h in KRB supplemented with amino acids, before treatment for a further 1h in KRB containing increasing concentrations of glucose in the presence or absence of GLP-1. Cells were also treated at 20mM glucose or 20mM glucose plus GLP-1 in the presence of AICAR. As expected, treatment of islets at high glucose concentrations and with GLP-1 led to the dephosphorylation of AMPK on Thr172. The activation of AMPK by AICAR inhibited glucose stimulated rpS6 phosphorylation on Ser235/236 and Ser240/244. GLP-1 stimulated rpS6 phosphorylation on Ser240/244 was inhibited by AICAR but had a modest effect on GLP-1 stimulated rpS6 phosphorylation on Ser235/236 (**Figure 4.18c**).

Collectively, our data shows that glucose stimulated rpS6 phosphorylation is blocked via the activation of AMPK which may act to negatively regulate mTOR signalling via TSC2 phosphorylation. GLP-1 stimulated rpS6 phosphorylation on Ser235/236 was unaffected in both MIN6 cells and islets, yet the phosphorylation on Ser240/244 was blocked by the activation of AMPK.

4.2.11 Identification of the GLP-1 Stimulated rpS6 Ser235/236 Kinase

4.2.11.1 Can p90RSK Directly Phosphorylate rpS6 on Ser235/236 in Response to GLP-1?

Two classes of protein kinases are known to phosphorylate rpS6 *in vitro* and *in vivo*, the S6K1/2 and the p90 ribosomal S6 kinase (RSK) family of serine/threonine kinases. I have shown that GLP-1 stimulated phosphorylation of rpS6 on Ser235/236 is independent of S6K1 and likely independent of p90RSK. Surprisingly, I find that GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is unaffected by PI3K inhibition and therefore independent of S6K, which suggests that inactivation of S6K reveals the presence of a GLP-1 stimulated kinase which is able to phosphorylate the first two serine residues of rpS6 in order to compensate for the loss of S6K. Therefore, it is possible that, in the absence of S6K1, p90RSK compensates to phosphorylate Ser235/236. In order to determine whether the Erk1/2 pathway acting via p90RSK is responsible for the phosphorylation of

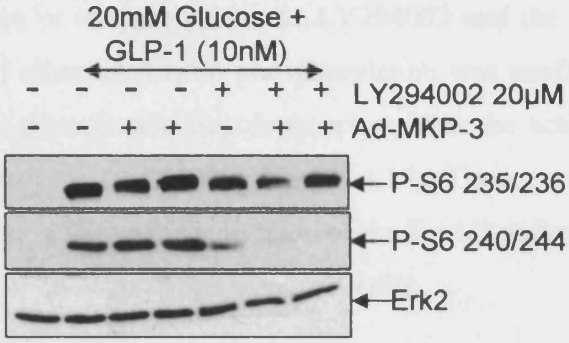


Figure 4.19: Compensatory rpS6 Phosphorylation on Ser235/236 is Independent of Erk1/2 and p90RSK

MIN6 cells were mock infected or infected with a recombinant adenovirus over-expressing MKP3 for 48h. Following infection, MIN6 cells were pre-incubated in KRB (plus 0.5X amino acids) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 20mM glucose plus 10nM GLP-1 in the presence or absence of LY294002 (20μM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244 and total Erk2 as a loading control. Results representative of 2 independent experiments.

Ser235/236 in response to GLP-1 in the presence of LY294002, MIN6 cells were infected with recombinant adenovirus encoding MKP3 and incubated with 20mM glucose plus GLP-1 in the presence or absence of 20μM LY294002 and the phosphorylation state of rpS6 assessed. GLP-1 stimulated rpS6 phosphorylation was unaffected in the presence of MKP-3 (dual-specific phosphatase that dephosphorylates the activation loop of ERK1/2) alone or in the presence of LY294002 (**Figure 4.19**). These results indicate that GLP-1 does not phosphorylate rpS6 via the activation of the Erk1/2 pathway and hence is unlikely to be phosphorylated by p90RSK in pancreatic β-cells.

4.2.12 Does mTOR Inhibition Activate a Phosphatase?

The rapamycin sensitivity of the GLP-1 stimulated phosphorylation on Ser235/236 could be attributed to the activation/inactivation of a phosphatase which dephosphorylates rpS6 on Ser235/236. Indeed, in yeast and mammals the presence of nutrients mTOR stimulates the phosphorylation the alpha-4 regulatory subunit of PP2A (phosphatase). Phosphorylation induces a direct interaction with alpha-4 subunit with the catalytic subunit of PP2A. This interaction prevents association with the additional subunits required for activation and thus PP2A is inhibited and prevents dephosphorylation of downstream targets. In the absence of nutrients, or in cells treated with rapamycin, mTOR no longer phosphorylates the alpha-4 regulatory subunit of PP2A resulting in dephosphorylation and loss of PP2AC (catalytic) binding activity. PP2AC is then free to associate with the A and B subunits and to dephosphorylate downstream targets. I have shown that GLP-1 stimulated phosphorylation of rpS6 on Ser235/236 is sensitive to rapamycin; however, it is possible that mTOR inhibition may lead to the activation of a phosphatase which could dephosphorylate rpS6 on Ser235/236. To examine the possibility that rapamycin could activate a phosphatase, I tested the effects of okadaic acid (specific inhibitor of protein phosphatases 1 and 2A) (**Figure 4.20a**) and cantharidin (inhibitor of protein phosphatase 2A) (**Figure 4.20b**) on rapamycin inhibition of Ser235/236 phosphorylation in response to GLP-1. As expected, rapamycin completely abolished the GLP-1 induced rpS6 phosphorylation. However, okadaic acid had little effect on the phosphorylation of rpS6 on Ser235/236. However, in the presence of rapamycin, okadaic acid stimulated a significant restoration of rpS6 phosphorylation on Ser235/236. Treatment of cells with the PP2A inhibitor, cantharidin also resulted in a small restoration of rpS6 phosphorylation on Ser235/236 in the presence of rapamycin. Both inhibitors had no effect on rpS6 phosphorylation at Ser240/244. Therefore these results indicate that, in GLP-1 treated cells, rapamycin leads to the activation of PP2A, which dephosphorylates rpS6 on

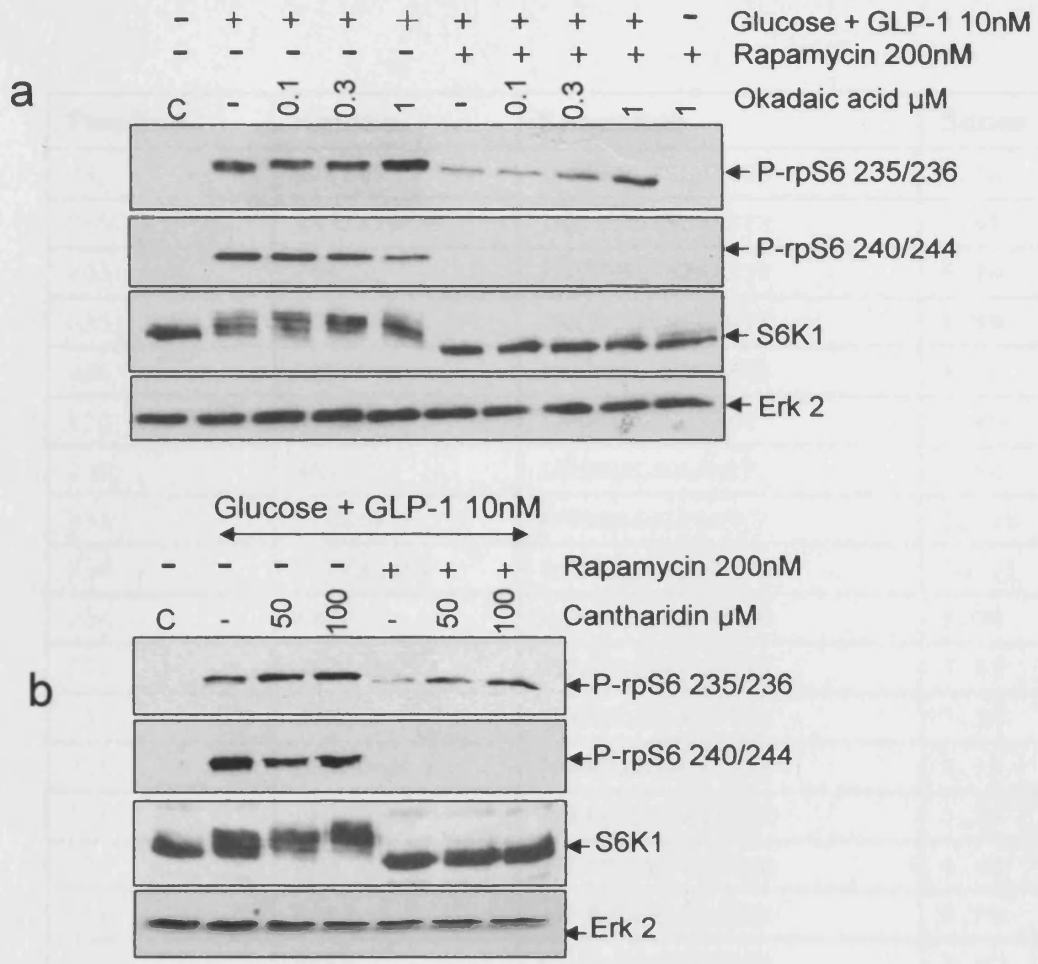


Figure 4.20: Can mTOR Inhibition Lead to Activation of a Phosphatase?

MIN6 cells were pre-incubated in KRB supplemented with amino acids (0.5X) for 1h. Cells were then treated for a further 1h in KRB with 20mM glucose plus 10nM GLP-1 in the presence or absence of (a) Oxadaic acid or (b) cantharidin, (both inhibitors of PP2A) in the presence or absence of rapamycin (200nM). Proteins were resolved by SDS-PAGE followed by immunoblot analysis using antibodies directed against phosphorylated rpS6 at Ser235/236 or Ser240/244 using phosphospecific antibodies. The cell lysates were also immunoblotted for S6K1 and Erk2 as a loading control. Results are representative of 2 independent experiments.

Position	Kinase	Sequence	Score
235	p70S6K	IAKRRRLSSLRASTS	9.00
235	PKC/Alpha	IAKRRRLSSLRASTS	7.61
235	PKA	IAKRRRLSSLRASTS	6.26
235	RSK5	IAKRRRLSSLRASTS	4.89
235	PKC/Delta	IAKRRRLSSLRASTS	4.50
235	RSK2	IAKRRRLSSLRASTS	3.60
235	AKT	IAKRRRLSSLRASTS	3.54
236	p70S6K	AKRRRLSSLRASTSK	12.25
236	PKC/Alpha	AKRRRLSSLRASTSK	10.21
236	AKT2	AKRRRLSSLRASTSK	9.00
236	PKC/Eta	AKRRRLSSLRASTSK	7.89
236	SGK	AKRRRLSSLRASTSK	7.58
236	PKC/Delta	AKRRRLSSLRASTSK	6.73
236	PKC/Iota	AKRRRLSSLRASTSK	5.36
236	PKA	AKRRRLSSLRASTSK	4.68
236	RSK2	AKRRRLSSLRASTSK	3.70
240	RSK5	RLSSLRASTSKSESS	7.22
240	PKC/Alpha	RLSSLRASTSKSESS	5.13
240	p70S6K	RLSSLRASTSKSESS	4.50
240	RSK2	RLSSLRASTSKSESS	4.40
240	PKC/Eta	RLSSLRASTSKSESS	2.77
244	PKC/Eta	LRASTSKSESSQK**	8.22
244	p70S6K	LRASTSKSESSQK**	4.00
244	PKC/Alpha	LRASTSKSESSQK**	3.40
244	RSK	LRASTSKSESSQK**	2.19

Figure 4.21 Motif Scan Search of rpS6
The primary amino acid sequence of ribosomal protein S6 was analyzed using computational method named GPS: group-based phosphorylation site predicting and scoring platform. For each predicted phosphorylation site, the position, kinase, flanking peptide and GPS score value are presented. A higher score indicates that the peptide is more probable to be a real phosphorylation site.

Ser235/236. This would indicate that GLP-1 stimulates a kinase which directly phosphorylates rpS6 which, in the presence of rapamycin is dephosphorylated due to the activation of a phosphatase.

4.2.13 Motif Scan for rpS6

Given that the phosphorylation of rpS6 on Ser235/236 in response to GLP-1 appears to be independent of S6K and p90RSK we sought to identify possible candidate kinases that can phosphorylate rpS6 on Ser235/236 using a program which recognises conserved kinase recognition sites (group-based phosphorylation site predicting and scoring platform; available at http://973-proteinweb.ustc.edu.cn/gps/gps_web/) (Zhou, Xue et al. 2004). Several kinases were identified that could potentially phosphorylate rpS6 on Ser235/236 (**Figure 4.21**). The kinases include the two classes of protein kinases that have been found to phosphorylate rpS6 *in vitro*, the S6K1/2 and the p90RSK family as well as PKA, SGK (Serum and glucocorticoid-inducible kinase), PKB and PKC, all members of the AGC family of kinases. However, we have already shown that PKB/S6K1, S6K2 and p90RSK are not involved in GLP-1 stimulated rpS6 phosphorylation on Ser235/236 (**Figures 4.13, 4.15** and **4.16**). Therefore the potential Ser235/236 rpS6 kinases are PKA, SGK and PKC.

4.2.14 PKC Activation Results in rpS6 Phosphorylation

To investigate whether PKC activation results in rpS6 phosphorylation, MIN6 cells were incubated in KRB supplemented with serum and/or PMA, a direct activator of both classical and novel PKC isoforms. It had previously been reported that, in order to activate PKCδ, PMA was added in the presence of serum, resulting in the rapid and robust phosphorylation of PKCδ (Parekh, Ziegler et al. 1999). Serum and PMA or PMA alone both resulted in an increase in rpS6 phosphorylation on Ser235/236 (**Figure 4.22**). Serum and PMA stimulated an increase in rpS6 phosphorylation on Ser240/244, whereas PMA alone resulted in a small increase in rpS6 phosphorylation on Ser240/244. In order to assess whether PMA stimulated rpS6 phosphorylation occurs via a similar mechanism than GLP-1 mediated signalling to rpS6 on Ser235/236, MIN6 cells were treated with PMA or PMA plus serum in the presence or absence of the PI3K inhibitors, LY294002 and wortmannin, or the mTOR inhibitor rapamycin (**Figure 4.22a/b**). Like GLP-1 stimulated rpS6 phosphorylation, PMA-stimulated rpS6 phosphorylation at Ser235/236 was resistant to inhibitors of PI3K. Serum plus PMA stimulated rpS6 phosphorylation on Ser235/236 was reduced by LY294002 and wortmannin to the same level as PMA alone. Rapamycin

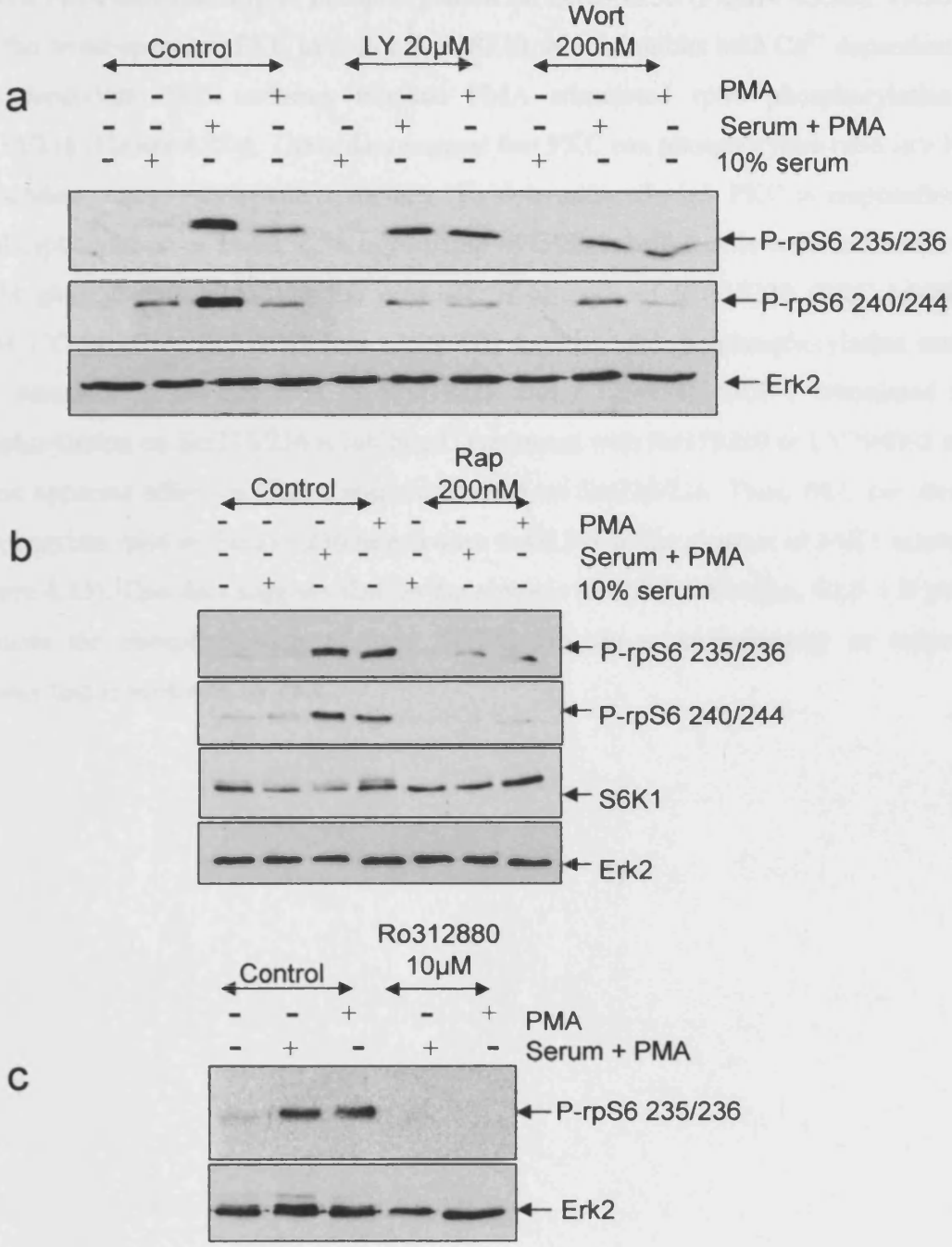


Figure 4.22: PKC Activation Results in Ribosomal Protein S6 Phosphorylation

MIN6 cells were pre-incubated in KRB (plus 0.5X amino acids) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 10% serum, 10% serum and 1μM PMA or 1μM PMA alone in the (a) presence or absence of the PI3K inhibitors LY294002 (20μM) or wortmannin (200nM) or (b) 200nM rapamycin or (c) 10μM Ro312880 a broad spectrum PKC inhibitor. Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, S6K1 and total Erk2 as a loading control. Results are representative of 3 independent experiments.

blocked PMA-stimulated rpS6 phosphorylation on Ser235/236 (**Figure 4.22b**). Treatment with the broad-spectrum PKC inhibitor Ro318220, which inhibits both Ca^{2+} dependent and Ca^{2+}independent PKC isoforms blocked PMA stimulated rpS6 phosphorylation on Ser235/236 (**Figure 4.22c**). These data suggest that PKC can phosphorylate rpS6 in a PI3K independent, rapamycin sensitive manner. To determine whether PKC is responsible for the phosphorylation of Ser235/236 in response to GLP-1, MIN6 cells were incubated with 20mM glucose plus GLP-1 in the presence or absence of Ro318220 (PKC inhibitor), 20μM LY294002 or Ro318220 and LY294002 together and the phosphorylation state of rpS6 assessed. In the presence of Ro318220 and LY294002, GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is inhibited. Treatment with Ro318220 or LY294002 alone had no apparent effect on GLP-1 stimulated rpS6 on Ser235/236. Thus, PKC can directly phosphorylate rpS6 on Ser235/236 in response to GLP-1 in the absence of S6K1 activation (**Figure 4.23**). This data suggests that, in the absence of S6K1 activation, GLP-1 is able to stimulate the phosphorylation of rpS6 Ser235/236 via a compensatory or redundant pathway that is mediated by PKC.

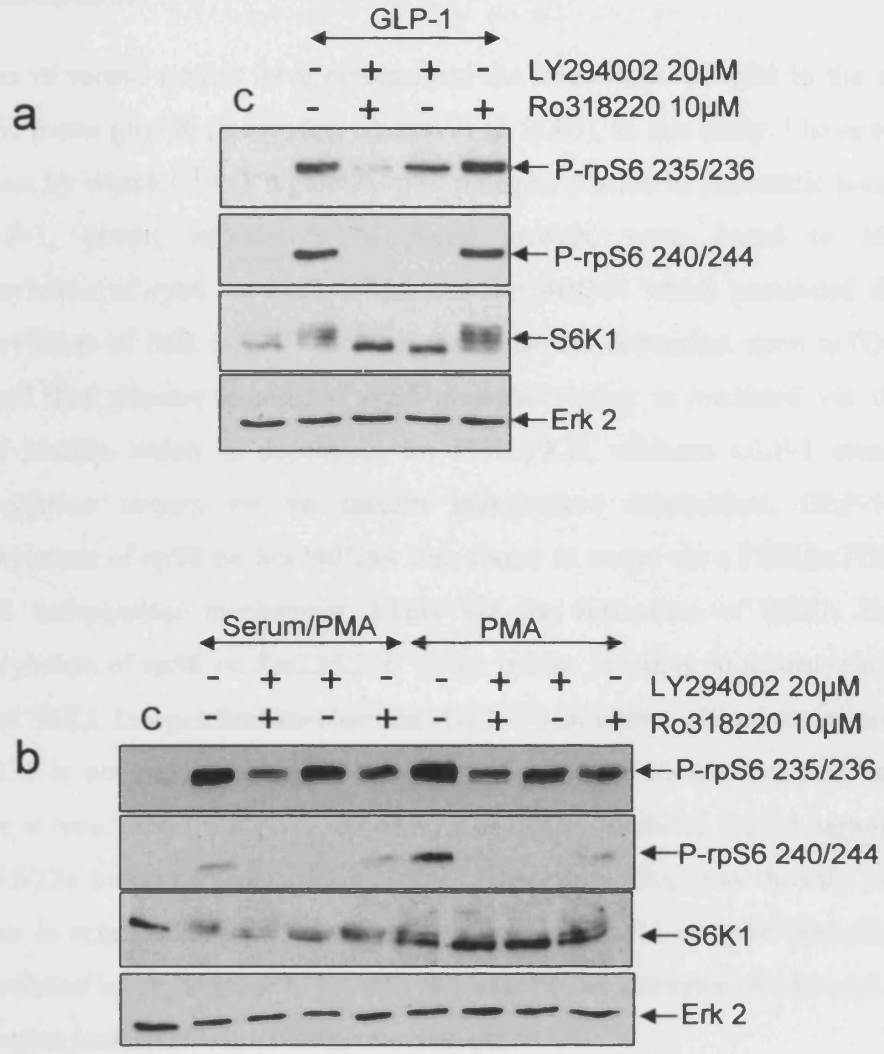


Figure 4.23: Role of PKC in Ribosomal Protein S6 Phosphorylation

MIN6 cells were pre-incubated in KRB (plus amino acids 0.5X) for 1h. Cells were then incubated for a further 1h in KRB supplemented with 20mM glucose plus 10nM GLP-1, 10% serum and 1μM PMA or 1μM PMA. To identify if PKC was responsible for the PI3K independent rpS6 phosphorylation on Ser235/236, cells were also incubated in the presence or absence of the PKC inhibitor Ro318220 (10μM) alone or in combination with LY294002 (20μM). Proteins were resolved by SDS-PAGE and Western blotted using antisera against phosphorylated rpS6 at Ser235/236 or Ser240/244, S6K1 and total Erk2 as a loading control.

4.3 Discussion

A number of recent studies have emphasized the importance of rpS6 in the regulation of pancreatic β-cell growth (Ruvinsky, Sharon et al. 2005). In this study, I have examined the mechanism by which GLP-1 regulates rpS6 phosphorylation in pancreatic β-cells. Glucose and GLP-1, potent stimulators of β-cell growth, were found to stimulate the phosphorylation of rpS6 on Ser235/236 and Ser240/244 which paralleled the increased phosphorylation of S6K and 4E-BP1 via a mechanism dependent upon mTOR activity. I also found that glucose stimulated rpS6 phosphorylation is mediated via the autocrine effect of insulin which is dependent on PI3K/PKB, whereas GLP-1 stimulated rpS6 phosphorylation occurs via an insulin independent mechanism. GLP-1 stimulated phosphorylation of rpS6 on Ser240/244 was found to occur via a PI3K/mTOR dependent but PKB independent mechanism, likely via the activation of S6K1. However, the phosphorylation of rpS6 on Ser235/236 which is also sensitive to rapamycin occurs via a PI3K and S6K1 independent mechanism. GLP-1 stimulated phosphorylation of rpS6 on Ser235/236 is not mediated by the two currently known S6 kinases, S6K and p90RSK. However, a broad spectrum PKC inhibitor, Ro318220, inhibited GLP-1 signalling to rpS6 on Ser235/236 in the presence of LY294002. Therefore, PKC may directly phosphorylate these sites in response to GLP-1. All these effects of GLP-1 on rpS6 phosphorylation are likely mediated by an increase in cAMP, as forskolin, an activator of adenylyl cyclase, can also phosphorylate rpS6 via a similar mechanism.

4.3.1 Glucose Regulates the Phosphorylation of Ribosomal Protein S6 by the Autocrine Effect of Insulin

I found that glucose promotes the phosphorylation of rpS6 at Ser235/236 and Ser240/244 which correlates with the activation of S6K1 via a rapamycin sensitive pathway. These effects are mediated via the autocrine effect of insulin as diazoxide or nifedipine (inhibitors of insulin secretion) completely blocks glucose stimulated rpS6 phosphorylation (**Figure 4.12b**). This is in agreement with Xu *et al.* and Gleason *et al.* in RINm5F cells, an alternative insulin-secreting β-cell line (Xu, Kwon et al. 1998; Gleason, Lu et al. 2007). I show that S6K1 phosphorylation is dependent on both PI3K and PKB activation. In other cell types it has been shown that PKB can directly phosphorylate TSC2 and inhibit the GAP activity towards Rheb and hence increase mTOR activity (Inoki, Li et al. 2002).

Therefore, it is likely that glucose increases mTOR activity and activates downstream signalling to rpS6 in β-cells via the PI3K/PKB pathway acting on TSC2 (**Figure 4.24**). AMPK also plays an important role in glucose stimulated rpS6 activation, since I show that artificial activation of AMPK using AICAR blocks glucose stimulated rpS6 phosphorylation (**Figure 4.18**). AMPK is known to regulate signalling through mTOR via the phosphorylation of TSC2 on Ser1270 and Ser1388, which enhances the ability of the TSC1/TSC2 complex to act as a Rheb-GAP, and therefore blocks Rheb-dependent mTOR activation (Inoki, Li et al. 2002). In summary, I hypothesise that, in pancreatic β-cells, glucose metabolism leads to an influx of calcium through L-type VGCC and subsequent insulin secretion. Insulin acts on the insulin receptor on the β-cell which in turn activates a downstream signalling cascade involving PI3K and PKB. Activated PKB phosphorylates and inhibits TSC2. Concomitantly, an increase in glucose metabolism leads to a decrease in the intracellular AMP level, thus inactivating AMPK which results in the dephosphorylation of TSC2. Together, this signalling pathway leads to the inactivation of TSC2 and hence increased mTOR activity. An increase in mTOR activity ultimately results in the activation of S6K1 and subsequent phosphorylation of rpS6 (**Figure 4.24**).

4.3.2 Phosphorylation of Ribosomal Protein S6 by GLP-1

I provide evidence that the ability of GLP-1 to signal to mTOR and rpS6 is dependent on an increase in cAMP, as artificial activation of adenylyl cyclase with forskolin or inhibition of PDEs using IBMX results in an increase in rpS6 phosphorylation. Furthermore, inhibition of GLP-1 and forskolin stimulated increases in cAMP production using MDL-12330A blocked rpS6 phosphorylation (**Figure 4.6 and 4.7**). Elevations in cAMP can lead to the activation of two downstream effectors, PKA and Epac. As H89, a PKA inhibitor, can inhibit GLP-1 and forskolin stimulated rpS6 phosphorylation and 8-CPT-2-Me-cAMP, an activator of Epac, had no effect on rpS6 phosphorylation, it is likely that PKA mediates the actions of cAMP in response to GLP-1. However, this interpretation is complicated by the fact that H89 can also inhibit S6K and p90RSK (Davies, Reddy et al. 2000).

Unlike glucose stimulated rpS6 phosphorylation, GLP-1 stimulated rpS6 phosphorylation is independent of the autocrine effect of insulin (**Figure 4.12**). Indeed, it had previously been published that agents that increase cAMP such as GLP-1, exenatide or forskolin can activate mTOR independently of insulin secretion in rat islets (Kwon, Marshall et al.

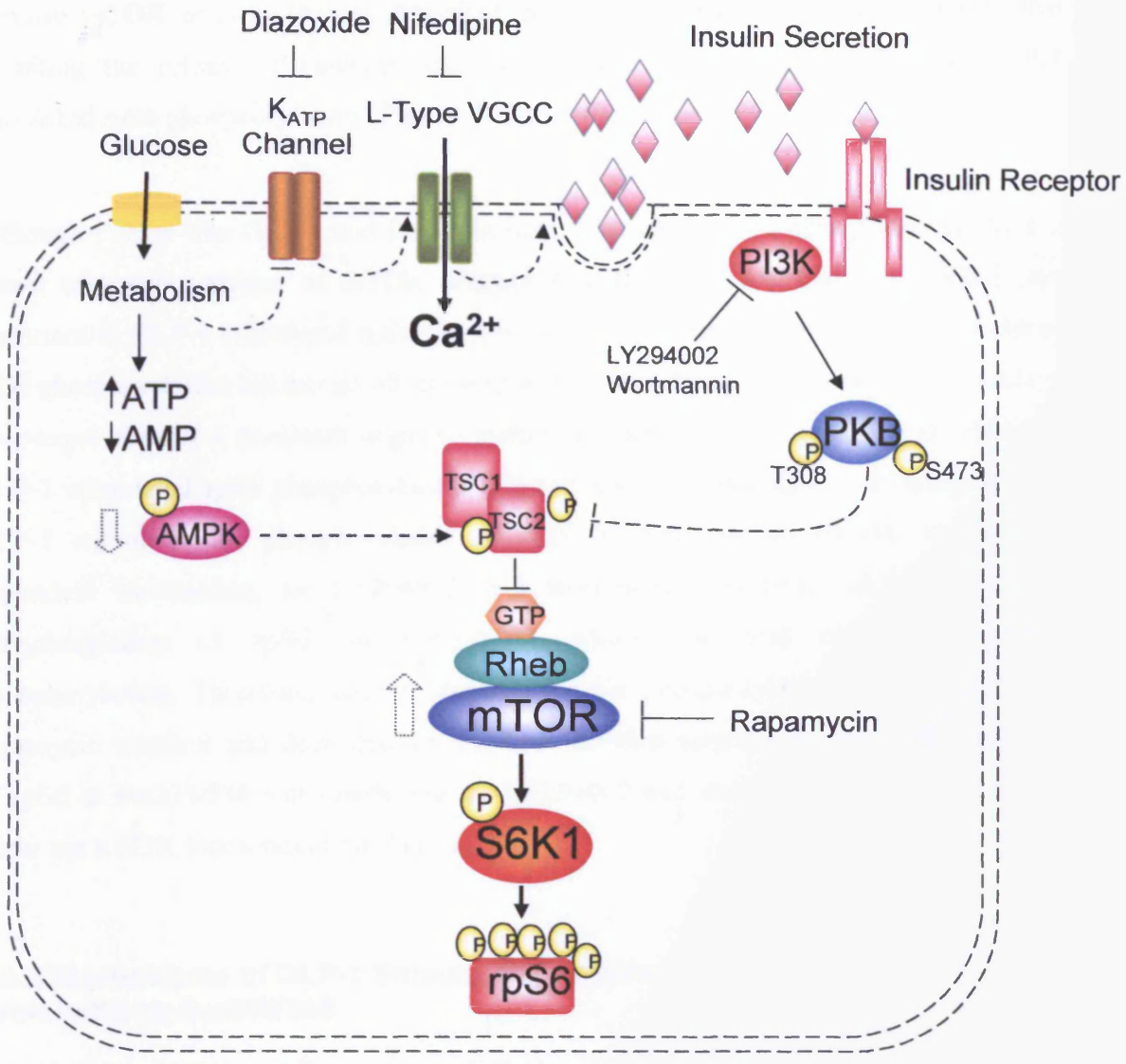


Figure 4.24: Proposed Model for Glucose Stimulated rpS6 Phosphorylation in Pancreatic β-cells

In pancreatic β-cells, glucose metabolism leads to an increase in the ATP/ADP ratio, the closure of ATP-sensitive K+ channels, the depolarization of the cell membrane, the opening of L-type VGCC and the subsequent influx of extracellular calcium into the cell. This influx of calcium leads to the exocytosis of insulin containing granules. Insulin acts in an autocrine manner to activate the insulin receptor and downstream signalling to PKB. PKB increases mTORC1 activity via the phosphorylation and inactivation of TSC1/2. An increase in glucose metabolism leads to a decrease in the levels of AMP which inactivates AMPK stimulating mTOR signalling.

2004). These authors hypothesised that cAMP stimulates the release of intracellular calcium that activates mitochondrial dehydrogenases which increase ATP that in turn increase mTOR activity (Kwon, Marshall et al. 2004). However, I demonstrate that inhibiting the release of calcium from intracellular stores has no effect on GLP-1 stimulated rpS6 phosphorylation (**Figure 4.13b**).

Although I show that GLP-1 and forskolin both stimulate the phosphorylation of PKB, a potent upstream activator of mTOR (**Figure 4.6, 4.7**), I demonstrate that PKB is not important in GLP-1 stimulated rpS6 phosphorylation as diazoxide blocks GLP-1 induced PKB phosphorylation but has no effect on rpS6 phosphorylation (**Figure 4.12**). In addition, over-expression of a dominant negative mutant of PKB in MIN6 cells had no effect on GLP-1 stimulated rpS6 phosphorylation (**Figure 4.13**). Interestingly, I demonstrate that GLP-1 stimulates the phosphorylation of rpS6, at least on Ser240/244, via a PI3K dependent mechanism, as LY294002 and wortmannin inhibited GLP-1 stimulated phosphorylation of rpS6 on Ser240/244 which paralleled changes in S6K1 phosphorylation. Therefore, GLP-1 stimulated rpS6 phosphorylation on Ser240/244 is rapamycin sensitive and dependent on PI3K. Somewhat surprisingly the phosphorylation of rpS6 at Ser235/236 was unaffected by LY294002 and wortmannin and therefore can occur via a PI3K independent mechanism.

4.3.3 Mechanisms of GLP-1 Stimulated Phosphorylation of Ribosomal Protein S6 on Ser240/244

4.3.3.1 Role of PI3K and Downstream Effectors in GLP-1 Signalling to rpS6

I found that GLP-1 promotes rpS6 phosphorylation at Ser240/244 using a PI3K, S6K1 and S6K2 dependent pathway (**Figure 4.15,16**). The phosphorylation at Ser240/244 is also inhibited by rapamycin, indicating the involvement of mTOR. Our previous data revealed that the phosphorylation of rpS6 is independent of the autocrine effect of insulin and PKB. However, I demonstrate that the phosphorylation of rpS6 on Ser240/244 in response to GLP-1 is dependent on the activity of PI3K as LY294002 and wortmannin completely block GLP-1 stimulated rpS6 phosphorylation on Ser240/244 (**Figure 4.15**). Therefore, GLP-1 activates PI3K via a mechanism independent of insulin secretion as PKB, which is downstream effector of PI3K, is inhibited by diazoxide whilst the phosphorylation of rpS6 on Ser240/244 is unaffected. Furthermore, inhibition of PI3K also inhibits the activation of two isoforms of S6K (S6K1 and S6K2) induced by GLP-1 (**Figure 4.16**). Therefore, the

GLP-1 induced phosphorylation of rpS6 on Ser240/244 is dependent on PI3K which activates S6K via an mTOR sensitive mechanism. Surprisingly, I find that GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is unaffected by PI3K inhibition and is therefore independent of S6K, which suggests that inactivation of S6K reveals the presence of a GLP-1 stimulated kinase which is able to phosphorylate the first two serine residues of rpS6 in order to compensate for the loss of S6K.

4.3.3.2 Potential Role of Atypical Protein Kinase C

Activation of PI3K leads to its recruitment to the plasma membrane, enabling the catalytic subunit to phosphorylate its lipid substrate PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3. Newly synthesized PtdIns(3,4,5)P_3 leads to the recruitment of downstream effector proteins that contain a PH domain, including atypical PKCs, to the plasma membrane where the combination of lipid binding and phosphorylation by PDK-1 activates the enzymes. Given that the phosphorylation of rpS6 on Ser240/244 is dependent upon PI3K, atypical PKCs are one possible effector of PI3K action.

The PKC superfamily is subdivided into three subfamilies according to their activation profiles. Conventional PKCs (cPKC, α, βI, βII, γ,) are sensitive to calcium and diacylglycerol and can be activated by phorbol esters, novel PKCs (δ,ε,θ,η) are sensitive to diacylglycerol and phorbol esters only and the atypical PKCs (aPKC,ζ,ι,/λ) are insensitive to all three regulators (Parker and Murray-Rust 2004). All PKC isoforms require phosphorylation by PDK-1 for activation. As PDK-1 is recruited to the membrane following agonist stimulation of cells, it is likely that atypical PKCs are also translocated to the membrane. Phosphorylation of PKCζ by PDK-1 is sensitive to changes in PtdIns-3,4,5-P_3 levels. It is thought that PtdIns-3,4,5-P_3 binds to the regulatory domain of this enzyme, relieving autoinhibition. PDK-1 then phosphorylates Thr410 leading to PKCζ activation (Chou, Hou et al. 1998). In pancreatic β-cells, it has been shown that GLP- activates PKCζ, resulting in its nuclear translocation (Buteau, Foisy et al. 2001). A more recent study revealed that PKCζ markedly enhances ß-cell proliferation via a mechanism which may be dependent on mTOR (Vasavada, Wang et al. 2007). The atypical PKC isoforms have not been shown to be direct activators of the mTOR pathway. However, it has been demonstrated that PKCζ can stimulate general protein synthesis in response to insulin in WEHI-3 cells (leukemia cell line) (Mendez, Kollmorgen et al. 1997). It is also possible that PKC can directly phosphorylate TSC2 to inhibit its activity. PKC could potentially phosphorylate the R*X*R*XX*(S/T) consensus sites within TSC2 (Tee, Anjum et al.

2003). However, to date there is no evidence showing that PKC directly phosphorylates TSC2.

PKCζ can also activate and phosphorylate both Raf-1 and MEK (Toker 2000) and the MAPK kinase pathway can play an important role in regulating mTOR activity via TSC2. This is unlikely to be the mechanism by which GLP-1 stimulats rpS6 on Ser240/244 as inhibition of Erk1/2 activation via the over expression of MKP3 had no inhibitory effect on GLP-1 stimulated rpS6 phosphorylation on Ser240/244.

4.3.3.3 Role of p38 Mitogen Activated Protein Kinase

Another potential mechanism for GLP-1 stimulated PI3K dependent rpS6 phosphorylation on Ser240/244 involves another downstream target of PI3K, the p38 mitogen activated protein kinase (MAPK) (Buteau, Foisy et al. 2001) which is dependent on Rac1 (Shin, Kim et al. 2005). The Rac subfamilies of Rho GTPases are important downstream targets of PI3K signalling. Indeed, studies have shown that PI3K can activate Rac indirectly via PtdIns(3,4,5)P_3-sensitive Rac-GEFs (Welch, Coadwell et al. 2003). Rac-GEFs are targeted to the membrane via their PH domain where they stimulate the exchange of GDP for GTP on Rac. Once active, Rac induces p38 activation via the stimulation of p21-activated protein kinases (PAKs) (Bornfeldt 2000). In the pancreatic β-cell line INS (832/13), GLP-1 has been reported to activate p38 MAPK and inhibition of p38 MAPK suppresses the proliferative effects of GLP-1 on the β-cell (Buteau, Foisy et al. 2001). p38 MAPK is a serine/threonine kinase and one of its downstream targets include the kinase p38-activated kinase MK2 (also known as MAPKAPK2). Recent studies have suggested that MK2 can increase the activity of mTOR by a mechanism dependent on TSC2. Following serum stimulation, MK2 was shown to phosphorylate TSC2 on Ser1210 (Li, Inoki et al. 2003). MK2 dependent phosphorylation of TSC2 increases its interaction with 14-3-3 and prevents its interaction with TSC1, thereby increasing the levels of Rheb.GTP and mTOR activity (**Figure 4.25**).

4.3.3.4 Role of hVps34

It has been shown that a major pathway by which amino acids stimulates mTOR activity is through a class 3 PI3K, hVps34 (Byfield, Murray et al. 2005; Nobukuni, Kozma et al. 2007). It has been reported that LY294002 and wortmannin can inhibit hVps34 (Byfield, Murray et al. 2005). Therefore, it is possible that the effects of LY294002 and wortmannin

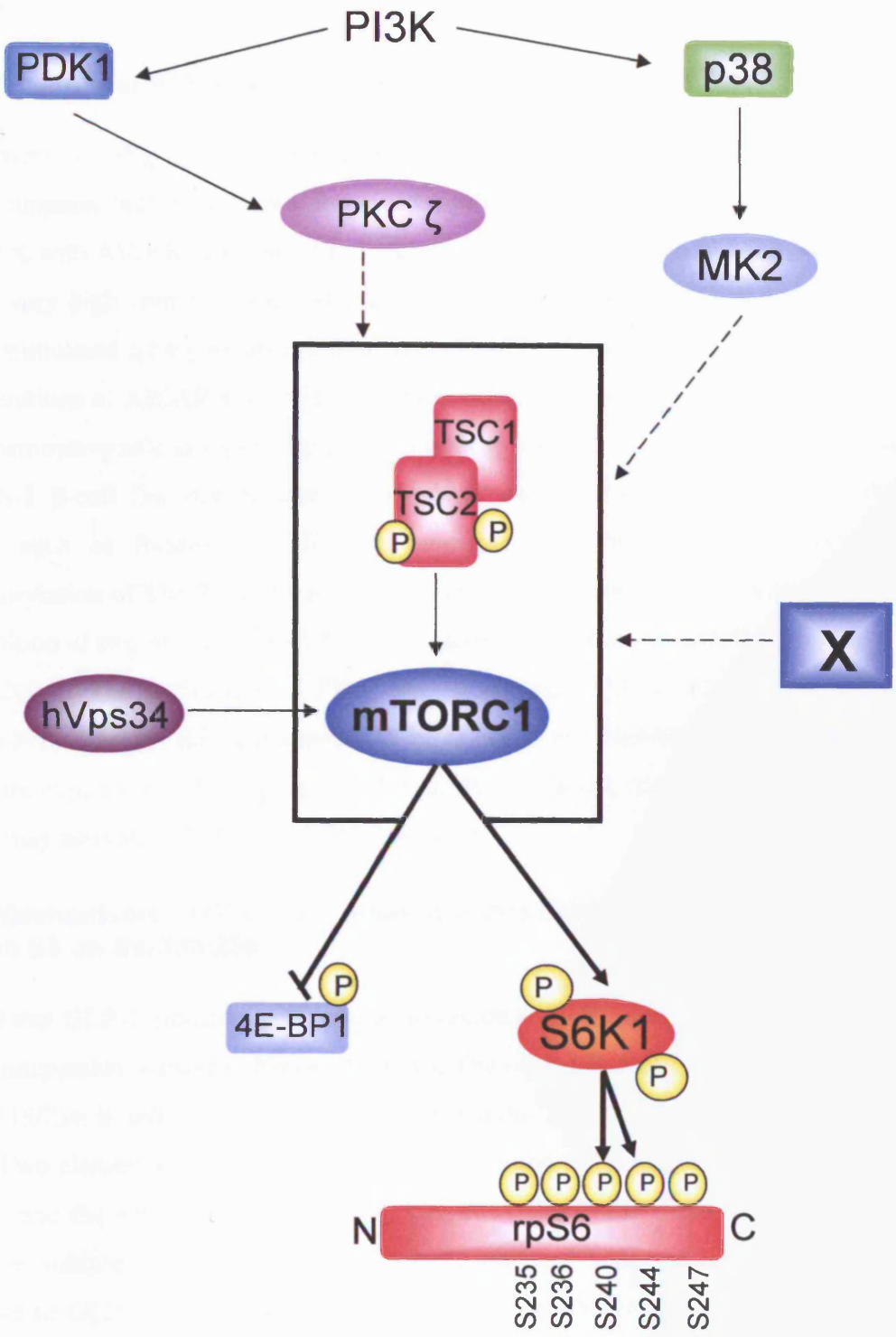


Figure 4.25: Proposed Mechanism for mTOR Activation by PI3K

The upstream kinase responsible for the activation of mTOR is likely to be regulated by PDK-1 as a consequence of PI3K activation. The atypical PKC isoform (PKCζ) have been reported to play an important role in regulating mTOR. Additionally PI3K dependent p38 MAPK activation regulates mTOR via the activation of MK2 which phosphorylates and inhibits TSC2. GLP-1 stimulation may also lead to the activation of the Class III PI3K, hVps34.

on GLP-1 stimulated rpS6 phosphorylation on Ser240/244 are via the inhibition of hVps34.

4.3.3.5 The Role of AMPK in GLP-1 Stimulated rpS6 on Ser240/244

In pancreatic β-cells, AMPK is activated by a decrease in glucose concentration which is able to suppress mTOR activity (Gleason, Lu et al. 2007). I show that artificial activation of AMPK with AICAR reduced GLP-1 stimulated phosphorylation of rpS6 on Ser240/244 only at very high concentrations. However, expression of a CA-AMPK had no effect on GLP-1 stimulated rpS6 phosphorylation on Ser235/236 (**Figure 4.18**). Only at the maximal concentrations of AICAR was rpS6 phosphorylation reduced, indicating that AMPK plays a non-permissive role in regulating rpS6 phosphorylation in response to GLP-1. A study in the INS-1 β-cell line demonstrated that cAMP can regulate AMPK. cAMP elevating agents, such as forskolin or IBMX, suppressed AMPK activity by reducing the phosphorylation of Thr172 in the activation loop. This decrease in AMPK activity was due to inhibition of two of four known AMPK kinases, CaMKKα and CaMKKβ (Hurley, Barre et al. 2006). The activation of PKA also modulates AMPK via phosphorylation on Ser485/491, although this site appears to be required for inhibition of AMPK, additional inputs are required to fully suppress AMPK activity (Hurley, Barre et al. 2006). Therefore, GLP-1 may activate mTOR via AMPK inhibition.

4.3.4 Mechanisms of GLP-1 Stimulated Phosphorylation of Ribosomal Protein S6 on Ser235/236

I found that GLP-1 promotes rpS6 phosphorylation at Ser235/236 using a PI3K, S6K1 and S6K2 independent pathway (**Figure 4.15,16**). The GLP-1 induced phosphorylation of rpS6 at Ser235/236 is inhibited by rapamycin, indicating the involvement of mTOR (**Figure 4.11**). Two classes of protein kinases have been found to phosphorylate rpS6 *in vitro*, the S6K1/2 and the p90RSK family (Pende, Um et al. 2004; Ruvinsky and Meyuhas 2006). However, inhibition of PI3K using LY294002 inhibited both S6K1 and S6K2 activity in response to GLP-1. Furthermore, in the presence of LY294002, the over-expression of MKP3, a dual-specific phosphatase that dephosphorylates the activation loop of Erk1/2, had no effect on rpS6 phosphorylation on Ser235/236 in response to GLP-1, indicating that p90RSK is not responsible for the phosphorylation of rpS6 on Ser235/236 (**Figure 4.19**). Additionally, I also found that GLP-1 is unable to activate p90RSK by immunoprecipitating the enzyme and using an activity assay towards GST-rpS6 (data not shown).

4.3.4.1 Is Rapamycin Sensitivity Conferred by the Activation of a Phosphatase?

In yeast and mammals the presence of nutrients results in the phosphorylation of the alpha-4 regulatory subunit of PP2A phosphatase by mTOR. Phosphorylation induces a direct interaction between the alpha-4 subunit and the catalytic subunit of PP2A. This interaction prevents association with the additional subunits required for PP2A activation and thus PP2A is inhibited and dephosphorylation of downstream targets is prevented. In the absence of nutrients or in cells treated with rapamycin, mTOR no longer phosphorylates the alpha-4 regulatory subunit of PP2A. This results in dephosphorylation of the alpha-4 subunit and prevents the binding to the catalytic subunit of PP2A (PP2AC). PP2AC is then free to associate with the A and B subunits, and to dephosphorylate downstream targets, resulting in translation inhibition (Gingras, Raught et al. 2001) (**Figure 4.26a**). I show that a PP2A-selective inhibitor, cantharidin, or the general phosphatases inhibitor, okadaic acid, restores the phosphorylation of rpS6 only at Ser235/236 and not Ser240/244 in the presence of rapamycin. This data suggests that mTOR regulates the site specific dephosphorylation of rpS6 on Ser235/236 via the regulation of a phosphatase. Therefore, GLP-1 likely stimulates the phosphorylation of rpS6 235/236 via an mTOR independent mechanism (**Figure 4.20**).

4.3.4.2 Role of PKC in rpS6 Phosphorylation on Ser235/236

The site surrounding Ser235/236 conforms to the consensus target site of PKC. Stimulation of MIN6 cells with phorbol esters, which activate the classical and novel PKC isoforms, is able to stimulate the phosphorylation or rpS6 on Ser235/236 and this phosphorylation is independent of PI3K, but rapamycin sensitive. Inhibition of PKC using Ro312880 in the presence of LY294002 abolishes the GLP-1 LY294002 resistant phosphorylation on Ser235/236 (**Figure 4.22**), indicating that GLP-1 phosphorylates rpS6 via PKC. However, early reports revealed that Ro312880 not only inhibits PKC isoforms but also inhibits S6K and the RSK family of kinases (Alessi 1997). Our data revealed that at the concentrations of Ro312880 used, S6K1 was unaffected (**Figure 4.22**). The impact of Ro312880 on RSK is not currently known. Yet we provide evidence that RSK is not involved in the direct phosphorylation of rpS6 on Ser235/236 since the LY294002 resistant phosphorylation is not blocked by over-expression of MKP3, an inhibitor of Erk1/2 and hence RSK (**Figure 4.19**).

Recent work in our laboratory by Dr T P Herbert and Jianling Xie have demonstrated that the classical or novel PKC isoforms are not responsible for the phosphorylation of rpS6 on Ser235/236 in response to GLP-1 as BIM I and Go6967, which inhibit the classical or novel PKC isoforms, or the over-expression of dominant negative forms of PKCδ or PKCε had no effect on rpS6 Ser235/236 phosphorylation induced by GLP-1. However, they have strong data indicating that GLP-1 exerts its effects on the phosphorylation of rpS6 on Ser235/236 via PKA.

4.3.4.3 Role of PKA in rpS6 Phosphorylation on Ser235/236

The site surrounding Ser235/236 conforms to the consensus target site for the AGC kinase PKA. Therefore PKA is a possible and likely candidate for the GLP-1 induced phosphorylation of rpS6 on Ser235/236. Early studies showed that rpS6 can be phosphorylated by a number of the AGC family of kinases *in vitro* (Flotow and Thomas 1992). GLP-1 causes a rapid increase in the production of cAMP in pancreatic β-cells. My results indicate that increases in cAMP alone by forskolin can activate mTOR and its downstream targets. Forskolin or GLP-1 stimulated phosphorylation of rpS6 on Ser235/236 was insensitive to PI3K inhibitors, LY294002 and wortmannin and was shown to occur independently of PI3K and S6K1. Therefore GLP-1 may also phosphorylate rpS6 via a similar mechanism. In cells treated with forskolin, I also observed a partial rapamycin independent phosphorylation on Ser235/236. cAMP exerts its effects via two main effectors, PKA and Epac. H89, a PKA inhibitor blocked GLP-1 and forskolin stimulated rpS6 phosphorylation, supporting the conclusion that cAMP is acting via PKA. It is possible that PKA directly phosphorylates rpS6 on Ser235/236 and, in the presence of rapamycin, an increase in phosphatase activity dephosphorylates these sites. Indeed, according to the scansite motif search, I have shown that PKA is predicted to only phosphorylate Ser235/236 on rpS6 (**Figure 4.21**).

In summary, I provide evidence that, in the pancreatic β-cell line MIN6, GLP-1 dependent activation of rpS6 is independent of insulin secretion and PKB. The phosphorylation of rpS6 on Ser240/244 is dependent on PI3K and S6K1. In contrast, GLP-1 stimulates the direct phosphorylation of rpS6 on Ser235/236 via PKA. Phosphorylation at this site is inhibited by rapamycin, indicating the involvement of mTOR which was found to regulate the activation/inactivation of a phosphatase which specifically dephosphorylates rpS6 on

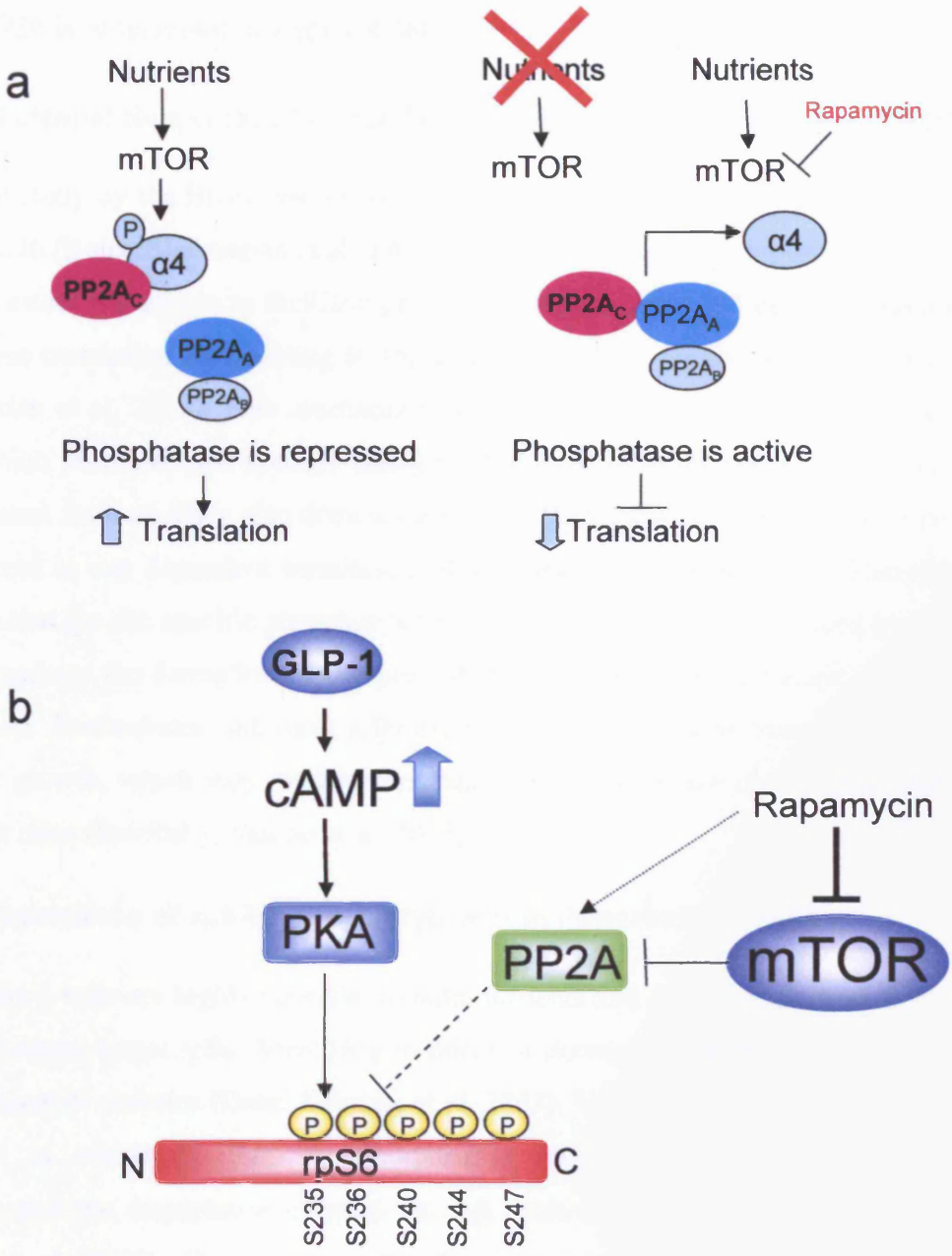


Figure 4.26: Proposed Mechanism for GLP-1 Stimulated rpS6 Phosphorylation on 235/236

a) ***Regulation of PP2A by mTOR***. In the presence of nutrients mTOR phosphorylates alpha-4 regulatory subunit of PP2A which induces a direct interaction with the catalytic subunit. Conversely, rapamycin treatment and the dephosphorylation of the alpha-4 subunit enables the catalytic subunit to interact with A and B subunits required for phosphatase activity.

b) ***GLP-1 stimulated rpS6 Ser235/236 phosphorylation***. GLP-1 stimulates rpS6 phosphorylation through a mechanism dependent upon mTOR but independent of S6K1 and PI3K signalling. The mTOR sensitivity of this phosphorylation may be due to the activation of a phosphatase. GLP-1R signalling and the activation of PKA leads to the phosphorylation of rpS6 on Ser235/236.

Ser235/236. The proposed model for GLP-1 stimulated rpS6 phosphorylation on Ser235/236 is summarised in **Figure 4.26b**.

4.3.4.4 Potential Role of the Site Specific Phosphorylation of rpS6 on Ser235/236

A recent study by the Blenis group showed a functional role for rpS6 phosphorylation on Ser235/236 (Roux, Shahbazian et al. 2007). Site specific phosphorylation at Ser235/236 by phorbol esters was shown to facilitate protein synthesis in HEK293 cells by increasing cap dependent translation by assisting in the formation of the pre-initiation complex (Roux, Shahbazian et al. 2007). This mechanism was dependent on Erk1/2 signalling acting via RSK which promoted site specific phosphorylation of rpS6 on Ser235/236 in an mTOR independent fashion. They also demonstrated that PMA induced RSK activation promoted an increase in cap dependent translation (Roux, Shahbazian et al. 2007). Therefore, it is possible that the site specific phosphorylation of rpS6 on Ser235/236 induced by PKA, like RSK, regulates the formation of the pre-initiation complex and enhances cap dependent translation. Furthermore, the most affected mRNAs maybe those encoding proteins that regulate growth, which may explain the reduction in β-cell size observed in the $rpS6^{P-/-}$ knock-in mice (Ruvinsky, Sharon et al. 2005).

4.3.5 Importance of rpS6 Phosphorylation in Pancreatic β-cells

Pancreatic β-cells are highly sensitive to both nutrients and growth factors, as is S6K1 and its downstream target rpS6. Mutations in S6K1 or downstream effectors contribute to the development of diabetes (Dann, Selvaraj et al. 2007). The generation of the $rpS6^{P-/-}$ knock-in mice in which all five phosphorylatable serines were substituted to alanines demonstrated the importance of rpS6 phosphorylation in β-cell physiology (Ruvinsky, Sharon et al. 2005). These mice suffer from diminished levels of pancreatic insulin, hypoinsulinemia and impaired glucose tolerance suggesting that rpS6 phosphorylation is important in regulating these processes. Mouse embryonic fibroblasts (MEFs) containing the $rpS6^{P-/-}$ mutation were significantly smaller compared to the $rpS6^{P+/+}$ MEFs. The small cell phenotype is not limited to MEFs but also pancreatic β-cells (Ruvinsky, Sharon et al. 2005). Thus, the reduction in β-cell size can account for both the reduction in insulin content and in insulin secretion. This decrease in insulin secretion and reduced β-cell mass contribute to the later stages of type 2 diabetes (Ruvinsky, Sharon et al. 2005).

4.3.6 Future Directions

Role of rpS6 Phosphorylation in Pancreatic β-cells

As mentioned previously, the phosphorylation of rpS6 is important in regulating β-cell growth. A recent study revealed that the mTOR independent site specific phosphorylation on Ser235/236 by RSK played an important role in regulating the formation of pre-initiation complex. The phosphorylation of Ser235/236 on rpS6 by RSK led to the recruitment of rpS6 to the 7-methyl guanosine cap binding complex and an increase in cap dependent translation (Roux, Shahbazian et al. 2007). Therefore it is possible that the PKA dependent site specific phosphorylation of rpS6 on Ser235/236 like RSK regulates the formation of the pre-initiation complex and enhances cap dependent translation. Furthermore, the mRNAs most affected maybe those encoding proteins that regulate growth which may explain the reduction in β-cell size observed in the $rpS6^{P-/-}$ knock-in mice (Ruvinsky, Sharon et al. 2005). Therefore it would be interesting to investigate if the over-expression of a wild type GST-rpS6 is recruited to the 7-methylguanosine cap complex in response to GLP-1 in pancreatic β-cells and to observe if this effect is blocked by the over-expression of GST-rpS6 with alanine substitutions at position Ser235 and Ser236. It would also be interesting to investigate if GLP-1 increases cap dependent translation by a bicistronic reporter. Briefly, a bicistronic reporter plasmid that directs cap-dependent translation of the Renilla luciferase (*RL*) gene and cap-independent HCV IRES-mediated translation of the firefly (*FL*) gene can be expressed in MIN6 cells and the level of RL/FL luminescence quantified using a luminometer.

How does GLP-1 Stimulate the Phosphorylation of rpS6 on Ser240/244 in a PI3K Dependent Mechanism Independent of PKB.

As discussed in section 4.3.3 there are several potential targets that could be responsible for the activation mTOR, including the atypical PKC isoforms. In order to investigate the role of atypical PKCs, peptide based inhibitors rather than the classical inhibitors which have limited specificity could be used. Another potential kinase which has been reported to regulate mTOR signalling is p38 MAPK which activates MK2 resulting in TSC2 inhibition. p38 MAPK can be inhibited by a specific inhibitor SB203580, which has been used to show p38 MAPK involvement in GLP-1 induced β-cell proliferation (Buteau, Foisy et al. 2001). Another interesting area would be to investigate the phosphorylation state of TSC2 to investigate if the phosphorylation of rpS6 on Ser240/244 is dependent on TSC2 regulation of mTOR. One way to achieve this would be to radiolabel the cells and immunoprecipitate endogenous or over-expressed TSC2 to investigate if GLP-1 treatment

leads to an increase in TSC2 phosphorylation. If TSC2 is phosphorylated in response to GLP-1 further analysis to identify the specific residues on TSC2 could be carried out by tryptic/chymotryptic digest and any proteins of interest can then separated by 2D electrophoresis followed by Edman degradation to identify the phosphorylated residues.

Chapter 5: Regulation of PI3K and PKB by Glucagon Like Peptide-1 in Pancreatic β-cells

5.1 Introduction

5.1.1 Phosphoinositide-3 Kinase (PI3K)

5.1.1.1 Structure and Function of PI3Ks

Phosphatidylinositol lipids (PtdIns) consists of a glycerol backbone with fatty acids attached at position 1 and 2 and an inositol group located at position 3. PI3K, a lipid kinase, phosphorylates the 3'OH position of the inositol ring (Vanhaesebroeck, Leevers et al. 2001). There are three classes of PI3K, Class I, Class II and Class III, which are classified according to their substrate specificity, mode of activation and subunit composition.

5.1.1.2 Class 1 PI3K

The class 1 PI3Ks are composed of a regulatory adaptor subunit and a catalytic subunit; these subunits are tightly bound to give rise to a heterodimer. Class 1 PI3Ks are further subdivided into the Class 1_A and Class 1_B PI3K, which signal downstream of receptor tyrosine kinases and GPCR, respectively (Stephens, Jackson et al. 1993). The Class 1_A PI3Ks are composed of one of three catalytic subunits p110α, p110β and p110δ and one of three regulatory subunit, p85α, p85β and p55γ (Hawkins, Anderson et al. 2006). The catalytic subunits of the Class 1_A consists of 4 domains: a kinase domain, a Ras binding domain, a C2 domain and a helical (PIK) domain (Oudit, Sun et al. 2004) (**Figure 5.1**). The latter two are important in lipid binding and protein-protein interactions respectively. The Class 1_A PI3K regulatory adaptor subunits each encode two SH2 domains, which bind to phosphotyrosines on activated receptors (Hawkins, Anderson et al. 2006). This is important in bringing the kinases to the plasma membrane where they can phosphorylate lipid substrates (Hawkins, Anderson et al. 2006). Another important factor for the efficient activity of Class 1_A PI3K is the direct association with Ras via their Ras binding domain. A study has shown that over-expression of Ras GTPases are able to stimulate the lipid kinase activity as measured by the levels of their 3′ phosphorylated lipid products. The activity of all the class I PI3K isoforms, except p110β, is increased upon co-expression of constitutively active Ras (Rodriguez-Viciana, Sabatier et al. 2004).

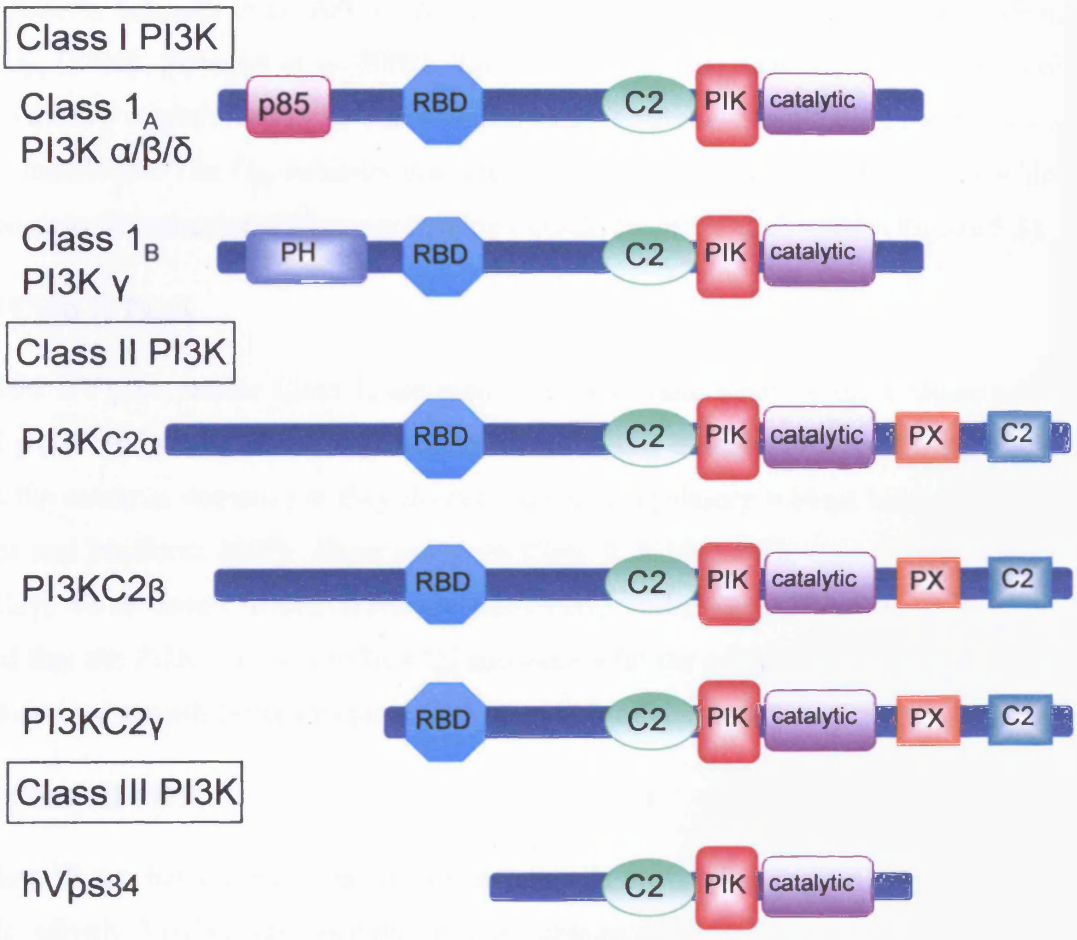


Figure 5.1: Domain Structure of PI3 Kinase Family.

The domain structures of Class I, II and III PI3K. Class I_A PI3K are heterodimers consisting of a regulatory subunit (p85, p50, p55) and one of three catalytic subunits, p110α, p110β and p110δ. The class I_B are heterodimers consisting of a regulatory subunit (p101 or p85) and the p110γ catalytic subunit. The class II PI3K have not been reported to require a regulatory subunit, whilst Class III PI3K (hVps34) is a heterodimer consisting of a catalytic hVps34 subunit and the p150 regulatory subunit. RBD: Ras binding domain; PX: Phox domain; PH: Pleckstrin homology.

The Class 1_B PI3Ks are composed of a catalytic subunit (p110γ) and one of two regulatory subunits (p84, p101). The structure of the Class 1_B catalytic subunit is similar to that of Class 1_A, except p110γ contains two $G_{\beta\gamma}$ interaction sites which facilitate activation by GPCR (Brock, Schaefer et al. 2003). The regulatory subunit of the Class 1_B can also bind to the $G_{\beta\gamma}$ (Brock, Schaefer et al. 2003). Following agonist stimulation, the p101 subunit binds to the $G_{\beta\gamma}$ subunit leading to translocation of the catalytic subunit from the cytosol to the cell membrane. The $G_{\beta\gamma}$ subunits also interact with the catalytic p110γ subunit which contributes to the subsequent kinase activation (Brock, Schaefer et al. 2003) (**Figure 5.2**).

5.1.1.3 Class II PI3K

The Class II PI3Ks, unlike Class 1, are monomers and share many of the features of the Class 1 p110 catalytic subunit. They contain a Ras binding domain, PIK and C2 domain as well as the catalytic domain but they do not contain a regulatory subunit binding domain (Falasca and Maffucci 2007). There are three Class II PI3Ks, PI3K-C2α, PI3K-C2β and PI3K-C2γ, whose mode of activation are not clearly understood. However, it has been reported that the PI3K-C2α and PI3K-C2β associate with the epidermal growth factor and platelet-derived growth factor receptors (Falasca and Maffucci 2007).

5.1.1.4 Class III PI3K

The Class III are heterodimeric proteins consisting of an adaptor protein (p150) and the catalytic subunit (Vps34). The catalytic subunit contains a lipid kinase domain as well as a C2 and PIK domain but lacks the Ras binding domain. hVps34 uses PtdIns as a substrate to produce PtdIns(3)P_1 (Nobukuni, Kozma et al. 2007). It has been shown that hVps34 activity is increased by amino acids which contributes to the regulation of mTORC1 by nutrients, as the over-expression of hVps34 potentiates amino acid induced S6K1 phosphorylation and this effect is blocked by siRNA mediated knockdown of hVps34 (Nobukuni, Joaquin et al. 2005). It has also been shown that hVps34 plays an important role in autophagy (Nobukuni, Kozma et al. 2007). In nutrient-deprived cells, autophagy degrades cytoplasmic contents by engulfing and forming vesicules which fuse with lysosomes, enabling degradation of the vesicule contents. hVps34 has been shown to interact with the autophagic gene beclin, which interacts with Bcl-2 and the over-expression of Bcl-2 leads to a decrease in hVps34-beclin interaction (Nobukuni, Kozma et al. 2007). Therefore, in nutrient deprived cells, Bcl-2 inhibits autophagy by preventing interaction of beclin with hVps34 (Nobukuni, Kozma et al. 2007).

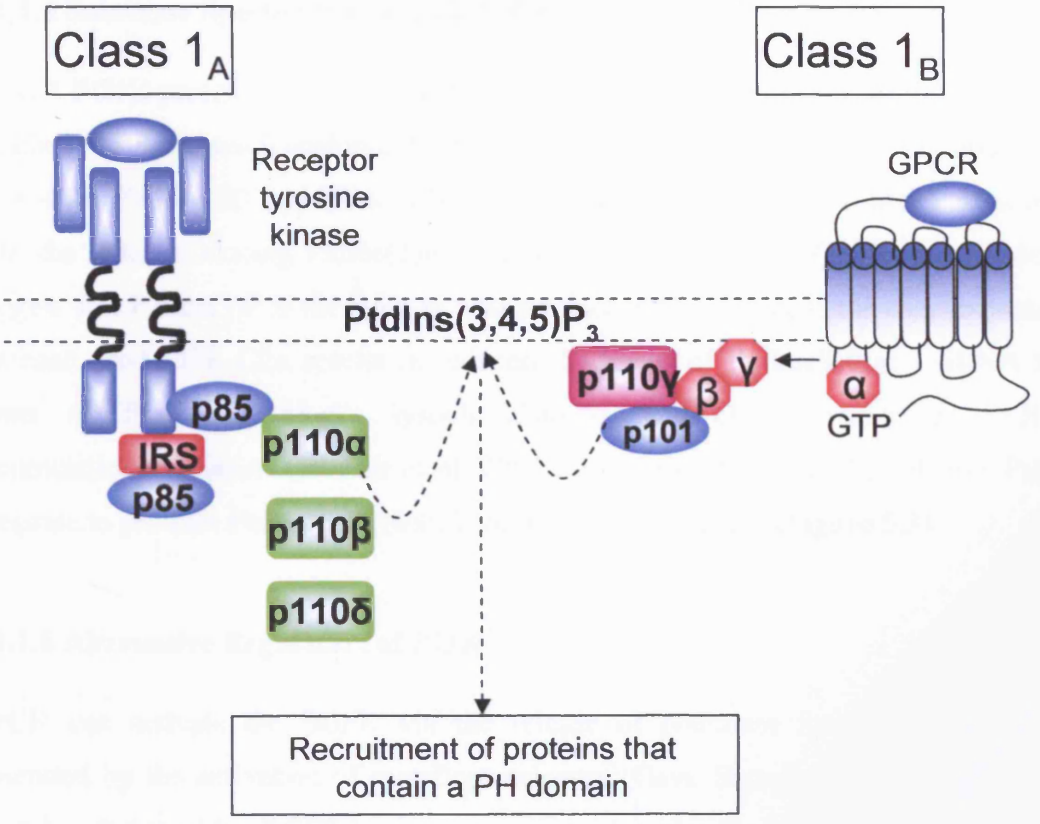


Figure 5.2: Activation of Class 1 PI3K

Activation of Class 1_A PI3K by receptor tyrosine kinase or Class 1_B PI3K by GPCR. Class 1_A PI3K catalytic subunits (p110α,β,δ) bind to the adaptor subunit p85 which in turn is recruited by its phosphotyrosine binding domain to phosphorylated receptors or adaptor proteins like IRS. Class 1_B PI3Kγ binds to free βγ subunits following GPCR activation. All class 1 PI3Ks phosphorylate PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3. The generation of PtdIns(3,4,5)P_3 leads to the recruitment of effector proteins which bind via their PH domain.

5.1.1.5 Substrate Specificity of the PI3K Classes

Class 1 PI3Ks preferentially phosphorylate PtdIns(4,5)P_2, which leads to the generation of PtdIns(3,4,5)P_3 *in vivo* (Hawkins, Welch et al. 1997). The three members of class II PI3Ks, PI3K-C2α, PI3K-C2β and PI3K-C2γ phosphorylate both PtdIns-4-P and PtdIns *in vitro* with the latter producing PtdIns(3)P (Arcaro, Volinia et al. 1998). There is evidence to suggest that PtdIns(3)P is the main *in vivo* product as in PC12 cells the over-expression of a kinase dead PI3K-C2α results in reduced the levels of PtdIns(3)P and siRNA knock-down of PI3K-C2β blocks lysophosphatidic acid (LPA) stimulated PtdIns(3)P accumulation (Meunier, Osborne et al. 2005). The class III PI3K hVps34 uses PtdIns as substrate to produce PtdIns(3)P (Nobukuni, Kozma et al. 2007) (**Figure 5.3**).

5.1.1.6 Alternative Regulators of PI3K

GPCR can activate the EGFR via the release of precursor forms of EGFR ligands generated by the activation of metalloproteinases (Gavi, Shumay et al. 2006) or via the phosphorylation of the EGFR by the non-receptor tyrosine kinase c-src on Tyr845, thereby creating docking sites for proteins containing SH2 domains (Biscardi, Maa et al. 1999). Therefore, EGFR activation/phosphorylation can lead to the recruitment and subsequent activation of Class 1 PI3Ks.

PI3K can also be activated by small GTPase molecules such as Ras and Rac1 (Chan, Rodeck et al. 2002). PI3K activation by Ras correlates with its binding to p110 while PI3K activation by Rac1 may be mediated by its binding to p85 (Chan, Rodeck et al. 2002).

5.1.1.7 Negative Regulators of PI3K Signalling

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumour suppressor gene and acts as both a protein and a lipid phosphatase. The lipid phosphatase activity of PTEN can dephosphorylate the D3 position of PtdIns (3,4) P_2 and PtdIns (3,4,5) P_3. Indeed, cells lacking PTEN function exhibit a two fold increase in PtdIns(3,4,5)P_3 levels (Sun, Lesche et al. 1999). Therefore, PTEN can attenuate PI3K signalling (Paez and Sellers 2003).

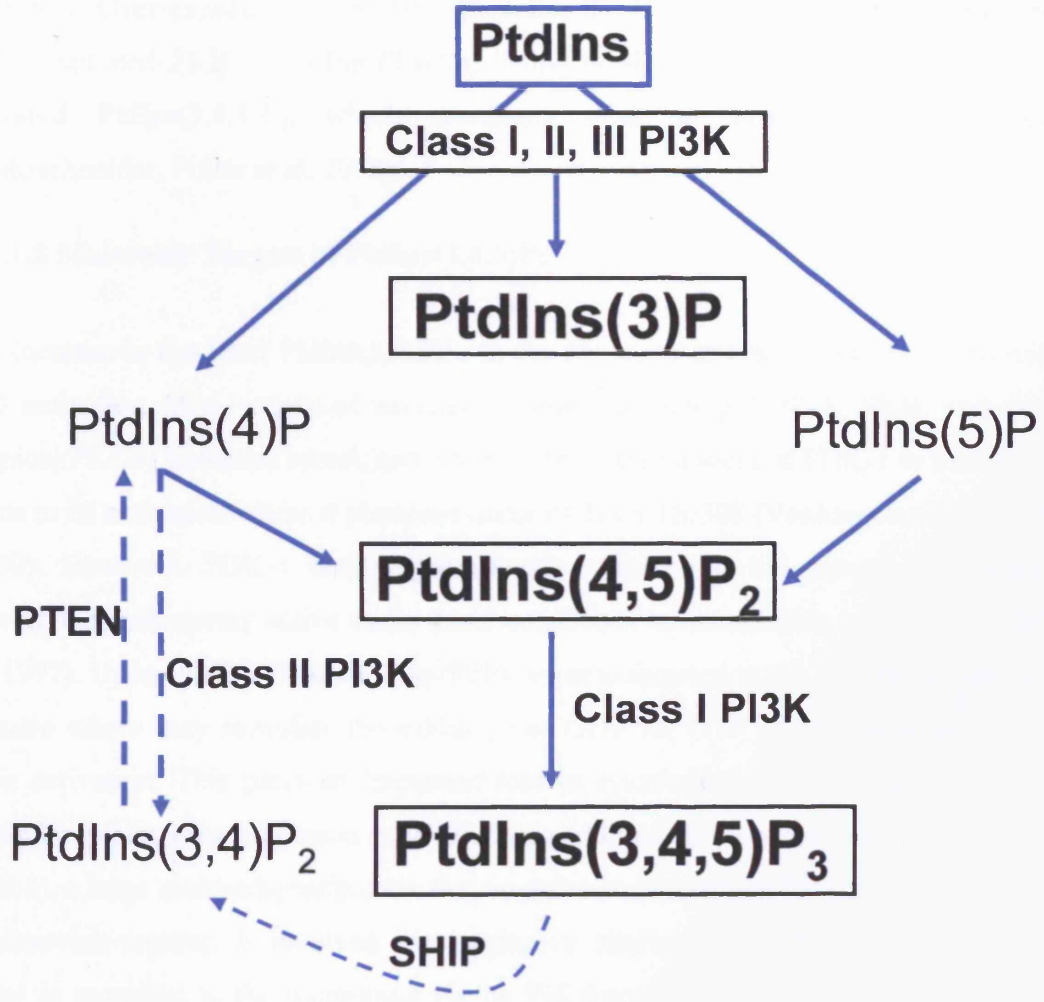


Figure 5.3: Substrate Specificity of the PI3 kinases.

SHIPs (Src homology 2-containing inositol 5' phosphatases) are lipid phosphatases, which preferentially hydrolyze PtdIns (3,4,5) P_3 and PtdIns(1,3,4,5)P_4 (Rohrschneider, Fuller et al. 2000). SHIP1 and 2 contain an SH2 domain enabling interaction with phosphorylated receptors. Over-expression of SHIP2 attenuates the levels of PtdIns(3,4,5)P_3 and reduces PI3K mediated PKB activation (Taylor, Wong et al. 2000). SHIP knock-out mice have elevated PtdIns(3,4,5)P_3, which correlates with an increase in PI3K activity (Rohrschneider, Fuller et al. 2000).

5.1.1.8 Molecular Targets of PtdIns(3,4,5)P_3

An increase in the level PtdIns(3,4,5)P_3 in the plasma membrane leads to the recruitment and activation of a variety of effector proteins, including PDK-1, PKB, and possibly atypical PKCs (Vanhaesebroeck and Alessi 2000). Recruitment of PDK-1 to the membrane leads to its activation where it phosphorylates PKB on Thr308 (Vanhaesebroeck and Alessi 2000). However, PDK-1 maybe permanently resident at the plasma membrane and therefore constitutively active under basal conditions in mammalian cells (Alessi, Deak et al. 1997). Upon PI3K activation, Rac-GEFs are also targeted to the membrane via their PH domain where they stimulate the exchange of GDP for GTP on Rac proteins, leading to their activation. This plays an important role in cytoskeletal remodelling and membrane trafficking (Hawkins, Anderson et al. 2006). In addition, Grb2-associated binding protein 1 (Gab1), a large multi-adapter protein that contains an N-terminal PH domain as well as two proline-rich regions, is involved in constitutive binding to Grb2 upon PI3K activation. Gab1 is recruited to the membrane via its PH domain (Dance, Montagner et al. 2006). These proteins play an important role in efficiently transducing signals downstream.

5.1.2 Protein Kinase B (PKB/Akt)

PKB is a Ser/Thr kinase and there are three mammalian isoforms, PKBα, PKBβ and PKBγ, which are all expressed in a broad range of tissues (Jones, Jakubowicz et al. 1991; Cheng, Godwin et al. 1992; Brodbeck, Cron et al. 1999). PKB consists of a PH domain located at the N-terminal domain, a kinase domain and a C-terminal regulatory domain (Alessi, Andjelkovic et al. 1996). PKB is a major downstream effector of the Class 1 PI3Ks (Hawkins, Anderson et al. 2006). Activation of PI3K and the formation of PtdIns(3,4,5)P_3 recruits PDK-1 and PKB from the cytosol to the plasma membrane via their PH domain, and PDK-1 phosphorylates PKB on Thr308 within the activation loop. Full activation of PKB requires the phosphorylation of Ser473 within the hydrophobic motif by mTORC2

(Sarbassov, Guertin et al. 2005). The fully active kinase is then able to phosphorylate a number of targets located in the cytosol and nucleus.

5.1.2.1 PI3K Independent Activation of PKB

Several reports have suggested that PKB can also be activated in a PI3K independent manner. In various cell types, it has been shown that cAMP is able to activate PKB (Zmuda-Trzebiatowska, Manganiello et al. 2007). cAMP, the product of adenylyl cyclase activation, exerts a multitude of effects within the cell via the activation of its downstream effectors, PKA and Epac. Studies using forskolin, the artificial activator of adenylyl cyclase, revealed that elevations in cAMP and the activation of PKA can activate PKB independently of PI3K in adipocytes (Sable, Filippa et al. 1997; Filippa, Sable et al. 1999). Recent studies in adipocytes have shown that cAMP can either activate or inhibit PKB depending on which effector cAMP is signalling through (Zmuda-Trzebiatowska, Manganiello et al. 2007). For example activation of Epac by cAMP leads to a decrease in insulin stimulated PKB activity (Brennesvik, Ktori et al. 2005) whilst PKA activation increases signalling by PKB (Zmuda-Trzebiatowska, Manganiello et al. 2007).

5.1.3 Downstream Targets of PKB

PKB phosphorylates many proteins important in controlling cell survival and metabolism, in both the cytoplasm and the nucleus. The minimum recognition motif required for PKB phosphorylation is Arg-X-Arg-Y-Z-Ser/Thr-Hyd, where X is any amino acid, Y and Z are small amino acid residues and Hyd is a bulky hydrophobic residue (Alessi, Caudwell et al. 1996). Known PKB substrates include GSK3, FOXO1, BAD, caspase 9, TSC2, PRAS40, p27 and p21 (**Figure 5.4b**).

GSK3- GSK3 is one of the best characterised substrates of PKB. GSK3 is responsible for phosphorylating and inactivating glycogen synthase and phosphorylation of GSK3 by PKB leads to its inactivation (Cross, Alessi et al. 1995). PKB dependent inhibition of GSK3 also plays an important role in regulating proliferation by inhibiting the degradation of cyclin D1 and Myc, which are important regulators of cell cycle progression (Liang and Slingerland 2003).

FOXO1- FOXO1 is a member of the forkhead transcription factor family, which controls the expression of genes important in inducing apoptosis, such as Fas ligand. PKB

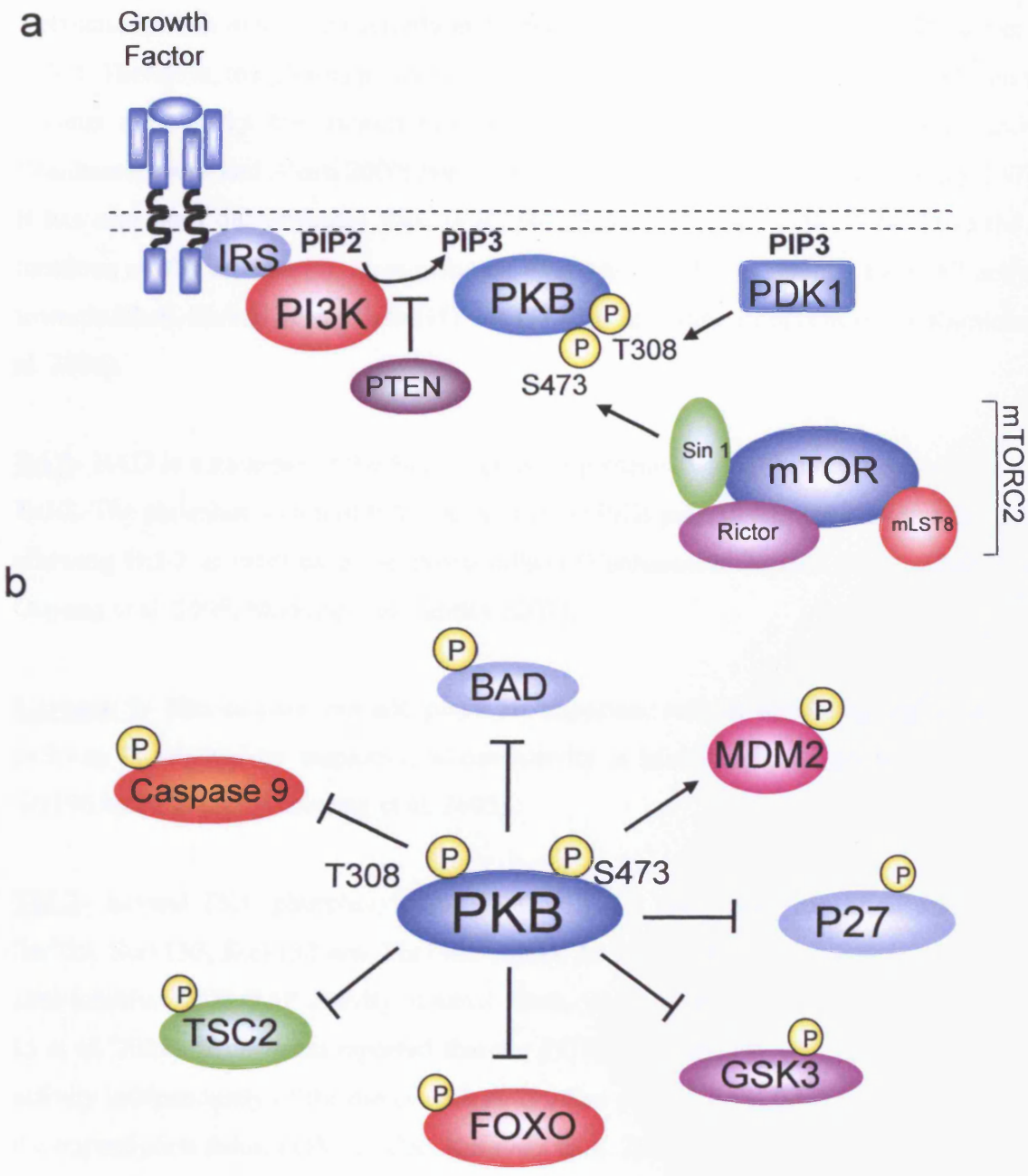


Figure 5.4: Activation of PKB by Growth Factors

a) Upstream activation of PKB by growth factors. Activation of receptor tyrosine kinase activates Class 1 PI3K through direct binding to the receptor or via IRS proteins, which bind and activate PI3K. PI3K phosphorylates PIP2 to form PIP3 leading to the recruitment of PDK-1 and PKB to the membrane. PDK-1 phosphorylates Thr308 on PKB whilst mTORC2 phosphorylates Ser473, resulting in full activation of PKB.

b) Downstream targets of PKB. PKB mediated phosphorylation of these proteins leads to their activation (arrows) or inhibition (blocking arrows).

phosphorylates FOXO1 in the nucleus at Thr24, Ser256 and Ser319 (Tran, Brunet et al. 2003). Phosphorylation at Thr24 and Ser256 induces the association of FOXO1 with 14-3-3 proteins, which inhibits its activity and targets it for nuclear export (Tran, Brunet et al. 2003). Therefore, the phosphorylation of FOXO by PKB prevents entry of FOXO into the nucleus and blocks the transcription of pro-apoptotic genes promoting cell survival (Vanhaesebroeck and Alessi 2000; Song, Ouyang et al. 2005; Manning and Cantley 2007). It has also been demonstrated that, in its phosphorylated state, FOXO1 binds to the C-terminus of TSC2, reducing its association with TSC1 and thus inhibits the GAP activity towards Rheb. Elevations in Rheb.GTP leads to the activation of mTOR (Cao, Kamioka et al. 2006).

BAD- BAD is a member of the Bcl-2 family of proteins, which forms a heterodimer with Bcl-2. The phosphorylation of BAD on Ser136 by PKB prevents its association with Bcl-2, allowing Bcl-2 to exert its pro-survival effects (Vanhaesebroeck and Alessi 2000; Song, Ouyang et al. 2005; Manning and Cantley 2007).

Caspase 9- The caspase cascade plays an important role in regulating apoptosis. The pathway is initiated by caspase-9, whose activity is inhibited by its phosphorylation on Ser196 by PKB (Song, Ouyang et al. 2005).

TSC2- Several PKB phosphorylation sites on TSC2 have been identified and include Ser939, Ser1130, Ser1132 and Thr1462 (Inoki, Li et al. 2002). Phosphorylation at these sites inhibits TSC2 GAP activity towards Rheb, which in turn activates mTORC1 (Inoki, Li et al. 2002). It has been reported that the PI3K/PKB pathway can also regulate TSC2 activity independently of the direct phosphorylation of TSC2, via PKB phosphorylation of the transcription factor FOXO1 (Cao, Kamioka et al. 2006).

PRAS40- PRAS40 is a novel mTOR binding partner which binds to the mTOR kinase domain during nutrient or serum deprivation, conditions that inhibit mTOR signalling (Vander Haar, Lee et al. 2007). It has been demonstrated that PKB can phosphorylate PRAS40 on Thr246, which is reported to release the inhibitory effect of PRAS40 on mTORC1 (Kovacina, Park et al. 2003).

p27- p27 is a cell cycle inhibitor which acts by disrupting cyclin and CDK complexes. PKB phosphorylates p27 at Thr157 thereby preventing its nuclear entry and blocking cell cycle arrest (Lawlor and Alessi 2001). In addition, the p27 gene is controlled by the FOXO transcription factor. Therefore, phosphorylation of FOXO by PKB prevents p27 gene expression.

p21- p21 is another member of the cell cycle inhibitors. Its activity is indirectly activated via the PKB dependent phosphorylation of MDM2 on Ser166 and Ser186, resulting in down regulation of p53 mediated expression of p21 (Manning and Cantley 2007).

5.1.4 Role and Regulation of the PI3K/PKB Pathway in Pancreatic β-cells

Human islets express PI3K Class 1_A and Class 1_B isoforms as well as class II PI3KC2α and PI3KC2γ (Muller, Huang et al. 2006). It is unknown whether β-cells express the class III PI3K, hVps34. In addition, human islets also express all 3 isoforms of PKB and PDK-1 together with both isoforms of the insulin receptor A and B and IRS1 and IRS2 proteins (Muller, Huang et al. 2006). In pancreatic β-cells, insulin like growth factor 1 (IGF-1) or insulin stimulation results in an increase in tyrosine phosphorylation of the β-chain of the insulin receptor that in turn tyrosine phosphorylates IRS2, leading to PI3K recruitment to the membrane and activation (Trumper, Trumper et al. 2000). Earlier studies demonstrated that elevated glucose stimulates the tyrosine phosphorylation of IRS1, which increases the association of IRS1 with the p85 regulatory subunit of PI3K (Rothenberg, Willison et al. 1995). Similar results were also obtained in isolated rat islets (Velloso, Carneiro et al. 1995). Therefore, in pancreatic β-cells, nutrients and growth factors recruit many of the key signalling elements downstream of the insulin receptor, which ultimately lead to PI3K activation.

5.1.4.1 GLP-1 and PI3K Activation

Activation of the PI3K signalling pathway by glucose, hormones and growth factors plays an important role for the enhancement of β-cell proliferation and survival (Dickson and Rhodes 2004). GLP-1 has been shown to stimulate the activation of PI3K in many β-cell lines and human islets (Dickson, Lingohr et al. 2001; Buteau, Foisy et al. 2003; Trumper, Ross et al. 2005). In human islets, GLP-1 stimulated PI3K activity co-immunoprecipitates with Rap1, indicating that Rap1 may activate PI3K in pancreatic β-cells via a similar mechanism by which Ras activates PI3K (Trumper, Ross et al. 2005). However,

transactivation of the epidermal growth factor receptor (EGFR) by GLP-1 has also been reported to activate PI3K in the pancreatic β-cell line INS(832/13) (Buteau, Foisy et al. 2003). This is mediated by the activation of c-src and subsequent activation of metalloprotease which cleaves membrane bound betacellulin (BTC) (endogenous EGF-like ligands) (Buteau, Foisy et al. 2003). This GLP-1 mediated transactivation of the EGFR was shown to play an important role in GLP-1 stimulated β-cell proliferation (Buteau, Foisy et al. 2003), which is likely mediated by the PI3K/PKB dependent phosphorylation of FOXO1 (Buteau, Spatz et al. 2006). GLP-1 stimulation has also been reported to increase β-cell proliferation via PI3K dependent activation of the atypical PKC isoform PKCζ (Buteau, Foisy et al. 2001).

GLP-1 stimulated PI3K activation is also an important component in the regulation of glucose stimulated insulin secretion. GLP-1 stimulated PI3K activation and subsequent activation of atypical PKCζ results in the antagonism of Kv current (MacDonald, Wang et al. 2003). Antagonism of β-cell Kv currents prevents repolarization, causing a prolonged depolarized state and an increase in Ca^{2+} influx, which enhances insulin secretion (MacDonald, Wang et al. 2003). Moreover, PI3Kγ (Class 1_B) specific knock-out mice exhibit a reduction in the glucose secretory response which is restored by the administration of exendin, a GLP-1R agonist, revealing that PI3Kγ plays an important role in regulating insulin secretion in pancreatic β-cells (Li, MacDonald et al. 2006).

5.1.4.2 PKB Activation and β-cell Function

PKB is rapidly phosphorylated on Ser473 and Thr308 in response to IGF-1 and insulin in INS-1 cells (Trumper, Trumper et al. 2001). However, in rat islets, only a high concentration of insulin (100nM) causes a modest activation of PKB (Wicksteed, Alarcon et al. 2003). Nutrients such as glucose also activate PKB in the β-cell line INS-1 (Lingohr, Dickson et al. 2002). This effect of glucose on PKB phosphorylation may be mediated by the up-regulation of IRS2 gene expression as a result of increased Ca^{2+} influx (Briaud, Lingohr et al. 2003). Elevations in Ca^{2+} can lead to the activation of Ca^{2+} regulated adenylyl cyclase, which raises cAMP levels leading to PKA activation and CREB phosphorylation, which enhances IRS-2 expression and hence PKB signalling (Jhala, Canettieri et al. 2003). However, it has also been proposed that glucose may activate PKB via the activation of cAMP-nucleotide exchange factor (GEF) and PKA in RINm5F cells or via the autocrine effect of secreted insulin in HIT-T15 β-cell line (Leibiger, Leibiger et al. 2001; Kwon, Pappan et al. 2004). Indeed, GLP-1, which leads to elevations in cAMP,

has also been reported to potentiate glucose stimulated PKB phosphorylation in INS-1 cells (Trumper, Trumper et al. 2000). It was reported but not shown that GLP-1 potentiates glucose stimulated PKB phosphorylation threefold at 2.5mM to 5mM glucose whereas at 20mM glucose only a twofold increase in GLP-1 stimulated PKB phosphorylation was observed (Trumper, Trumper et al. 2000).

The importance of PKB in β-cell function was demonstrated by the over-expression of a constitutively active form of PKB in transgenic mice. The mice display significant increases in both β-cell size and mass, resulting in hyperinsulinemia and hypoglycaemia (Bernal-Mizrachi, Wen et al. 2001; Tuttle, Gill et al. 2001). In mice and humans, the absence of PKBα results in a growth deficiency but normal glucose metabolism, whereas the loss of PKBβ causes insulin resistance and mild diabetes (Garofalo, Orena et al. 2003). Furthermore, adenoviral expression of a kinase-dead form of PKB in INS-1 cells decreased IGF-1-induced beta-cell proliferation (Dickson, Lingohr et al. 2001).

GLP-1 mediated activation of PKB has also been demonstrated to exert a protective effect against β-cell apoptosis (Li, El-Kholy et al. 2005). Treatment of INS-1 cells with exendin-4 alleviates cytokine induced β-cell apoptosis, which is reduced by over-expression of a kinase-dead mutant of PKB (Li, El-Kholy et al. 2005). Furthermore, in the obese *db/db* mouse, exendin-4 (a GLP-1R agonist) treatment resulted in the elevated expression of PKB, which increased both β-cell proliferation and reduced β-cell apoptosis (Wang and Brubaker 2002). Conversely, fatty acids decrease PKB phosphorylation, which correlates with an increase in apoptosis in INS-1 cells, which can be alleviated by the over-expression of a constitutively active PKB (Wrede, Dickson et al. 2002). These effects were likely mediated by the regulation of the pro-apoptotic downstream targets of PKB, as the phosphorylation and inhibition of GSK3 and FOXO also protects β-cells from fatty acid induced apoptosis (Wrede, Dickson et al. 2002).

5.1.5 Aims

The PI3K/PKB pathway plays an important role in regulating many cellular processes such as growth, survival, proliferation and metabolism. It has also been shown that PKB activation plays an essential role in mediating proliferative and anti-apoptotic effects in β-cells in response to GLP-1. Therefore, in this study, our aim was to investigate the mechanism by which GLP-1 receptor activation is coupled to the PI3K/PKB signalling pathway in pancreatic β-cells.

5.2 Results

5.2.1 The Phosphorylation of PKB by Glucose is Primarily Dependent on the Autocrine Effect of Insulin

In MIN6 cells, glucose stimulated PKB phosphorylation at Ser473 is not potentiated by GLP-1 (**Figure 5.5a, chapter 4, Figure 4.12**) and is blocked by diazoxide, an inhibitor of insulin secretion, indicating that glucose stimulated PKB phosphorylation is mediated via the autocrine effect of insulin (**Figure 5.5a**). Indeed, insulin is able to stimulate the phosphorylation of PKB on both Thr308 and Ser473 in MIN6 cells (**Figure 5.5b**). Interestingly, although glucose stimulated rpS6 phosphorylation is inhibited by diazoxide, GLP-1 stimulated rpS6 phosphorylation is unaffected by diazoxide (**Figure 5.5a**), yet the phosphorylation of rpS6 on Ser240/244 induced by GLP-1 is inhibited by two PI3K inhibitors, LY294002 and wortmannin (**Chapter 4 Figure 4.15a**). This data provides evidence that GLP-1 can stimulate a PI3K via an insulin independent mechanism. Yet this PI3K is unable to stimulate PKB.

To confirm that GLP-1 can stimulate PI3K via an insulin independent mechanism, I measured phospho-tyrosine (pY) associated PI3K activity in MIN6 cell lysates treated with 20mM glucose or 20mM glucose plus GLP-1 in the presence or absence of diazoxide. PI3K was activated by 20mM glucose which was inhibited by diazoxide (**Figure 5.5c**). In contrast, GLP-1 stimulated PI3K activity was unaffected by diazoxide, confirming that GLP-1 can stimulate PI3K activity independently of insulin secretion (**Figure 5.5c**).

5.2.2 Effects of Glucose/GLP-1 on PKB Activity in Pancreatic β-cells

I have shown that 20mM glucose stimulated an increase in PKB phosphorylation at 60min and that the addition of GLP-1 did not significantly potentiate glucose stimulated PKB phosphorylation at 60min (**Figure 5.5a**). Although I observed no potentiation of glucose stimulated PKB phosphorylation upon GLP-1 treatment there may, however, be changes in PKB activity. Therefore, I measured PKB activation in response to glucose and GLP-1 at 10 and 60min post-stimulation. PKB activity was increased in the presence of glucose at both 10 and 60min stimulation. The addition of GLP-1 resulted in a modest but statistically insignificant increase in PKB activity compared to glucose alone (**Figure 5.6**). Therefore, GLP-1 is unable to potentiate PKB activity stimulated by 20mM glucose in MIN6 cells.

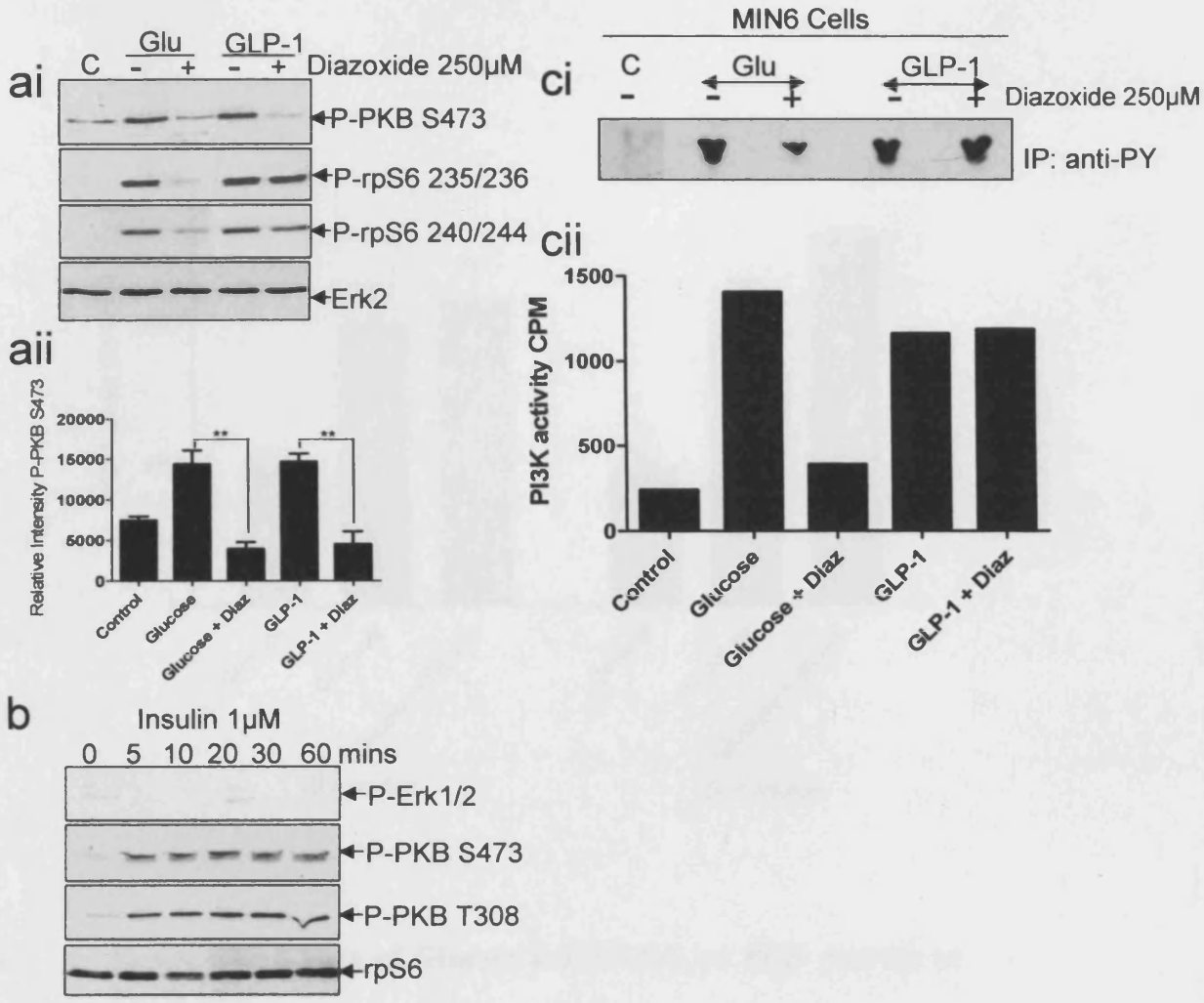


Figure 5.5: Glucose Stimulated PKB Phosphorylation is Primarily Dependent on the Autocrine Effect of Insulin in MIN6 Cells

In all cases, MIN6 cells were incubated in KRB supplemented with amino acid (0.5X) for 1h prior to treatment with a) 20mM glucose (Glu) or 20mM glucose plus 10nM GLP-1 (GLP-1) in the presence or absence of 250μM diazoxide for 1h or b) 1μM insulin for the times indicated in the figure. After treatment, proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-PKB (Thr308 and Ser473), phospho-Erk1/2 (Thr202/Tyr204), phosphorylated rpS6 (Ser235/236 or Ser240/244) and total Erk2 and rpS6 as loading controls. aii) Quantified data from phospho-PKB S473 blots in *ai,* shown as means ± SEM; $n = 3$, $^{**}P < 0.01$. P value obtained using a One-way ANOVA. ci) Cells were treated with 20mM glucose (Glu) or 20mM glucose plus 10nM GLP-1 (GLP-1) for 1h in the presence or absence of 250μM diazoxide. Equal amounts of cell lysates were subjected to immunoprecipitation with antibodies to pY. PI3K assays were performed as described in *Materials and Methods.* cii) ^{32}P-incorporation into phosphatidylinositol was quantified after separation by TLC. Results are representatives of 2 independent experiments.

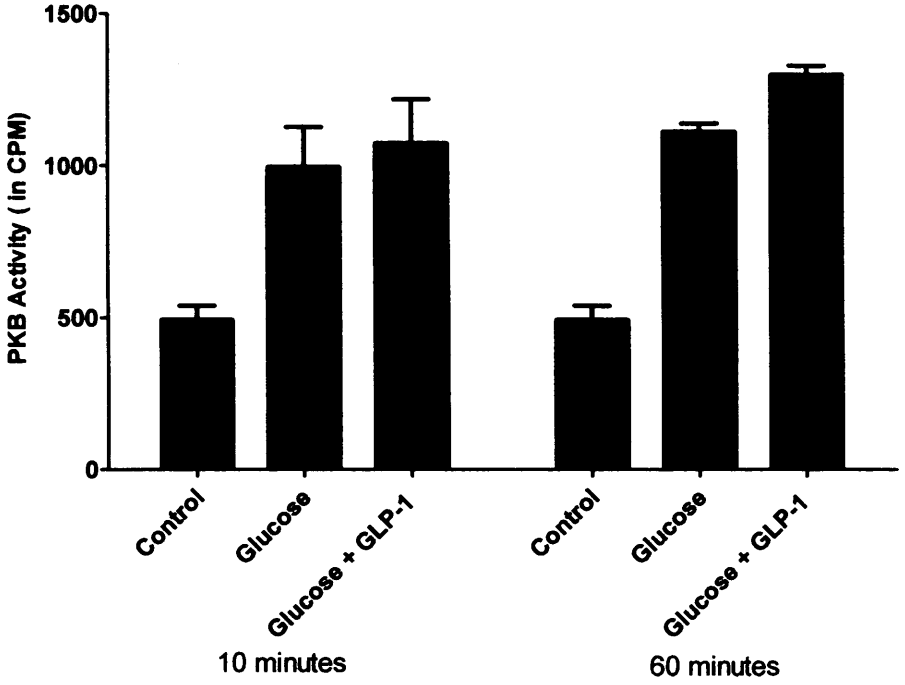


Figure 5.6: Effects of Glucose and GLP-1 on PKB Activity in MIN6 cells

MIN6 cells were infected with recombinant adenovirus over-expressing Ad-HA-PKB^{WT} for 48h. Following infection, MIN6 cells were pre-incubated in KRB supplemented with amino acid (0.5x) for 1h. Cells were incubated at 20mM glucose in the presence or absence of 10nM GLP-1 for 10 or 60min. HA tagged WT-PKB was immunoprecipitation using a monoclonal HA antibody and activation of HA tagged PKB was measured in an *in vitro* PKB kinase assay using Crosstide as substrate.

5.2.3 Activation of PI3K by GLP-1 is Independent of EGFR Transactivation

It has previously been reported in INS-1 cells that GLP-1 stimulates the activation of PI3K via the transactivation of the EGFR (Buteau, Foisy et al. 2003). Given that GLP-1 can activate PI3K via an insulin independent mechanism and that PI3K is required for GLP-1 stimulated rpS6 phosphorylation on Ser240/244, I sought to investigate the role of EGFR transactivation in PI3K activation. To determine whether EGF could stimulate PI3K activation, I looked at the phosphorylation of PKB, the downstream target of PI3K. MIN6 cells were pre-incubated in KRB for 1h prior to stimulation with EGF (20ng/ml). At specific times post-stimulation the phosphorylation states of PKB and Erk1/2, a downstream target of the EGFR were determined using phospho-specific antibodies directed towards Thr308 and Ser473 on PKB and Thr202/Tyr204 on Erk1/2. EGF treatment led to the rapid and sustained phosphorylation of Erk1/2. In contrast, EGF treatment resulted in a small and transient increase in PKB phosphorylation at Ser473 and had no detectable effect on Thr308 (**Figure 5.7a**). In order to assess the role of EGFR transactivation in PI3K activation and downstream signalling to rpS6, MIN6 cells were pre-incubated with 5nM of the selective EGFR inhibitor AG1478 prior to treatment with 20mM glucose plus GLP-1. To monitor the specificity of the inhibitor EGF was used as a positive control and insulin as a negative control. EGF dependent Erk1/2 activation was significantly blocked by AG1478 in MIN6 cells (**Figure 5.7bi**). Importantly, AG1478 had no effect on GLP-1 stimulated rpS6 phosphorylation on Ser240/244, indicating that EGFR transactivation is not required for GLP-1 stimulated PI3K activation (**Figure 5.7bii**). This was confirmed by measuring GLP-1 stimulated PI3K activity in the presence or absence of AG1478. AG1478 had no effect on GLP-1 stimulated PI3K activation (**Figure 5.7c**). Collectively, this data suggests that GLP-1 stimulated PI3K activation and subsequent rpS6 phosphorylation on Ser240/244 occurs via a mechanism independent of the transactivation of the EGFR in MIN6 cells.

5.2.4 Effects of Glucose/GLP-1 on PKB Phosphorylation in Isolated Islets of Langerhan

I show that GLP-1 is unable to potentiate glucose stimulated PKB activation in MIN6 cells (**Figure 5.5a**). However, whether GLP-1 is also unable to potentiate glucose stimulated PKB phosphorylation in islets is unknown. To investigate this, isolated rat islets were incubated in 20mM glucose or 20mM glucose plus GLP-1. GLP-1 was unable to potentiate PKB phosphorylation stimulated by 20mM glucose in rat islets (**Figure 5.8a**).

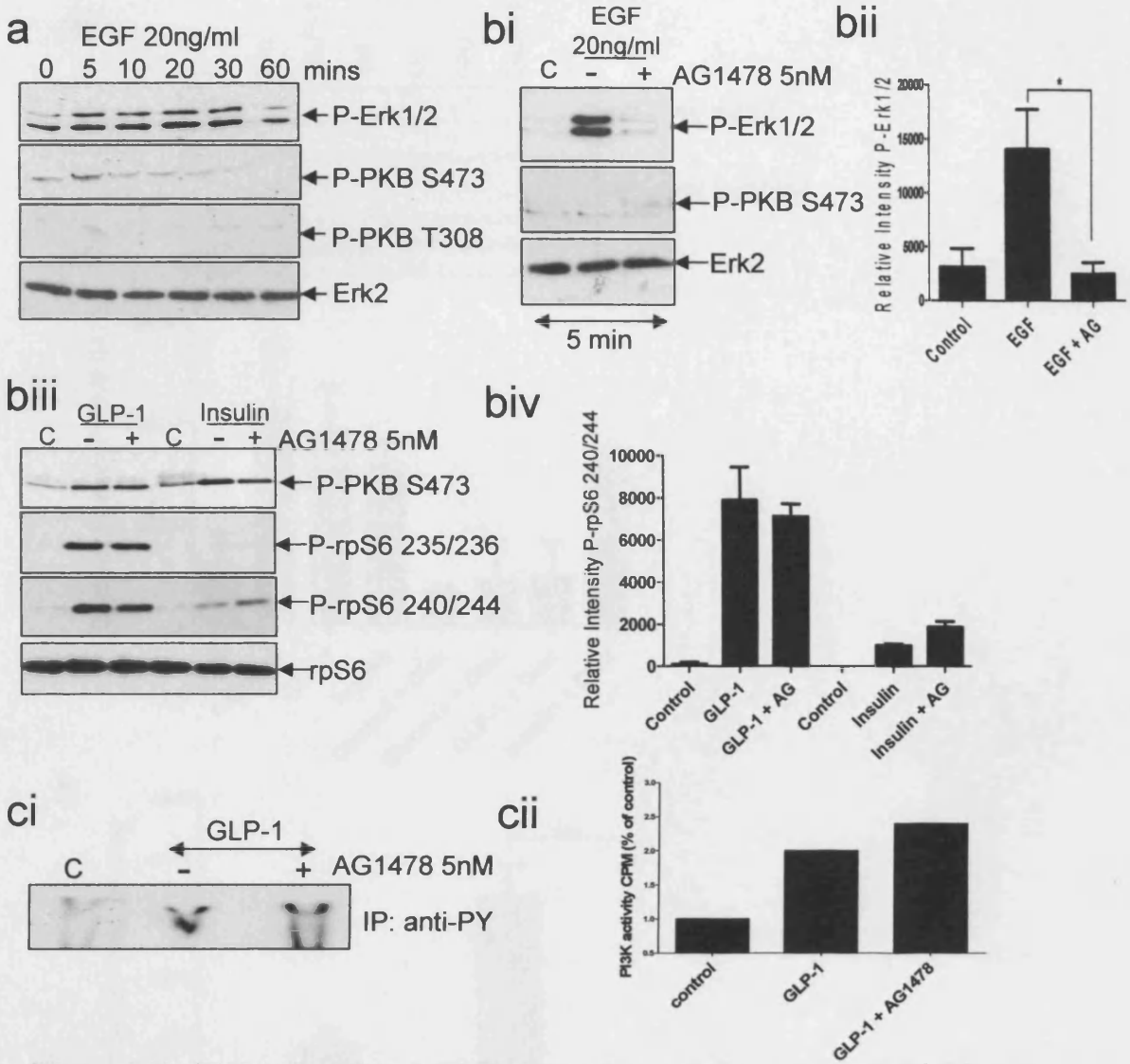


Figure 5.7: GLP-1 Stimulated PI3K Activation is Independent of EGF-R Transactivation in MIN6 Cells

In all cases, MIN6 cells were incubated in KRB supplemented with amino acid (0.5X) for 1h prior to treatment with a) 20ng/ml EGF for the times indicated in the figure. bi,iii) 20ng/ml EGF for 5min, 20mM glucose plus 10nM GLP-1 or 1μM insulin for 60min in the presence or absence of 5nM AG1478. After treatment, proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-PKB (Thr308 and Ser473) phospho-Erk1/2 (Thr202/Tyr204), phosphorylated rpS6 (Ser235/236 or Ser240/244) and total Erk2 and rpS6 as loading controls bii) Quantified data from phospho-Erk1/2 blots in *bi,* shown as means ± SEM; $n = 3$. $*P < 0.05$. P value obtained using a One-way ANOVA, biv) Quantified data from phospho-rpS6 Ser240/244 blots in *biii,* shown as means ± SEM; $n = 3$. ci) Equal amounts of cell lysates treated as in *biii* were subjected to immunoprecipitation with antibodies to pY. PI3K assays were performed as described in *Materials and Methods*. cii) ^{32}P-incorporation into phosphatidylinositol was quantified after separation by TLC.

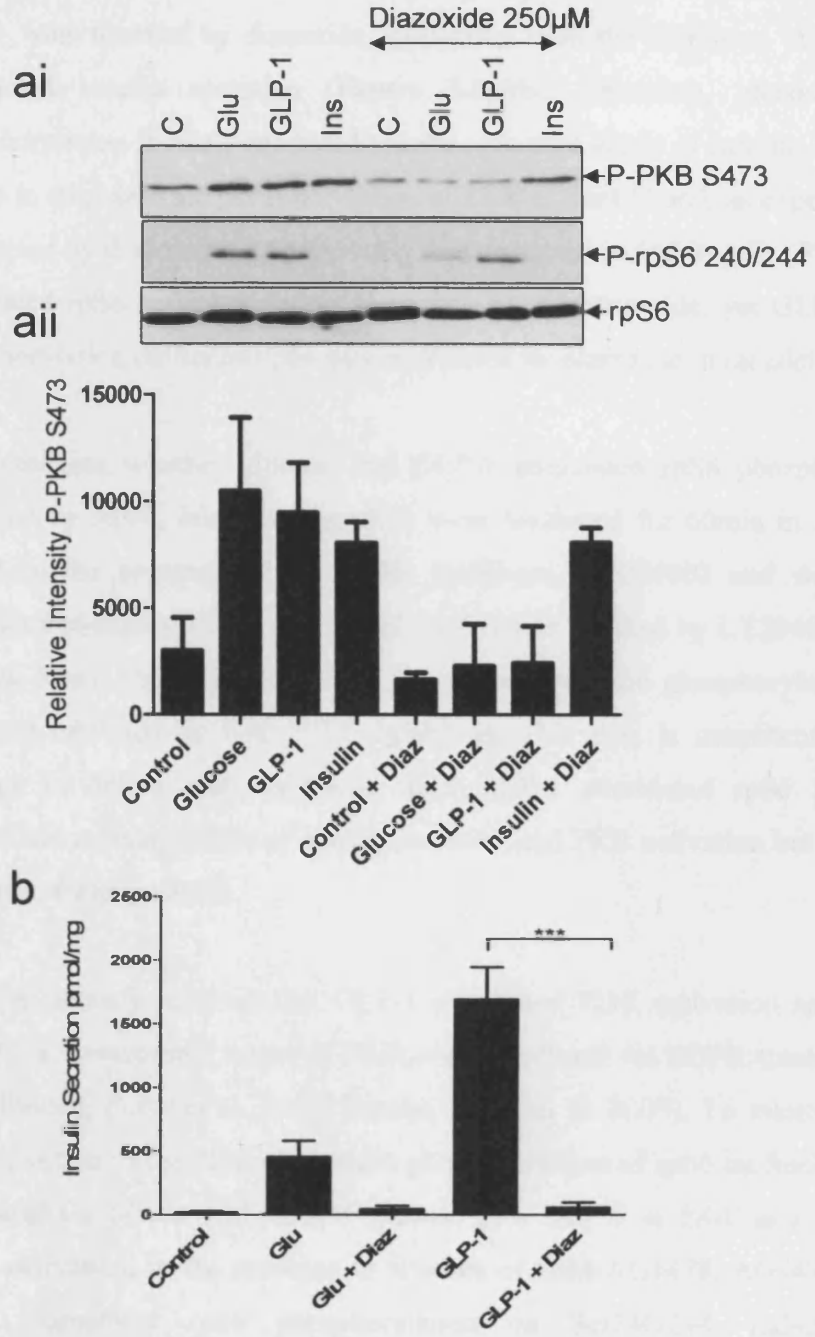


Figure 5.8: Glucose Stimulated PKB Phosphorylation is Dependent on Insulin secretion in Rat Islets of Langerhan

In all cases, islets of Langerhans were incubated in KRB supplemented with amino acids (0.5X) for 1h prior to treatment with 20mM glucose (Glu), 20mM glucose plus 10nM GLP-1 (GLP-1) or 1μM insulin (Ins) in the presence or absence of 250μM diazoxide; a) After treatment, proteins were resolved on SDS-PAGE and Western blotted using antisera against phospho-PKB (Ser473), phosphorylated rpS6 (Ser240/244) and total rpS6 as a loading control. aii) Quantified data from phospho-PKB S473 blots in *ai,* shown as means ± SEM; $n = 3$. b) The level of insulin secreted was determined using an ELISA assay, the results shown are means ± SEM; $n = 3$; $***P < 0.001$. P value obtained using a One-way ANOVA.

Furthermore, glucose stimulated PKB phosphorylation, either in the presence or absence of GLP-1, was blocked by diazoxide, in parallel with the inhibition of glucose and GLP-1 stimulated insulin secretion (**Figure 5.8ai/b**). Therefore, glucose stimulated PKB phosphorylation is likely mediated via the autocrine effect of insulin. Indeed, 1μM insulin is able to stimulate the phosphorylation of PKB at Ser473 and, as expected, this effect was unaffected by diazoxide. As previously demonstrated in MIN6 cells (**Figure 5.5a**), glucose stimulated rpS6 phosphorylation was inhibited by diazoxide, yet GLP-1 stimulated rpS6 phosphorylation on Ser240/244 was unaffected by diazoxide in rat islets (**Figure 5.8a**).

To investigate whether glucose and GLP-1 stimulated rpS6 phosphorylation was also mediated by PI3K, islets of langerhan were incubated for 60min in 20mM glucose plus GLP-1 in the presence of two PI3K inhibitors, LY294002 and wortmannin. Glucose stimulated phosphorylation of PKB at Ser473 was blocked by LY294002 and wortmannin (**Figure 5.9a**). Furthermore, the GLP-1 stimulated rpS6 phosphorylation on Ser240/244 was also inhibited by both PI3K inhibitors. This data is consistent with my previous findings in MIN6 cells indicating that GLP-1 stimulated rpS6 phosphorylation on Ser240/244 is independent of insulin secretion and PKB activation but dependent on PI3K (**Chapter 4 Figure 4.15**).

It was previously reported that GLP-1 stimulated PI3K activation and the activation of FOXO1, a downstream target of PKB, were mediated via EGFR transactivation in INS-1 cells (Buteau, Foisy et al. 2003; Buteau, Spatz et al. 2006). To assess the role of EGFR transactivation in the PI3K dependent phosphorylation of rpS6 on Ser240/244, islets were stimulated for 60min with 20mM glucose plus GLP-1 or EGF as a positive control of EGFR activation, in the presence or absence of 5nM AG1478. AG1478 had no effect on GLP-1 stimulated rpS6 phosphorylation on Ser240/244, indicating that EGFR transactivation is not required for GLP-1 stimulated PI3K activation. Therefore, GLP-1 stimulated phosphorylation of rpS6 on Ser240/244 is not dependent on EGFR transactivation in rat islets of langerhans.

I also investigated the role of EGFR transactivation in glucose stimulated PKB phosphorylation. Islets were pre-treated with AG1478 (5nM) prior to treatment with 20mM glucose, 20mM glucose plus GLP-1, EGF as a positive control and insulin as a negative control to monitor the specificity of the inhibitor. EGF dependent Erk1/2 activation was effectively blocked by AG1478 in rat islets. In addition, EGF stimulation resulted in a

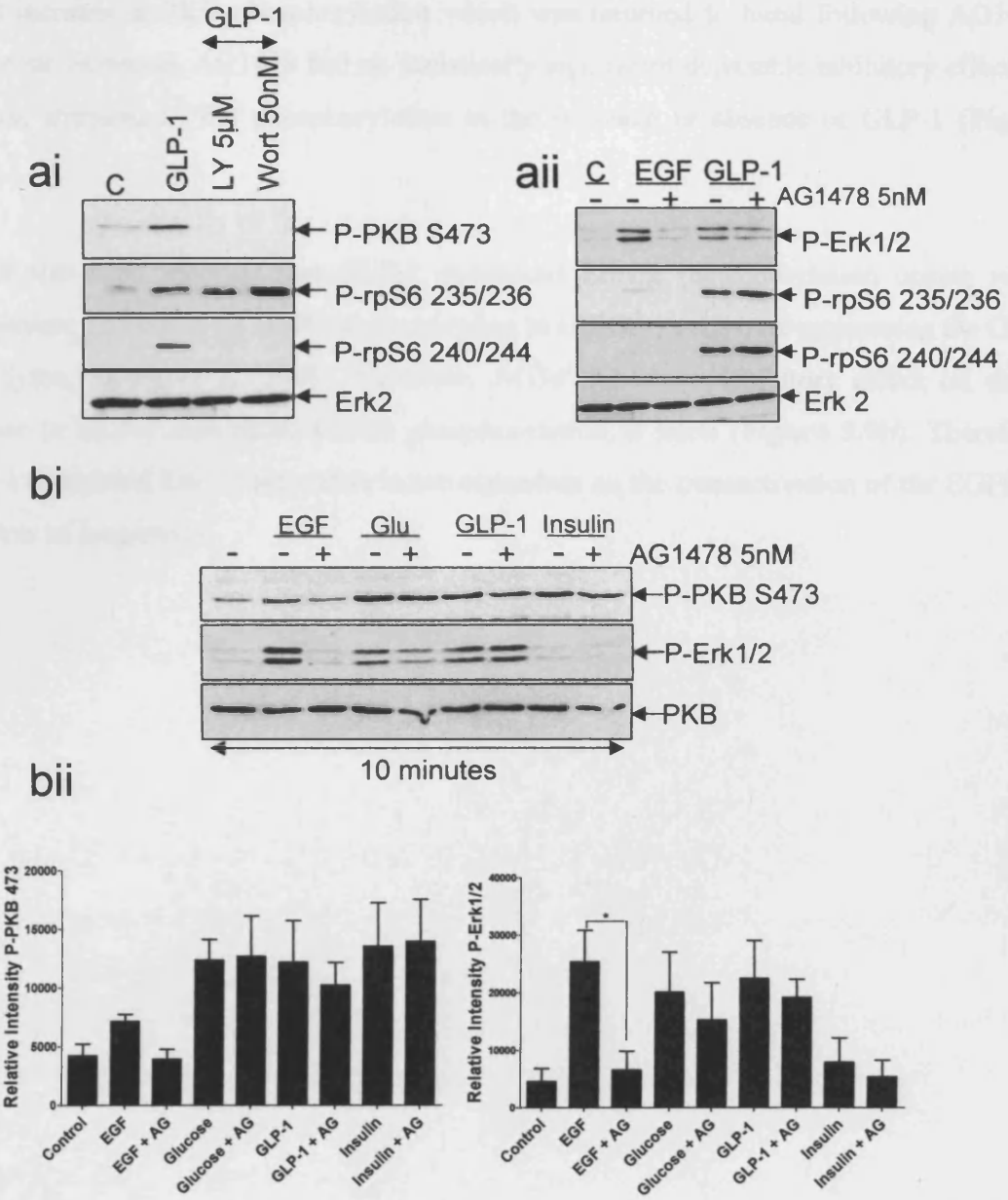


Figure 5.9: GLP-1 Stimulated rpS6 Phosphorylation is Independent of EGFR Transactivation in Rat Islets of Langerhan

In all cases, islets of Langerhans were incubated in KRB supplemented with amino acid (0.5X) for 1h prior to the addition of; ai) 20mM glucose plus 10nM GLP-1 in the presence or absence of 5μM LY294002 or 50nM wortmannin or aii) 20ng/ml EGF, 20mM glucose plus 10nM GLP-1 in the presence or absence of 5nM AG1478 for 60min or bi) 20ng/ml EGF, 20mM glucose (Glu), 20mM glucose plus 10nM GLP-1 (GLP-1) or 1μM insulin (Insulin) for 10min in the presence or absence of 5nM AG1478. After treatment, proteins were resolved by SDS-PAGE and Western blotted using antisera against phospho-PKB (Ser473), phosphorylated rpS6 (Ser235/236 or Ser240/244), total Erk2 and PKB as loading controls. bii) Quantified data from phospho-PKB S473 and P-Erk1/2 blots in *bi* shown as means ± SEM; $n = 3$, $^{*}P < 0.05$. P value obtained using a One-way ANOVA.

small increase in PKB phosphorylation which was returned to basal following AG1478 treatment. However, AG1478 had no statistically significant detectable inhibitory effect on glucose stimulated PKB phosphorylation in the presence or absence of GLP-1 (**Figure 5.9b**).

It has also been reported that GLP-1 stimulated Erk1/2 phosphorylation occurs via a mechanism dependent on EGFR transactivation in HEK293 cells over-expressing the GLP-1R (Syme, Zhang et al. 2006). However, AG1478 had no inhibitory effect on either glucose or GLP-1 stimulated Erk1/2 phosphorylation in islets (**Figure 5.9b**). Therefore, GLP-1 stimulated Erk1/2 activation is not dependent on the transactivation of the EGFR in rat islets of langerhans.

5.3 Discussion

In contrast to previous reports I could not detect any potentiation of glucose stimulated PKB phosphorylation by GLP-1. However, diazoxide, an inhibitor of insulin secretion, blocked PKB phosphorylation in cells treated with glucose and glucose in the presence of GLP-1. I also demonstrated that GLP-1 is able to stimulate an increase in PI3K activity independently of insulin secretion; interestingly this increase in PI3K activity is unable to activate PKB. It has been previously reported, and often cited, that GLP-1 activates PI3K via the transactivation of the epidermal growth factor receptor (EGFR) in the pancreatic β-cell line INS(832/13) (Buteau, Foisy et al. 2003). However, AG1478 (a selective EGFR inhibitor), at a dose that blocks EGF activation of Erk1/2, is unable to block PI3K activation in cells treated with glucose in the presence of GLP-1.

5.3.1 PKB Activation in Pancreatic β-cells

In the pancreatic β-cell line INS-1, it has been reported but not shown that GLP-1 potentiates glucose stimulated PKB phosphorylation threefold at 2.5mM to 5mM glucose, whereas at 20mM glucose, GLP-1 induced only a twofold increase in glucose stimulated PKB phosphorylation (Trumper, Trumper et al. 2000). However, whether GLP-1 potentiates glucose stimulated PKB phosphorylation in islets is poorly defined. A study in human islets reported that 15mM glucose and 10nM GLP-1 induced the phosphorylation of PKB at Ser473. However, there is very little potentiation of glucose stimulated PKB phosphorylation by GLP-1 and there is no statistical analysis performed on this data (Trumper, Ross et al. 2005). I have demonstrated that GLP-1 does not potentiate glucose stimulated PKB phosphorylation in MIN6 cells or rat islets (**Figure 5.5a, 5.9a**). In addition, I also demonstrated that GLP-1 did not significantly increase PKB activity in cells incubated at 20mM glucose (**Figure 5.6**).

5.3.2 Autocrine Effect of Insulin

In the beta TC3 insulin-secreting cell line, glucose rapidly induces the tyrosine phosphorylation of the insulin receptor beta-subunit and the inhibition of insulin secretion by removing the extracellular Ca^{2+} blocks this effect (Rothenberg, Willison et al. 1995). Elevated glucose also stimulates the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and increases the association of IRS-1 with the p85 regulatory subunit of PI3K (Rothenberg, Willison et al. 1995). Similar results were also obtained in isolated rat islets

(Velloso, Carneiro et al. 1995). In INS-1 cells, GLP-1 has been shown to increase the level of tyrosine phosphorylation on IRS-2, which co-immunoprecipitates with the regulatory subunit of PI3K, p85 (Trumper, Trumper et al. 2000); GLP-1 also significantly increased PI3K activity in Gab-1 immunoprecipitates (Trumper, Trumper et al. 2000). Gab-1 functions as an adaptor protein which is recruited to the membrane and undergoes tyrosine phosphorylation and the phosphorylation of Tyr472 regulates its binding to p85 subunit of PI3K (Rocchi, Tartare-Deckert et al. 1998). Therefore, GLP-1 recruits many of the downstream elements essential in insulin signaling. My data demonstrates that inhibition of insulin secretion using diazoxide significantly blocked PKB phosphorylation in cells treated with 20mM glucose in rat islets and MIN6 cells in either the presence or absence of GLP-1 (**Figure 5.5, 5.9**). However, at this glucose concentration GLP-1 did not potentiate glucose stimulated PKB activation but at this concentration of glucose GLP-1 is unlikely to potentiate glucose stimulated insulin secretion. Therefore, by lowering the glucose concentration, GLP-1 may potentiate insulin secretion and hence potentiate glucose stimulated PKB phosphorylation (**Figure 5.10**). Indeed, in islets, I have observed that GLP-1 increases glucose stimulated PKB phosphorylation at 5.5mM glucose. However, I have been unable to consistently reproduce this finding (data not shown).

5.3.3 GLP-1 Stimulated PI3K Activation is Independent of EGFR Transactivation

In pancreatic β-cells, the activation of PI3K by GLP-1 has been shown to be dependent on the transactivation of the EGFR which is mediated by the activation of c-src and subsequent activation of metalloproteases which cleave membrane bound BTC (endogenous EGF-like ligands), allowing transactivation of the EGFR (Buteau, Foisy et al. 2003). This GLP-1 mediated activation of PI3K was shown to play an important role in β-cell proliferation (Buteau, Foisy et al. 2003). However, another study in INS-1 cells demonstrated that the inhibition of tyrosine kinase activity had no effect on GLP-1 induced β-cell proliferation (Wang, Li et al. 2004). Therefore, within the same cell type, there are different mechanisms of GLP-1 induced PI3K activation. It has also been suggested that GLP-1 stimulates the activation of PI3K via Rap1 in human islets, since the immunoprecipitation of Rap1 correlated with an increase in PI3K activity in GLP-1 stimulated islets. It was proposed that the mechanism of activation is similar to that of Ras mediated PI3K activation in other cell types (Trumper, Ross et al. 2005).

My data demonstrates that glucose stimulated PKB phosphorylation is dependent on the upstream activator PI3K, since inhibition of PI3K using LY294002 and wortmannin completely blocks glucose stimulated PKB phosphorylation (**Chapter 4 Figure 4.15a,b**). Although, glucose stimulated rpS6 phosphorylation was inhibited by diazoxide, GLP-1 stimulated rpS6 phosphorylation was unaffected by diazoxide (**Figure 5.5a**). Moreover, the phosphorylation of rpS6 on Ser240/244 was inhibited by the two PI3K inhibitors, LY294002 and wortmannin. This data provides evidence that GLP-1 could stimulate PI3K via an insulin independent mechanism and that the activation of PI3K by GLP-1 is unable to couple to PKB. Furthermore, I show that inhibition of EGFR signalling with AG1478, a previously reported mechanism for GLP-1 stimulated PI3K activity, had no effect on GLP-1 stimulated PI3K activity or the phosphorylation of rpS6 on Ser240/244 (**Figure 5.7c**).

One potential reason for the differences in my data compared to those published may be due to the different cell lines used in this study. However, I have shown that glucose stimulated PKB phosphorylation also occurs via the autocrine effect of insulin in isolated rat islets of langerhan and not via EGFR transactivation. Another possibility for the differences between the studies may be due to the specificity of the EGFR inhibitor. In INS-1 cells, 250nM of AG1478 was used to block PI3K activity (Buteau, Foisy et al. 2003). However, I show that 5nM is sufficient to block EGFR signalling to Erk1/2 (**Figure 5.7a**). Furthermore, increases in AG1478 up to 250nM (data not shown) resulted in a decrease in insulin stimulated PKB phosphorylation, indicating that the inhibitor becomes less specific at high concentrations.

In addition, we also demonstrate that EGFR transactivation is not responsible for GLP-1 stimulated Erk1/2 activation, since concentrations of AG1478 that effectively block EGF induced Erk1/2 activation had no effect on GLP-1 stimulated Erk1/2 activation (**Figure 5.8c**). The mechanism by which GLP-1 stimulates the activation of Erk1/2 is discussed in chapter 3.

5.3.4 GLP-1 Activates PI3K Independently of Insulin Secretion

Interestingly, we show that inhibition of insulin secretion using diazoxide, which effectively blocked PKB phosphorylation in cells treated with glucose and GLP-1 had no effect on GLP-1 stimulated PI3K activity (**Figure 5.5c**). Therefore GLP-1 is able to stimulate an increase in PI3K activity independently of insulin secretion. Interestingly, this increase in PI3K activity is unable to activate PKB. Furthermore, given that I observed an

increase in GLP-1 stimulated PI3K associated activity using a phospho-tyrosine (pY) antibody and that pY antibodies are routinely employed to assess PI3K stimulation by tyrosine kinases, it is likely that GLP-1 stimulates a Class 1 PI3K. Therefore, it is possible that GLP-1 stimulates a distinct pool PI3K which is unable to activate PKB.

In pancreatic β-cells, the PI3K pathway has been implicated in many β-cell functions, including growth, survival and metabolism. Of the three classes of PI3K, it has been shown that human islets express the Class 1_A and Class 1_B isoforms as well as the class II PI3KC2α and PI3KC2γ (Muller, Huang et al. 2006). The class I_B isoform PI3Kγ is activated by GPCR ligand binding (Oudit, Sun et al. 2004). It is possible that GLP-1R activation leads to the activation of PI3Kγ via the βγ subunit which is released upon GLP-1R activation. In pancreatic β-cells, stimulation with GIP induces a rise in cAMP and intracellular calcium, which facilitates glucose dependent insulin secretion (Fehmann, Goke et al. 1995). In INS-1 cells, GIP was shown to increase the activity of a PI3K as assessed by immunoprecipitation using a phospho-tyrosine antibody, but the authors also show a significant increase in PI3Kγ activity in response to GIP (Trumper, Trumper et al. 2001). PI3Kγ specific knock-out mice exhibit a reduction in the glucose secretory response, which is restored by the administration of exendin, a GLP-1R agonist, revealing that this isoform plays an important role in regulating insulin secretion in pancreatic β-cells (Li, MacDonald et al. 2006). Another interesting characteristic of these PI3Kγ deficient mice is that the phosphorylated or total forms of PKB were unaltered in the pancreas suggesting that the other isoforms of PI3K can compensate for the activation of PKB (MacDonald, Joseph et al. 2004; Li, MacDonald et al. 2006). Therefore, it is possible that GLP-1 signals to both isoforms of Class 1 PI3K, with Class 1_A activating PKB via insulin signalling and Class 1_B activated as a result of GLP-1R activation which activates PI3Kγ but not PKB.

It has been reported that there are spatially distinct pools of PI3K located in the cytoplasm (close to the plasma membrane) and nucleus (Martelli, Bortul et al. 2001). Both class 1_A and class 1_B have been shown to translocate to the nucleus following stimulation (Martelli, Bortul et al. 2001). The PI3Kγ isoform has also been shown to be nuclear in aortic smooth muscle whilst the class II C2β PI3K isoform has been identified in the nucleus of rat hepatocytes (Bacqueville, Deleris et al. 2001; Sindic, Aleksandrova et al. 2001). It is also worth noting that the nucleus also contains other components of the PI3K signalling pathways, such as PKB (Dai, Wei et al. 2007). Within the nucleus, PI3K activity is

regulated by the PI3K enhancer (PIKE), a GTPase which is in turn regulated by PLCγ1, a guanine nucleotide exchange factor for PIKE (Ahn, Rong et al. 2004). PIKE binds to the p85 regulatory subunit of PI3K, resulting in activation of the catalytic subunit (Martelli, Bortul et al. 2001). It has also been reported that, in HEK-293 cells and N1E-115 (neuroblastoma cells), Gs coupled receptor signalling which elevates cAMP activates Epac1, catalyzes the loading of GTP onto Rap2B, leading to PLCε activation (Keiper, Stope et al. 2004). Furthermore, in INS-1 cells, GLP-1 has also been shown to activate PLC, as assessed by pharmacological inhibition using U73122 (Suzuki, Zhang et al. 2006).

A recent study in 3T3-L6 myoblasts revealed that there are two separate pools of PI3K lipids which are dephosphorylated differentially. A pool of freshly generated lipids is dephosphorylated by PTEN whilst a stable pool of PI3K lipids is dephosphorylated by Ship2 (Mandl, Sarkes et al. 2007). Furthermore, these authors hypothesised that the newly generated lipids can diffuse to areas of the membrane where PTEN is absent to form a stable pool of lipids but this stable pool is unable to encounter PDK1 or PKB (Mandl, Sarkes et al. 2007).

It has recently been shown that a major pathway by which amino acids stimulates mTOR activity is through a class III PI3K, hVps34 (Byfield, Murray et al. 2005; Nobukuni, Kozma et al. 2007). hVps34 signals to mTOR independently of PKB and is not stimulated by insulin, but it is inhibited by amino acid starvation as well as LY294002 and wortmannin. It is therefore possible that the effects of LY294002 and wortmannin on GLP-1 stimulated rpS6 phosphorylation on Ser240/244 are via the inhibition of hVps34. I demonstrated that GLP-1 increases PI3K activity as assessed by measuring phospho-tyrosine (pY) associated PI3K activity, using PtdIns as a substrate (**Figure 5.5c**). Given that major substrate specificity of hVps34 is also PtdIns, it is possible that GLP-1 stimulates an increase in hVps34 activity and this would not lead to PKB activation. However, it is unknown whether in pancreatic β-cells express hVps34 or whether there is a link between GPCR activation and an increase in hVps34 activity. Furthermore, it is unclear why a class III activitys should be immunprecipitated using an anti-phospho tyrosine antibody.

How might the GLP-1R activate hVps34? A possible mechanism for GPCR dependent hVps34 activation may involve the small G-protein Rab5 which regulates hVps34 targeting to endosomes (Windmiller and Backer 2003). Rab5 is a member of Rab small G-

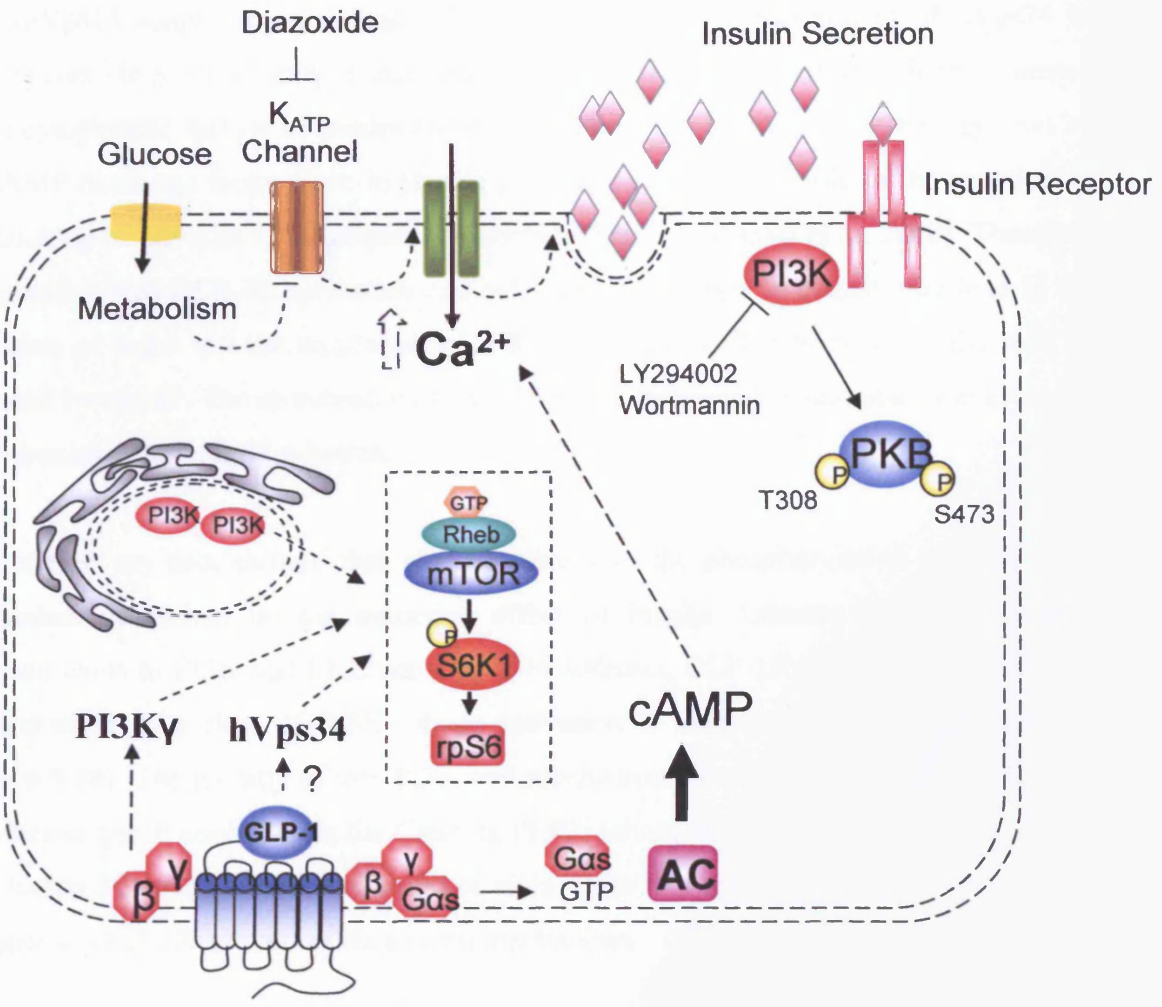


Figure 5.10: Proposed Model for Glucose Stimulated PKB Phosphorylation in Pancreatic β-cells

In pancreatic β-cells, glucose metabolism leads to an increase in the ATP/ADP ratio, the closure of ATP-sensitive K+ channels, the depolarization of the cell membrane, the opening of L-type VGCC, and the subsequent influx of extracellular calcium into the cell. Elevations in cAMP in response to GLP-1R activation enhance Ca^{2+} influx leading to the exocytosis of insulin containing granules. Insulin acts in an autocrine manner to activate the insulin receptor and downstream signalling to PKB. In addition, GLP-1R activation also leads to the activation of a distinct pool or isoform of PI3K, independently of insulin secretion.

protein family which comprises approximately 70 members (Stenmark and Olkkonen 2001). Rab5 mediates the membrane recruitment of various effector proteins which include p150 (hVps15 adaptor protein) and hVps34. The membrane recruitment of hVps34 to Rab5 occurs via p150 (Murray, Panaretou et al. 2002). The Rab3 isoform which is located on the cytoplasmic surface of insulin secretory granules has been shown to be regulated by the cAMP exchange factor Epac in β-cells and plays an important role in the recruitment and docking of granules at the plasma membrane (Ozaki, Shibasaki et al. 2000). Therefore it is possible that GLP-1R activation and subsequent increases in cAMP may lead to the activation of Rab5 via the regulation of a Rab5 specific GEF which like Rab3 will be regulated by cAMP. The recruitment of hVps34 to the membrane would place the kinase in close proximity to its lipid substrate.

In summary, my data showed that glucose stimulates the phosphorylation of PKB via a mechanism dependent on the autocrine effect of insulin. Glucose stimulated insulin secretion leads to PI3K and PKB activation. In addition, GLP-1R activation also leads to the activation of a class of PI3K whose activation is independent of insulin secretion (**Figure 5.10**). The identity of this PI3K and mechanism of activation are unknown, yet I hypothesise that it could be via the Class 1_B PI3Kγ whose activity will be regulated by the βγ subunits following GPCR activation or via a Class III PI3K which will be activated in response to GLP-1R activation via a novel mechanism.

5.3.5 Future Directions

The presented data demonstrated that GLP-1 can activate PI3K independent of insulin secretion which is unable to couple to PKB (Figure 5.1c). Therefore further studies are required to identify the PI3K isoform which is activated in response to GLP-1. One possibility is to immunoprecipitate with antibodies directed towards specific PI3K isoforms and measuring the associated PI3K activity. If GLP-1 couples to the class III PI3K future studies should explore the mechanism by which GLP-1 activates this isoform. In yeast it has been reported that the Vps34 regulatory protein, Vps15p can associate with the Gα G-protein (Slessareva, Routt et al. 2006). The Gα G-protein binds to Vps34 and Vps15 in a GTP dependent manner and increases PtdIns 3-kinase activity (Slessareva, Routt et al. 2006). Therefore, future studies could be directed towards investigating potential protein-protein interactions between the GLP-1R and hVps34 in pancreatic β-cells.

Chapter 6: Final Discussion

6.1 Overview

The gut hormone GLP-1 (Glucagon-like peptide-1) is secreted in a nutrient dependent manner from endocrine L-cells (Drucker 2001). A major target for GLP-1 is the pancreatic β-cell where its main action is to potentiate glucose stimulated insulin secretion, it also stimulates β-cell proliferation, differentiation and insulin gene transcription (Doyle and Egan 2007). A number of kinases are activated in response to GLP-1R activation, including extracellular regulated kinases (Erk1/2), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB) and the downstream target of mTOR, S6K1. At present the exact mechanisms by which GLP-1 activates these signalling pathways in pancreatic β-cells is not fully understood. However, it has been shown that glucose and GLP-1 causes a significant increase in Erk1/2 activation in a Ca^{2+} dependent manner (Gomez, Pritchard et al. 2002; Arnette, Gibson et al. 2003). In this thesis I provide evidence that this increase in Ca^{2+} influx is likely to phosphorylate Erk1/2 via the activation of the MEK kinase, Tpl2. This is mediated by the activation of a Ca^{2+} dependent phosphatase, calcineurin which activates IKK. An increase in IKK activity promotes the phosphorylation and dissociation of p105 from Tpl2 resulting in Tpl2 activation. The ability of glucose and GLP-1 to stimulate an increase in $[Ca^{2+}]_i$ also plays an important role in stimulating insulin secretion, which feeds back on to the insulin receptor and activates PI3K and PKB. GLP-1 can also activate PI3K independently of insulin secretion but is unable to activate PKB. However, this GLP-1 stimulated PI3K activity can stimulate the phosphorylation of rpS6 on Ser240/244 via an mTOR dependent mechanism. This effect is likely mediated by an increase in cAMP, as forskolin, an activator of adenylyl cyclase can phosphorylate rpS6 via similar mechanism. GLP-1 also stimulates the phosphorylation of rpS6 on Ser235/236 which is mediated directly by PKA (**Figure 6.1**).

6.1.1 GLP-1 Based Therapy for the Treatment of Type 2 Diabetes

The multiple actions of GLP-1 on the β-cell make GLP-1 an excellent therapeutic candidate for the treatment of type 2 diabetes. The glucose lowering effects of GLP-1 suggests that it may be used as a treatment for diabetes. Indeed, continuous infusion of exogenous GLP-1 increases insulin secretion to normal levels and effectively lowers blood

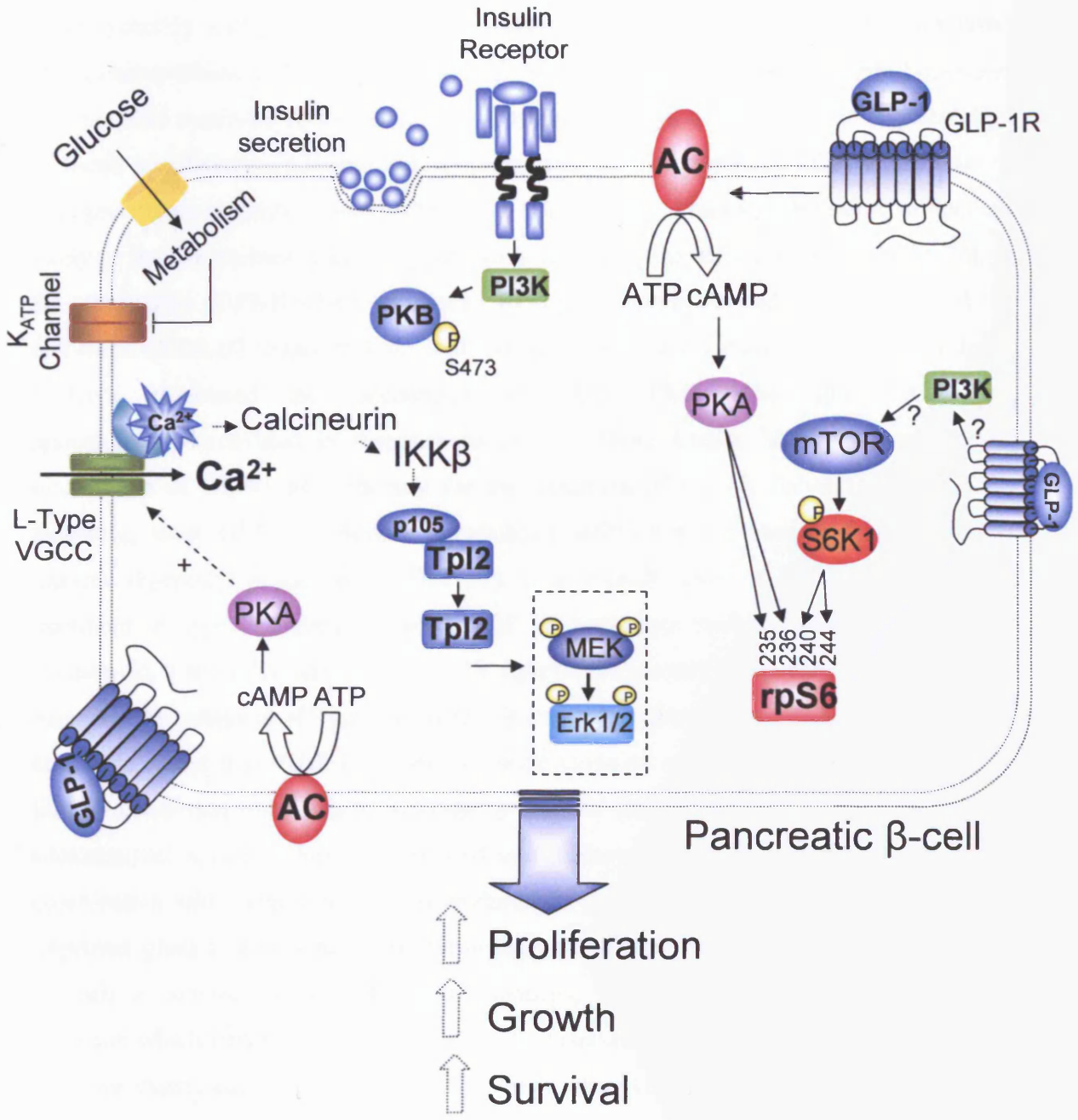


Figure 6.1: GLP-1 Signalling in Pancreatic β-cells

Binding of GLP-1 to the receptor is coupled to adenylyl cyclase activation, intracellular cAMP levels are increased leading to PKA and Epac activation. Following GLP-1R activation are number of signalling pathways are activated which include the Erk1/2 pathway, PI3K/PKB pathway and the mTOR signalling pathway which leads to an increase in β-cell proliferation, growth and survival.

glucose in type 2 diabetics (Ahren 1998; Todd and Bloom 2007). Furthermore, given that the insulinotropic action of GLP-1 is glucose dependent there is a minimal risk of hypoglycaemia during exogenous GLP-1 administration. Additionally, GLP-1 stimulates insulin biosynthesis as well as increases in β-cell mass which is reduced in type 2 diabetes. The possible mechanisms by which GLP-1 regulates β-cell number include augmentation of β-cell proliferation, inhibition of apoptosis and the differentiation of exocrine cells or islet precursors towards a β-cell phenotype. The GLP-1 induced proliferative effects are likely to involve multiple signalling pathways, including the activation of mTOR via rpS6, the stimulation of PKB which increases CREB phosphorylation and IRS2 levels, and the activation of Erk1/2 (Egan, Bulotta et al. 2003; Brubaker and Drucker 2004). In this thesis I have elucidated the mechanism by which PKB, rpS6 and Erk1/2 are phosphorylated/activated in response to GLP-1. These studies highlight the potential importance of GLP-1 as a therapy for the treatment of type 2 diabetes (**Figure 6.2**). However, since GLP-1 is rapidly metabolised within a few minutes (~ 2min) by the enzyme dipeptidyl peptidase-IV (DPP-IV), GLP-1 itself is not an ideal therapy for the treatment of type 2 diabetes. Indeed, GLP-1R analogues such as exenatide (synthetic exendin-4), a naturally occurring GLP-1R agonist is currently being used as a therapeutic agent for the treatment of Type 2 diabetes (Wang and Brubaker 2002). The benefit of using exenatide rather than GLP-1 is that exenatide contains a glycine residue at position 2, thereby conferring resistance to cleavage by DPP-IV (Doyle and Egan 2007). Exenatide is administrated subcutaneously and is used as a therapy in patients with type 2 diabetes in combination with metformin or a sulfonylurea, or both (Iltz, Baker et al. 2006). Exenatide improves glucose homeostasis by mimicking the actions of naturally occurring GLP-1 through a combination of known mechanisms, including glucose dependent insulin secretion which restores the first phase insulin response, regulation of glucagon secretion, delaying gastric emptying, and decreasing food intake (Iltz, Baker et al. 2006). In animal models, exendin-4 causes a reduction in plasma glucose and weight gain and increases β-cell mass in *db/db* mice via enhanced β-cell proliferation and reduce apoptosis (Wang and Brubaker 2002).

Another GLP-1 analogue is liraglutide. Liraglutide is a long acting GLP-1 analogue with a substitution of Lys34 with Arg34, and an attachment of a C-16 free-fatty acid. The free fatty acid is thought to promote binding of liraglutide to albumin and increase the plasma half-life by reducing renal clearance (Knudsen, Nielsen et al. 2000; Rolin, Larsen et al. 2002). Animal studies have shown that liraglutide administration reduced plasma glucose

levels and increased insulin secretion (Bregenholt, Moldrup et al. 2005). Liraglutide was also shown to protect β-cells from cytokine and fatty acid induced apoptosis which was blocked by inhibiting the PI3K pathway using wortmannin or the Erk1/2 pathway by using PD98059. This data suggests that liraglutide has anti-apoptotic effects on both cytokine, and free fatty acid-induced apoptosis which may be useful for retaining β-cell mass in type 2 diabetic patients (Bregenholt, Moldrup et al. 2005).

An additional approach to enhancing the actions of GLP-1 involves the inhibition of DPP-IV, the GLP-1 degrading enzyme. Studies have shown, in DPP-IV knock-out mice, an increased glucose tolerance, elevated levels of GLP-1 as well as increased insulin sensitivity compared to their wild type counterparts (Conarello, Li et al. 2003). In patients with type 2 diabetes administration of DPP-IV inhibitors increases the levels of endogenous GLP-1 resulting in stimulation of insulin secretion (Baggio and Drucker 2006). Another report demonstrated that inhibition of DPP-IV for 7 weeks in rats with streptozotocin induced diabetes increased the number of β-cells (Pospisilik, Martin et al. 2003), this is consistent with the ability of GLP-1 to enhance β-cell proliferation (Drucker 2003). Sitagliptin is a selective DPP-IV inhibitor. In a diabetic mouse model, sitagliptin resulted in improved glucose stimulated insulin secretion, increased insulin content and increases in β-cell mass (Mu, Woods et al. 2006). The beneficial effects of sitagliptin on glucose homeostasis and β-cell mass are at least partially mediated via increased GLP-1 signalling. Vildagliptin, is a selective, reversible, and competitive inhibitor of DPP-IV. In neonatal rats, vildagliptin has been demonstrated to stimulate β-cell replication, inhibit apoptosis and augment β-cell mass by enhancing GLP-1 action (Wajchenberg 2007).

Therefore, the development of GLP-1 analogues and DPP-IV inhibitors offer an effective treatment option for type 2 diabetes. Another interesting aspect of incretin based therapies is the trophic and protective effects that GLP-1 analogues exert on the pancreatic β-cell. Therefore, incretin based therapy offer potential advantages to the existing treatments for type 2 diabetes, based on their effects on pancreatic β-cell growth and the potential to reduce body weight or prevent weight gain.

Phenotype	Actions of GLP-1
Impaired β-cell function	Increased insulin secretion/biosynthesis
	Increased β-cell function
	Increases expression of β-cell specific genes (GLUT2, Glucokinase)
Reduced β-cell mass	Increased β-cell proliferation
	Increased β-cell mass
	Decreased β-cell apoptosis
Glucagon hypersecretion	Decreased glucagon secretion
Obesity	Decreased gastric emptying
	Decreased appetite
Insulin Resistance	Increased insulin sensitivity

Figure 6.2: Anti-diabetic actions of GLP-1

Adapted from (Holst, Deacon et al. 2008).

My findings have demonstrated that GLP-1R activation stimulates a number of signalling pathways including PI3K-PKB, mTOR-rpS6 and Erk1/2. Because activation of mTOR and its downstream targets have been implicated to regulate β-cell size, GLP-1 administration represents a potential strategy to further enhance mTOR stimulated cell growth and proliferation. Erk1/2 activation not only increases insulin gene expression but it has also been shown to play an important role in regulating insulin exocytosis via the phosphorylation of synapsin 1 which increases insulin secretion (Longuet, Broca et al. 2005). Therefore, GLP-1 stimulated Erk1/2 activation may assist in restoring insulin secretion in type 2 diabetics. Another potential site of therapeutic intervention is PKB. Transgenic mice over-expressing a kinase dead form of PKB show impaired glucose tolerance and defective insulin secretion and develop type 2 diabetes (Bernal-Mizrachi, Fatrai et al. 2004). Therefore, PKB in addition to positively enhancing β-cell survival also plays an important role in the regulation of β-cell function suggesting that PKB activation

may be an important therapeutic target for improving insulin secretion and enhancing β-cell survival in type 2 diabetes.

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