Hormonal and nutrient signalling to protein kinase B and mammalian target of rapamycin in pancreatic beta-cells

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By

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Objectives: Protein kinase B (PKB) and mammalian target of rapamycin (mTOR) signalling pathways control several critical cellular processes including metabolism, protein synthesis, proliferation and cell survival. Glucagon-like peptide-1(GLP-1) stimulates β-cell proliferation and protects cells against cytokine induced apoptosis via activation of mTORC1 and PKB. Amino acids are also able to regulate mTOR though the role of amino acid transporters in the regulation of amino acid-dependent regulation of mTORC1 is unknown. The regulations of PKB and mTORC1 by GLP-1 and amino acids have been the subjects of intense study; despite this, many questions remain to be answered. Therefore, this study aims to investigate the transduction pathways by which GLP-1 and amino acids regulate PKB/ mTORC1 activation.

Results: I showed that GLP-1 in the presence of glucose induced activation of mTORC1 in rat islets of Langerhans and β-cell lines and this was sensitive to PKB inhibitor, AKTi. GLP-1 also potentiated glucose-stimulated mTORC1 activation via a calcium and PI3K dependent mechanism. Diazoxide, an inhibitor of exocytosis, significantly reduced GLP1-induced PKB and mTORC1 activation. siRNA mediated knock-down of insulin receptor expression was unable to inhibit GLP1-stimulated PKB or mTORC1 activation. In contrast, siRNA mediated knockdown of the IGF-1 receptor effectively blocked GLP1-stimulated PKB and mTORC1 activation. Incubation of rat islets in medium without Glutamine or System L amino acids blocked mTORC1 activation in response to insulin. Inhibition of SNAT2 with methylaminoisobutyrate or silencing of SNAT2 expression with small interfering RNAs inhibited mTOR activation. Inhibition of LAT1 by 2-aminobicyclo(2,2,1) hepatane carboxylic acid (BCH) blocked PKB and mTORC1 activation.

Conclusion: In pancreatic β-cells, acute activation of PKB and mTORC1 by GLP1 is mediated via the activation of the IGF1 receptor, presumably through the autocrine effect of IGF. The plasma membrane amino acid exchangers (SNAT2 and LAT1) can regulate mTORC1 activity suggesting that they can play a key role in control β-cell function.

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Abstract in a conference

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Abbreviations

4EBP1	eukaryotic initiation factor 4E binding protein 1
5'UTR	5'untranslated region
AA	Arachidonic acid
AC	adenylyl cyclase
Ach	Acetyl choline
ADSS-1	adenylosuccinate synthase
Ala	Alanine
AMPK	AMP-dependent protein kinase
ANOVA	analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATF4	activating transcription factor 4
ATP	Adenosine-5-triphosphate
BCH	2-aminobicyclo-(2,2,1)heptanecarboxylic acid
BSA	bovine serum albumin
CaM	Calmodulin
CaM-KK	calmodulin-dependent protein kinase kinase
cAMP	cyclic adenosine monophosphate
CCK	Cholecystokinin
CDKs	cyclin-dependent kinases
Chloroquine	CQ
CREB	cAMP response element binding protein
Cx	Connexins
Cys	Cystine
DAG	Diacylglycerol
DDP-IV	Dipeptidyl peptidase IV
DEPC	Diethylpyrocarbonate
DEPTOR	DEP domain-containing protein 6
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DON	6-diazo-5-oxo-L-norleucine
EBSS	Earle's Balanced Salt solution
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF2	Eukaryotic Elongation Factor 2
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
eIF2α	eukaryotic initiation factor 2 α
eIF3	eukaryotic initiation factor 3
EJC	exon junction complex
Epacs	Exchange proteins activated by cyclic AMP
ER	Endoplasmic reticulum
Erk	Extracellular regulated kinase
FCS	Foetal calf serum
FGF-2	fibroblast growth factor-2
GABA	γ-amino butyric acid
GAP	GTPase-activating protein

GCN2	general control non-derepressible -2
GDH	Glutamate dehydrogenase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
Glu	Glutamate
GLUT 2	Glucose transporter 2
Gly	Glycine
GPCR	G protein-coupled receptor
GRP	Gastrin releasing peptide
GSK3β	glycogen synthase kinase 3β
Gα	α-subunit of heterotrimeric G-proteins
Gβγ	β- and γ- subunits complex of heterotrimeric G-proteins
HbA1c	Haemoglobin A1c (Glycosylated haemoglobin)
HEK293	human embryonic kidney 293 cell line
His	Histidine
hnRNPs	Heterogeneous ribonucleoproteins
HPLC	High Performance Liquid Chromatography
HRF	histamine-releasing factor
IAP-2	Inhibitor of apoptosis protein-2
IBMX	isobutylmethylxanthine
IEC-6	epithelial cells from rat small intestine
IKKβ	inhibitory κB kinase β
Ile	Isoleucine
INS1E	Rat insulinoma cell lines
IPEC-J2	porcine intestine epithelial cell line
IR	insulin receptor
IRES	internal ribosome entry sequence
IRS	Insulin receptor substrate
KATP	Adenosine-5-triphosphate - sensitive K+
KIC	Ketoisocaproate
KIC	α-ketoisocaproate
Kv	voltage-dependent K^+
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDM2	The murine double minute 2
MeAIB	α-methylaminoisobutyric acid
MEM	Minimum Essential Medium
Met	Methionine
MIN6	Mouse Insulinoma 6
MKP3	mitogen-activated protein kinase phosphatase 3
mLST8	mammalian lethal with sec18 protein 8
mSin1	mammalian stress-activated protein kinase (SAPK)-interacting protein 1
mTOR	Mammalian target of rapamycin
NPY	neuropeptide Y
PA	phosphatidic acid
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	polymerase chain reaction

PDK1	phosphoinositide-dependant kinase 1
PDX-1	Pancreatic and duodenal homeobox gene-1
PGC	Peroxisome-proliferator-activated receptor coactivator
PH	pleckstrin – homology
Phe	Phenylalanine
PI	Phosphoinositides
PI3K	Phosphoinositide-3 kinase
PIK	phoshatidylinositol kinase
PIKK	phosphatidylinositol kinase-related kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PLC	Phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PRAS40	proline-rich Akt substrate 40 kDa
Pro	Proline
protor	protein observed with rictor
PRR5	proline-rich protein 5
PTB	phosphotyrosine – binding
PtdIns	Phosphatidylinositol
PVDF	Polyvinylidine fluoride
raptor	regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
rictor	rapamycin independent companion of mTOR
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
rpS6	ribosomal protein S6
S6K1	p70 S6 kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SGK1	serum- and glucocorticoid-induced protein kinase 1
SH2	Src - homology-2
SLC1A5	Solute carrier family 1 member 5
SNAREs	soluble N - ethylmaleimide - sensitive factor attachment protein receptor
SNAT	The sodium-coupled neutral amino acid transporters
STAT3	Signal transducer and activator of transcription 3
TCA	tricarboxylic acid cycle
TCTP	The translationally controlled tumour protein
Thr	Threonine
tmACs	transmembrane adenylyl cyclises
TNFα	tumor necrosis factor α
Trp	Tryptophan
TSC	Tuberous sclerosis
Tyr	Tyrosine
Val	Valine
VIP	Vasoactive intestinal polypeptide
Vps34	vacuolar protein-sorting defective 34
WHO	the World Health Organization
α-MHC	α-myosin heavy chain

Contents

CHAPTER 1

Chapter 1 General Introduction

1. Introduction

1.1. Diabetes Mellitus

Diabetes Mellitus is a chronic metabolic disorder which is characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism. It results from impaired insulin secretion, insulin action, or both. The classical clinical symptoms of diabetes include thirst, polyuria, blurring of vision and weight loss (Association, 2012). In most cases, these symptoms are mild or absent and mild hyperglycaemia persists with tissue damage developing while the person is asymptomatic. A definite diagnosis can be assumed if the venous plasma glucose level is greater than 11.1 mmol/L (200 mg/dL) and the fasting plasma glucose is greater than 7 mmol/L (126 mg/ml) (WHO, 2006). It is estimated that the world prevalence of diabetes reached 6.4% among adults (aged 20-79) to affect 285 million in 2010 and it is expected to rise to 7.7% to affect 439 million adults by 2030 (Shaw et al., 2009). Diabetes is associated with the development of macrovascular involving cardiovascular diseases and microvascular complications. The latter involves retinopathy which can lead to blindness, nephropathy with potential renal failure and neuropathy with risk of foot ulcers and amputations in addition to autonomic nerve dysfunction (Association, 2012).

According to the new classification suggested by WHO Expert Committee on Diabetes Mellitus and the American Diabetes Association, diabetes can be classified into Type 1 diabetes, Type 2 diabetes, gestational diabetes and other specific types Table 1.1 (Association, 2012) . Type 1 diabetes is caused by β-cell destruction which results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas thus insulin treatment is essential for survival. This form of diabetes encompasses 5-10% of those suffering from diabetes. Immune-mediated type 1 diabetes is characterised by loss of β-cell function caused by autoimmune destruction of pancreatic β-cells. On the other hand, some forms of type 1 diabetes have no etiologies and they are known as Idiopathic Diabetes.

Table 1.1 Etiologic classifications of disorders of glycaemia.

Types	Description and Subtypes
Type 1	β-cell destruction, usually leading to absolute insulin deficiency. ➢ Autoimmune diabetes mellitus ➢ Idiopathic
Type 2	Ranging from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance
Other specific types	➢ Genetic defects of β-cell function ➢ Genetic defects in insulin action ➢ Diseases of the exocrine pancreas ➢ Endocrinopathies ➢ Drug- or chemical-induced ➢ Infections ➢ Uncommon forms of immune-mediated diabetes ➢ Other genetic syndromes sometimes associated with diabetes
Gestational diabetes	Carbohydrate intolerance resulting in hyperglycaemia of variable severity first detected during pregnancy

Type 2 is the most common form of diabetes as it accounts for 90%-95% of diabetic cases globally. This class of diabetes is characterized by insulin resistance and usually relative insulin deficiency (i.e patients with Type 2 diabetes are able to secrete insulin but it is not enough to overcome insulin resistance). In addition to the insulin deficiency, there are other causes contributing to insulin resistance and hence leads to the development of type 2 diabetes including abnormalities in other hormones such as reduced secretion of the incretin glucagon-like peptide 1 (GLP-1), hyperglucagonaemia, and raised concentrations of other counter-regulatory hormones (Figure 1.1) (Tahrani et al., 2011). Typically, those patients do not require insulin treatment for survival but eventually they require insulin to maintain proper glycemic control. To date, the molecular mechanisms underlying the development of type 2 diabetes are not clear. The most promising finding until now has been the identification of TCF7L2 gene as a possible candidate in the pathogenesis of type 2 diabetes (Grant et al., 2006).

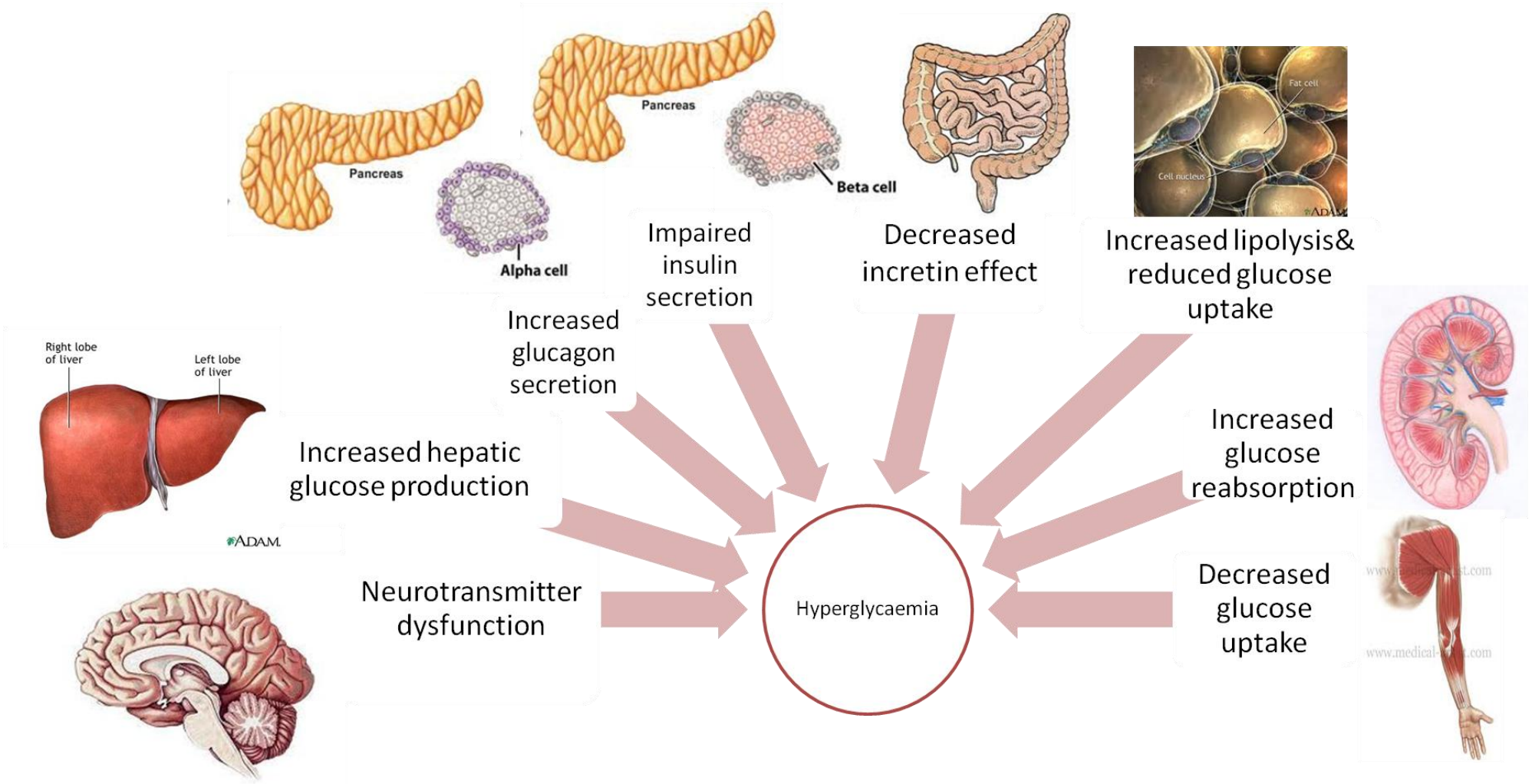


Figure 1.1. Typical pathogenic features of hyperglycaemia in type 2 diabetic patients

It is obvious that Type 2 diabetes is tightly linked to obesity, particularly visceral adiposity, physical inactivity and the westernization of lifestyles. The incidence of this form of diabetes also increases with age, which may be related to decrease in exercise and muscle mass. Both obesity and lack of physical activity cause insulin resistance which will result in diabetes in those with only a small capacity to increase insulin secretion. Weight loss and pharmacological treatments may help in overcome the insulin resistance (Association, 2012) .

1.1.1. Management of Type 2 diabetes

It has been revealed that tight blood glucose control can delay or prevent the development and progression of complications of diabetes. Thus, intensive blood glucose control, with a target of HbA1c level <7, for the management of diabetes has been recommended as well as reduction of potential risk factors such as hypertension, obesity and dyslipidaemia (Blonde, 2009). The initial approach after diagnosis of type 2 diabetes is to try non-pharmacological interventions involving modifications of life style including diet, exercise, smoking cessation, reducing alcohol intake and weight reduction, which are expected to enhance insulin sensitivity and improve glycaemic control. Pharmacological treatment for type 2 diabetes includes oral hypoglycaemic agents (Table 1.2) and insulin injection. The mechanism of action of oral hypoglycaemic agents to reduce plasma glucose levels is via increasing insulin secretion; reducing insulin resistance or; delaying glucose absorption by the gut (Blonde, 2009). In many cases monotherapy gradually fails to improve blood glucose control and combination therapy is required. The long-term success of these treatments varies substantially and when blood glucose control by oral hypoglycaemic agents become inadequate (HbA1c > 7.5) insulin therapy is introduced. However, intensive insulin therapy is associated with potential adverse effects including hypoglycaemia, weight gain, and increase in cardiovascular risk factors, such as hypertension and dyslipidaemia, and increased risk of colorectal cancer (Chiasson, 2009). Thus, there is an imperative need for novel therapeutic strategies for glycaemic control that can complement existing therapies and possibly attempt to preserve normal physiological response to meal intake. One such approach is based on the action of the incretin hormone; GLP-1.

Table 1.2 Summary of available hypoglycaemic agents

Drugs	Mechanism of action	Dosing	Advantages	Disadvantages
Sulphonylureas Gliclazide Glipizide Glimepiride Glibenclamide	bind to sulphonylurea receptor 1, resulting in depolarization and calcium influx that initiates insulin secretion	Once or twice a day	• Long-term safety • Low cost	• Hypoglycaemia • Weight gain • Possible need for self-monitoring • blood glucose
Biguanide Metformin	decreases hepatic glucose output Increases insulin sensitivity in muscle Interferes with glucose and lactate metabolism in the gut Might increase concentrations of endogenous GLP-1	Once or twice a day	• Long-term safety • Weight neutral • Low risk of hypoglycaemia • Low cost	• Gastrointestinal side-effects • Possible link to lactic acidosis • Avoid in deteriorating renal function or hypoxaemic states
Meglitinides Nateglinide Repaglinide	bind to sulphonylurea receptor 1 on the β-cell, but at a different site to sulphonylureas, resulting in a more rapid and shorter insulin response	With each meal	• Rapid, short acting • Suitable for prandial use	• Few data for long-term safety • Weight gain • Hypoglycaemia • Self-monitoring of blood glucose
α-glucosidase inhibitors Acarbose Miglitol	Inhibit carbohydrate degradation in gut	Up to three times a day	• Weight neutral • Low cost	• Gastrointestinal side-effects
Thiazolidinedione Pioglitazone Rosiglitazone	Peroxisome-proliferator activated-receptor-γ agonists act primarily in the adipose tissue to increase SC adipogenesis and reduce release of free fatty acids Increase insulin sensitivity in muscle and liver	Once a day	• Low risk of hypoglycaemia • Might reduce blood pressure	• Long-term safety not established • Risk of weight gain, oedema heart failure, and fractures

Glucagon-like peptide-1 mimetics Exenatide Liraglutide	Binds to GLP-1 receptor, causing increased glucose-dependent insulin secretion and glucagon suppression, delayed gastric emptying, and appetite suppression	Once or twice a day	• Weight loss • Low risk of hypoglycaemia • Possible effect on β-cell survival	• Long-term safety not known • Unconfirmed association with pancreatitis and medullary cell carcinoma • Gastrointestinal side-effects Avoid in renal failure
Dipeptidyl peptidase- IV inhibitors Sitagliptin Saxaglitpin	Increase endogenous incretin concentrations	Once a day	Weight neutral Low risk of hypoglycaemia Possible effect on β-cell survival and decline	• Long-term safety not known • Unconfirmed association with pancreatitis
Amylin analogue Pramlintide	A synthetic soluble analogue of human amylin, lowers postprandial glucose by centrally mediated satiety, suppressing postprandial glucagon secretion, and delaying gastric emptying	Three times a day	Weight loss	• Unknown long-term safety • Increases the risk of insulin-induced hypoglycaemia
Insulin	Directly activate the insulin receptor, decrease hepatic glucose output, increase peripheral use, and reduce lipolysis	Once to four times a day	More sustained glycaemic improvements compared with other drugs	• Weight gain • Hypoglycaemia • Need for self-monitoring of blood glucose • Fluid retention

Table adapted from (Tahrani et al., 2011).

1.1.2. Incretins in the treatment of type 2 diabetes

Incretins are gastrointestinal hormones that were found to have hypoglycaemic effects and were originally extracted from the intestine mucosa. The " incretin" concept was developed when it was demonstrated that oral administration of glucose promotes a threefold increase of plasma insulin compared to intravenously glucose infusion, despite the same plasma glucose response (McIntyre et al., 1964). The major incretins are glucose-dependent insulinotropic polypeptide (GIP) and GLP-1. Together, GIP and GLP-1 are believed to be responsible for approximately 70% of the insulin seceretion from the β-cells of the islets of Langerhans after meal intake. It was demonstrated that Type 2 diabetic patients have an impaired incretin effect, indicating a role for incretin hormones or their actions in the pathogenesis of type 2 diabetes (Figure 1.1) (Knop et al., 2007).

GIP is a 42-amino acid hormone and released from K-cells, which are enterochromaffin cells of the proximal small intestine (enteroendocrine duodenal and jejunal mucosa). The release of GIP is stimulated by enteral glucose in a concentration-dependent manner (Schirra et al., 1996). However, GIP has not been considered a good candidate for development for the therapy of type 2 diabetes since (i) the concentration of GIP in type 2 diabetic patients is normal or slightly increased after meal intake and (ii) GIP infusion does not reduce plasma glucose concentration in type 2 diabetic patients (Holst and Orskov, 2004). In contrast, GLP-1 was found to stimulate glucose dependent insulin secretion under conditions of hyperglycaemia in type 2 diabetic subjects (Holst et al., 2009). In addition, exogenous GLP-1 administration leads to improvement of glycaemic control in patients with type 2 diabetes (Ratner et al., 2010). Therefore, GLP-1 based therapies appear an interesting approach to address some components of the complex pathophysiology of type 2 diabetes.

1.2. Glucagon-like Peptide 1

GLP-1 is an incretin hormone secreted by the L cells of the distal ileum and colon in response to nutrient intake. It is a C-terminally amidated 30 amino acid peptide, which is processed from proglucagon. Although multiple immunoreactive forms of

GLP-1 are present *in vivo*, only two forms, GLP-$1_{(7\text{-}36)\ amide}$ (most predominant form) and GLP-$1_{(7\text{-}37)}$, have been shown to possess a biological activity. *In vivo*, GLP-1 is tremendously susceptible to the catalytic activity of the enzyme dipeptidyl peptidase IV (DDP-IV), which removes the two NH_2-terminal amino acids. This results in the generation of inactive metabolites; GLP-$1_{(9\text{–}36)\ amide}$ or GLP-$1_{(9\ \text{–}37)}$ (Holst, 2007).

The enzyme DPP-IV is widely expressed and can be found in multiple tissues such as central nervous system, kidney, lung, adrenal gland, liver, intestine, spleen, testis, and pancreas, as well as on the surface of lymphocytes and macrophages. DPP-IV also is found on the surface of endothelial cells, including those lining blood vessels that drain the intestinal mucosa, which are positioned directly adjacent to the sites of GLP-1 secretion (Hansen et al., 1999). Thus, most GLP-1 secreted from the intestine is already inactivated by DPP-IV upon entry into the capillaries, suggesting that GLP-1 may exert part of its effect before it enters the capillaries. Apart from this effect, it is likely that the activity of endogenous GLP-1 is also exerted through interaction with sensory afferent nerve fibres relaying in the brain and modulating efferent vagal fibers (Holst, 2007) that, in turn, regulate a number of its biological actions as described below.

1.2.1. Actions of Glucagon-like Peptide 1

GLP-1 effects are mediated via the interaction with the GLP-1 receptor, which is a 463-amino-acid member of class 2 G protein-coupled receptor (GPCR). The GLP-1 receptor is widely distributed in pancreas, heart, brain, kidney and the gastrointestinal tract.

Figure 1.2 presents the main physiological actions of GLP-1. One of the most important effects of GLP-1 is to act as incretin hormone. GLP-1 was found to stimulate insulin secretion in a glucose-dependent manner. Furthermore, it inhibits glucagon secretion and stimulates somatostatin secretion (Orskov et al., 1988). Thus, it can be concluded that GLP-1 plays an important role in postprandial insulin secretion and is essential for normal glucose tolerance. Moreover, GLP-1 stimulates insulin gene expression and insulin synthesis (Fehmann and Habener, 1992).

Further important effects of GLP-1 include inhibition of gastric emptying time and gastric acid and pancreatic exocrine secretion. These effects of GLP-1 on gastric function might be mediated by vagal pathways (Wettergren et al., 1994). Another physiological function of GLP-1 is its central action on the hypothalamic nuclei involved in the regulation of eating behaviour. GLP-1 infusion in healthy subjects and type 2 diabetic patients suppresses appetite, improved satiety and food intake, inducing weight loss (Flint et al., 1998; Gutzwiller et al., 1999) .

It is well established that there are GLP-1 receptors in the heart (Bullock et al., 1996), thus GLP-1 is likely to have physiological effects on the heart. In the basal state, GLP-1 may have a negative ionotropic effect, but after cardiac injury GLP-1 has constantly increased myocardial performance both in experimental animals (Zhao et al., 2006) and in patients (Nikolaidis et al., 2004). GLP-1 stimulates insulin secretion and may enhance cardiac functions via the combined effects of enhanced insulin secretion and action (Zhao et al., 2006)

GLP-1 may also possess neurotropic effects. It was demonstrated that intracerebroventricular administration of GLP-1 was associated with enhanced learning and memory in rats and also showed neuroprotective effects (During et al., 2003). Moreover, GLP-1 has been proposed as a potential therapeutic tool for neurodegenerative diseases, such as Alzheimer's disease (Li, 2007).

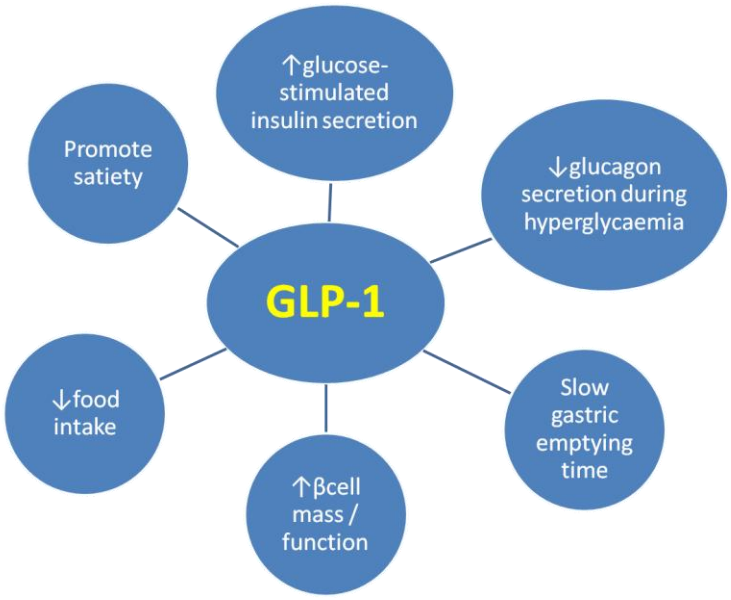


Figure 1 .2 Physiological actions of GLP-1

GLP-1 in the pancreas: insulin secretion

The beta cell is electrically excitable and its resting membrane potential is mainly controlled by the adenosine-5-triphosphate (ATP) - sensitive K^+ (K_{ATP}) channels. Under resting conditions, the K_{ATP} channels are spontaneously open and permit efflux of positively charged K^+ ions, thus maintain the membrane potential at approximately -70mV. Glucose acts as the triggering molecule when it is taken up into the cell through the glucose transporter 2 (GLUT2). When glucose is metabolized through glycolysis and in the Krebs cycle, it raises the intracellular energy levels by increasing the ATP/ADP ratio. This results in closure of ATP-sensitive K^+ channels and membrane depolarization, which opens the voltage-gated Ca^{+2} channels to increase the influx of Ca^{+2}. Elevation of intracellular Ca^{+2} elicits insulin secretion (Gromada et al., 2004) (Figure1.3).

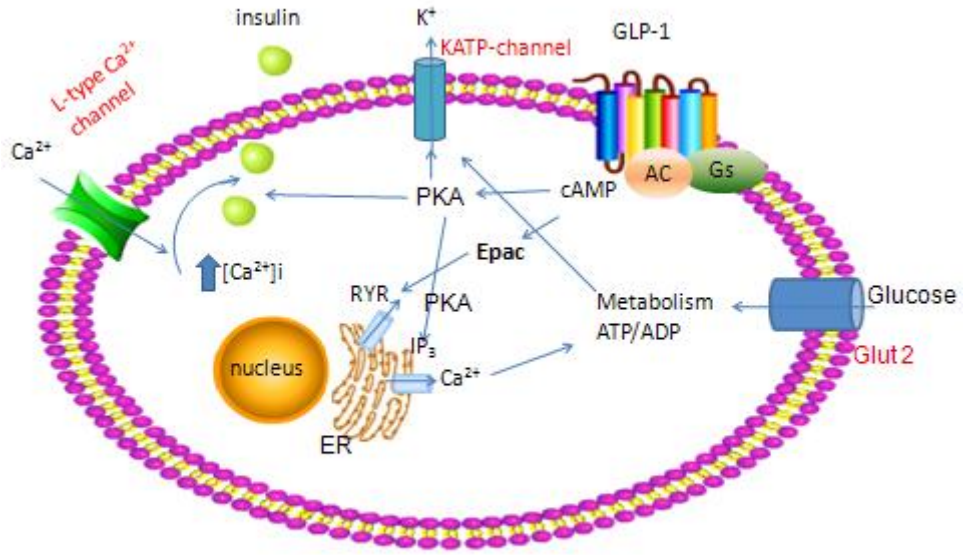


Figure 1.3 . Summary of the cellular actions of GLP-1 that lead to insulin secretion.

Binding of GLP-1 to the receptor enables coupling with an intracellular heterotrimer Gs protein. Thus, the stimulatory G protein activates adenylate cyclase resulting in the formation of cAMP, with subsequent activation of protein kinase A (PKA) and the Epac family of cAMP-regulated guanine nucleotide exchange factors, both of which have multiple downstream effectors. GLP-1 triggers glucose-dependent insulin secretion and Ca^{+2} signalling via both PKA and Epac as discussed below.

Firstly, GLP-1 augments the glucose effect to close ATP-sensitive K^+ channels. These channels are sensitive to the glucose metabolism of the beta cells since they are affected by the intracellular levels of ATP. In addition, they may also be closed by GLP-1-induced activation of PKA resulting in consequent depolarization of the plasma membrane and opening of voltage-sensitive calcium channels (Gromada et al., 1998). Besides PKA-dependent closure of K_{ATP}-channels, GLP-1 was demonstrated to cause a slight increase in the ATP sensitivity of the K_{ATP}-channel and subsequently an increased responsiveness of channel closure (Suga et al., 2000).

Secondly, GLP-1 receptor activation can regulate the activity of voltage-dependent K^+ (Kv) channels in pancreatic β-cells. Kv channels can control the electric activity of the beta cell as they open in response to glucose induced membrane depolarisation, thus promote membrane repolarisation by restoring the efflux of K^+ (Ashcroft and Rorsman, 1989). Antagonism of Kv currents in rat β-cells by GLP-1 has been reported (MacDonald et al., 2002), which results in prolongation of the interval of action potentials and strengthening excitability. Activation of both the cAMP/PKA and Phosphoinositide -3- kinase (PI3K)/PKCζ signalling pathways mediates GLP-1 effect on Kv channels (MacDonald et al., 2003).

Inhibition of the K_{ATP}- and Kv-channels by GLP-1 promotes the depolarisation of β-cell membrane and consequently activates the voltage-gated Ca^{+2}- channels with resulting Ca^{+2} influx and initiation of Ca^{+2}- dependent insulin exocytosis (Gromada et al., 2004).

Thirdly, Ca^{+2} influx through the Ca^{+2} channels in the presence of GLP-1 and a stimulatory concentration of glucose prompts the mobilization of Ca^{+2} from intracellular stores by Ca^{+2}- induced Ca^{+2} release (Gromada et al., 2004). Moreover, GLP-1 induces Ca^{+2} release from Ins $(1,4,5)P_3$ – sensitive Ca^{+2} store via a PKA-mediated event and from Ryanodine receptors via Epac. The mobilization of Ca^{+2} from intracellular stores promotes mitochondrial ATP synthesis, which results in more membrane depolarisation via ATP-dependent closure of K_{ATP} channels. In addition, ATP is required for the exocytosis of insulin containing secretory granules (Gromada et al., 2004).

All these multiple effects of GLP-1 mediated through activation of PKA and Epac account for the ability of GLP-1 to stimulate insulin secretion from pancreatic beta cell.

GLP-1 in the pancreas: β-cell mass

Several studies have indicated that GLP-1 and its analogues directly stimulate β-cell growth and proliferation to promote an increase in islets mass (Egan et al., 2003). In addition, there is some evidence that GLP-1 inhibits β-cell apoptosis (Li et al., 2005),

a further action that would promote expansion of β-cell mass. Together, these findings suggest that GLP-1 receptor agonists have potential implications in diabetes since β-cell loss is thought to play a crucial role in the development and progression of diabetes.

The intracellular events involved in the effects of GLP-1 on β-cell growth and apoptosis is currently a topic of intensive investigation. The proliferative effects of GLP-1 involves multiple signalling pathways including phosphoinositide-3 kinase (PI3K), protein kinase B (PKB), mitogen-activated protein kinase (MAPK) and protein kinase Cζ (Brubaker and Drucker, 2004) . For example, it was shown that GLP-1 stimulates proliferation in pancreatic β-cells and protects against staurosporine-induced cell death via PI3K/PKB dependent pathway (Wang et al., 2004). Another study (Buteau et al., 2006) pointed to the role of the transcription factor FoxO1 in mediating the proliferative and anti-apoptotic actions of GLP-1. It was shown in this study that GLP-1 inhibits the forkhead transcription factor FoxO1 through PKB-mediated nuclear exclusion. A study in INS 832/13 cells revealed that increased expression of dominant negative protein kinase Cζ blocked GLP-1- induced proliferation (Buteau et al., 2001). In addition, it was shown by the same researchers that inhibition of epidermal growth factor receptor (EGFR) transactivation either by the EGFR-specific inhibitor AG1478 or over-expression of a dominant negative EGFR significantly reduced GLP-1 β-cell proliferation in INS 832/13 (Buteau et al., 2003).

Several studies have shown that GLP-1 receptor signalling improves cell survival by inhibiting apoptosis of human and rodent β-cells exposed to high levels of glucose (Farilla et al., 2002) , inflammatory cytokines (Li et al., 2005) and free fatty acids (Kwon et al., 2004b). It was shown that exendin-4 mediates beta cell regeneration in streptozotocin-induced diabetic mice through upregulation of insulin receptor-2 (IRS-2) and nuclear exclusion of FoxO1 and hence increase pancreatic and duodenal homeobox gene-1 (PDX-1) expression (Kodama et al., 2005).

The antiapoptotic actions of GLP-1R agonists have been demonstrated by many researchers as evident by the decrease in the levels of proapoptotic proteins such as active caspase 3 (Farilla et al., 2002), as well as upregulation of prosurvival factors

such as Bcl-2 and inhibitor of apoptosis protein-2 (IAP-2) (Buteau et al., 2004). Elucidation of the molecular mechanism by which GLP-1 inhibits β-cell apoptosis has revealed that it may be mediated through activation of cAMP/ PKA signalling pathway with consequent phosphorylation and activation of cAMP response element binding protein (CREB), leading to activation of IRS-2 and activation of PKB pathway (Jhala et al., 2003).

Furthermore, Exendin-4, a potent glucagon-like peptide 1 agonist, was found to attenuate endoplasmic reticulum (ER) stress in pancreatic β-cells (Tsunekawa et al., 2007). It was demonstrated that diabetes is marked with the development of ER stress in β-cells (Yusta et al., 2006) and that GLP-1R signalling reduces ER stress via activation of PKA and induction of ATF4, an ER stress marker, translation in db/db diabetic mice (Yusta et al., 2006). Since ER stress may be implicated in β-cell dysfunction and death (Marchetti et al., 2007), these results hint towards a potential therapeutic value of GLP-1 for the treatment of diabetes.

1.2.2. GLP-1 Signalling in Pancreatic β-Cells

Activation of GPCR by GLP-1 induces a rapid elevation of cAMP via stimulation of transmembrane adenylyl cyclases (tmACs) (Ramos et al., 2008). cAMP is an important mediator of GLP-1 action on acute molecular events regulating β-cell function since it activates further signalling pathways, the two most significant ones being cAMP-dependent PKA and Epac. Both of them are essential for insulin exocytosis as described before. Moreover, PKA activates the transcription factor cAMP response element-binding protein (CREB) in β-cells, which is an essential transcription factor for glucose homeostasis and β-cell survival (Jhala et al., 2003).

In addition to the classical GPCR signalling mechanisms, it has been shown that GLP-1 can activate various signalling proteins (extracellular regulated kinase (Erk), protein kinase Cζ, PI 3-kinase and PKB) Figure 1.4.

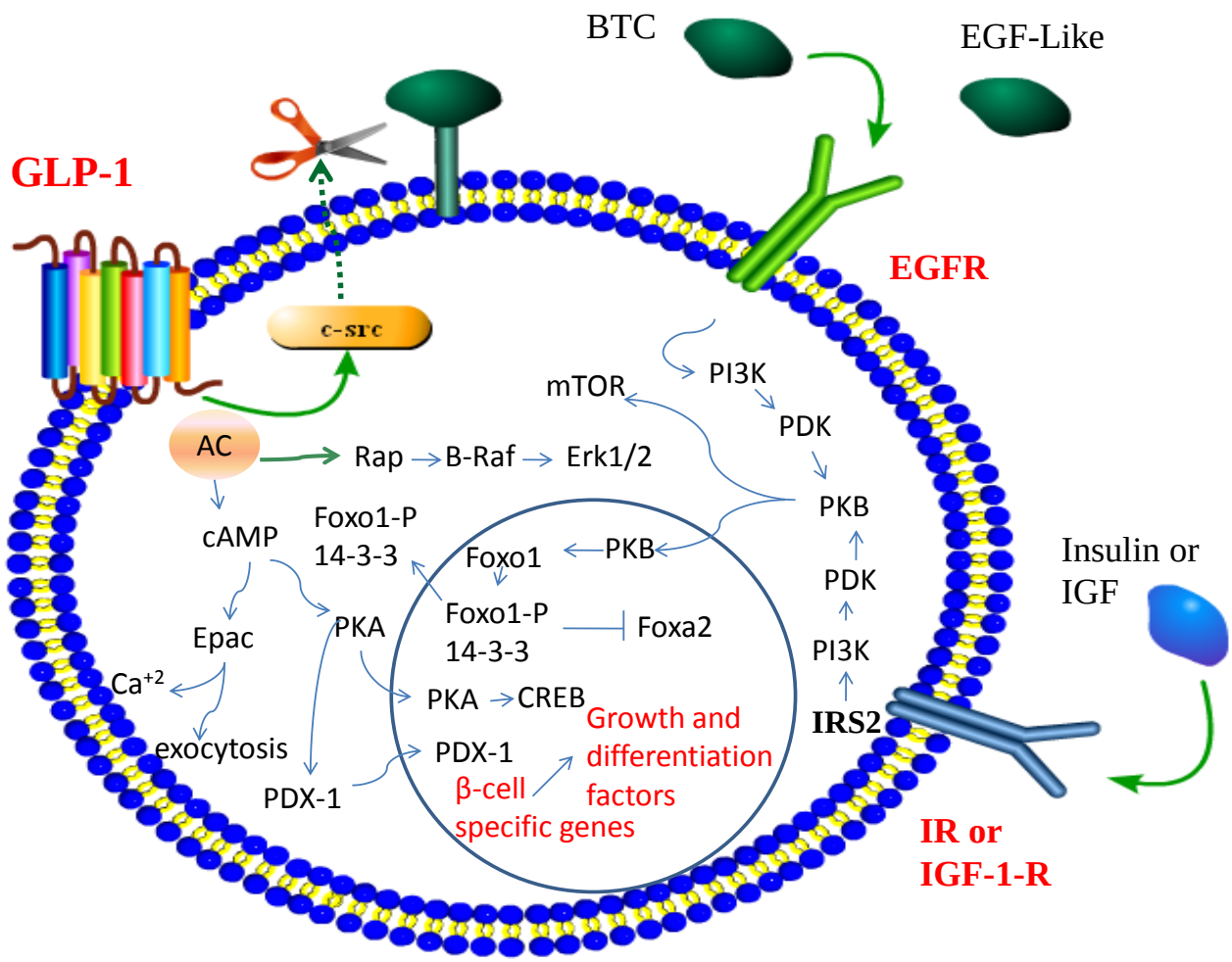


Figure 1.4. GLP-1 signalling in pancreatic β-cells

In MIN6 cells, GLP-1 was shown to induce rapid phosphorylation of Erk 1/2 in a glucose-dependent manner through a mechanism dependent on PKA and influx of extracellular calcium through L-type voltage-gated calcium channels (Gomez et al., 2002). Similarly, it was reported that GLP-1-potentiated glucose-induced Erk 1/2 phosphorylation in another pancreatic beta-cell line, INS1 cells, is mediated through cAMP/ PKA signalling pathway (Briaud et al., 2003). However, a study done using human islets revealed that glucose and GLP-1 promote the binding of Rap, which is activated by Epac, to B-Raf, which in turn mediates Erk activation (Trumper et al., 2005).

Binding of GLP-1 to its receptor activates PKA and results in an increase in the intracellular Ca^{+2} through its release from IP_3 sensitive Ca^{+2} channels. It has been proposed that this elevation of intracellular Ca^{+2} activates phospholipase C (PLC)

and subsequently results in degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3 (Suzuki et al., 2006). Consequently, DAG induces the translocation of PKCα and PKCε from the cytosol to the plasma membrane, a marker for PKC activation (Suzuki et al., 2006). Thus, it can be concluded from this study that GLP-1-evoked Ca^{+2} signal via PLC activation mediates the activation of PKCα and PKCε in INS1 cells.

Another study demonstrated that GLP-1 treatment of INS(832/13) cells activates PKCζ, an atypical isoform, indicated by its translocation from the cytoplasm to the nucleus (Buteau et al., 2001). The nuclear translocation of PKCζ is suggested to play a role in the proliferative effect of GLP-1 since over expression of dominant negative form of PKCζ abolished GLP-1 induced proliferation.

1.3. Islet Structure and Function

1.3.1. Islet Anatomy

A typical mammalian islet is composed of four main types of cells: the insulin-expressing β- cells (≈ 70-80% of adult human islet cells), glucagon-expressing α-cells (15-20%), somatostatin- expressing δ-cells (≈ 5%) and pancreatic polypeptide -expressing cells (< 5%) distributed all over the exocrine portion of pancreas (Kulkarni, 2004). Islets account for 2-3% of the total pancreatic mass and are organized into small spherical clusters of cells called islet of Langerhans. The islets have usually oval shape (Kulkarni, 2004) . They are highly vascularised as they receive ≈ 15% of the total pancreatic blood supply (Ballian and Brunicardi, 2007). In addition, islets are well supplied by autonomic nerve fibres (parasympathetic nerves, sympathetic nerves and sensory nerves) and terminals containing the classic neurotransmitters acetylcholine and norepinephrine, along with a variety of biologically active neuropeptides (Ahren, 2000).

The pancreatic islets are functionally coupled through a network of gap junctions made up of assemblies of transmembrane proteins called connexins (Cx) with evidence linking Cx36 in the regulation of insulin biosynthesis and secretion (Serre-Beinier et al., 2009). Furthermore, cell-cell communication through cell adhesion

molecule such as E-cadherin (Carvell et al., 2007) and ephrins(Konstantinova et al., 2007) have been shown to play a crucial role in modulating islet function.

Biologically active substances released from one islet may influence the functional status of adjacent cells through paracrine effect or of itself through autocrine effect. For example, it has been shown in human islets that insulin can exert an autocrine effect through acting on its own receptor to increase insulin gene expression and to maintain β-cell mass through both a proliferative and an anti-apoptotic effect (Persaud et al., 2008).

1.3.2. Islet Function

1.3.2.1. Insulin Biosynthesis

The main function of β-cell is to secrete sufficient amount of insulin in response to metabolic demand to maintain the plasma glucose level within the physiological range. The insulin molecule is made up of two polypeptide chains (A and B) which are connected by two intrachain disulphide bonds in addition to another intramolecular disulfide bond in the A chain. These two chains of insulin molecule are not integrated from separate polypeptide chains but they are generated by the action of some proteolytic enzymes from a single polypeptide, proinsulin. The C-peptide joins the A and B chains of insulin in the proinsulin so that the molecule is correctly folded for cleavage (Steiner et al., 2009). Proinsulin is initially translated as preproinsulin which is rapidly cleaved into proinsulin within the endoplasmic reticulum. Proinsulin is then transferred in microvesicles to the Golgi apparatus where it is converted to insulin by the action of two endopeptidases (prohormone convertases 2 and 3) and carboxypeptidases E. Insulin and C-peptide are stored together in the secretory granules and released in equimolar amounts during exocytosis (Hutton, 1994).

1.3.2.2. Insulin exocytosis

The major components of insulin storage granule are insulin and C peptide, along with zinc, calcium, adenine nucleotides, biogenic amines and some enzymes such as proinsulin converting enzymes, acid phospatase and protein kinases (Howell, 1984). Insulin is released from their storage sites within the cells by exocytosis, a

process in which the granule membrane and the plasma membrane fuse together, releasing the granule content into the interstitial space. This process is mediated via the formation of multimeric complex of proteins known as SNAp Receptors (SNAREs) [soluble N - ethylmaleimide - sensitive factor attachment protein receptor] complex, which consists of proteins associated with secretory granules and the plasma membrane, and soluble fusion proteins (Easom, 2000). The SNARE proteins are responsible only for fusion of docked granules with the membrane. However, the release of granules in the presence of elevated levels of intracellular calcium is sensed by synaptotagmins, a class of calcium - binding granule proteins (Rorsman and Renstrom, 2003). The secretory granules in beta cells can be differentiated according their release competence into: a) the readily release pool of granules (RRP) which constitutes 1-5% and are immediately available for release thus the exocytosis of these granules accounts for the rapid first-phase release of insulin response to glucose stimulation, b) the non-releasable pool which represents the majority of the granules (95-99%) and the discharge of these granules is ATP-, Ca^{+2}, time- and temperature dependent and accounts for second-phase insulin secretion (Rorsman and Renstrom, 2003).

1.3.2.3. Regulation of insulin secretion

The regulation of insulin secretion is critical for the ability of the β-cell to finely control body glucose homeostasis. The primary physiological stimulator of insulin secretion is the circulating concentration of glucose and other nutrients, including amino acids and fatty acids. Glucose is transported into β–cells via high capacity glucose transporters (GLUT; GLUT2 in rodents, GLUT1, 2 and 3 in humans). When glucose is metabolized through glycolysis and in the Krebs cycle, it raises the intracellular energy levels by increasing the ATP/ADP ratio. This results in closure of ATP-sensitive K^+ channels and membrane depolarization, which opens the voltage-gated Ca^{+2} channels to increase the influx of Ca^{+2}. Elevation of intracellular Ca^{+2} elicits insulin secretion (Gromada et al., 2004).

Several amino acids were found to stimulate insulin secretion *in vivo* and *in vitro* (Newsholme et al., 2007). Most of these amino acids require glucose to mediate insulin secretion; however some amino acids such as leucine, lysine and arginine

can stimulate insulin secretion in the absence of glucose and thus can be considered as initiators of secretion. Leucine crosses β-cells by System L transporter and stimulates a biphasic increase in insulin release by acting as a metabolic fuel thus its metabolism to α-ketoisocaproate (KIC) decreases the potassium permeability, causing depolarization and activation of L-type calcium channels through which calcium enters the pancreatic β-cells that then triggers insulin secretory granules exocytosis (Yang et al., 2010). Leucine is also able to activate the amplifying pathway of insulin secretion by acting as an allosteric activator of glutamate dehydrogenase (GDH), a crucial enzyme controlling the oxidation of glutamate to enhance glutaminolysis (Yang et al., 2010). The charged amino acids such as lysine and arginine enter the β-cell plasma membrane via the specific transport system. It is usually assumed that the accumulation of these charged molecules can directly depolarize the pancreatic β-cell membrane leading to calcium influx and subsequently insulin release (Newsholme et al., 2007).

The pancreatic β-cells can respond to non-nutrient potentiators of insulin secretion including a wide range of hormones and neurotransmitters. In most cases, these non-nutrient modulators act on β-cells by binding and activating specific receptors on the extracellular surface of cell membrane. Therefore, the β-cell expresses receptors for a variety of biological active peptides, glycoproteins and neurotransmitters. Glucagon is a 29 amino acid peptide secreted from the α-cells of the pancreas (Gromada et al., 2007). Post translational processing of proglucagon, the precursor of glucagon, gives rise to different peptides with different receptors and biological activities. These include GLP-1, an incretin hormone that was discussed before and GLP-2, which enhances the growth of the intestinal mucosa. Glucagon enhances insulin secretion through coupling with the stimulatory G-protein and the consequent activation of adenylate cyclase that result in the increase of cyclic AMP (Gromada et al., 2007).

Glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) is an amino acid polypeptide that is released in response to the absorption of glucose, amino acids and long- chain fatty acids (Karhunen et al., 2008). GIP binds to Gs-coupled receptors on the β-cell plasma membrane, stimulates adenylyl cyclase, elevates intracellular cAMP levels and increases intracellular calcium levels that

consequently results in an increase of insulin secretion. It is also reported that GIP can increase insulin release through generation of arachidonic acid (AA) via phospholipase A 2 activation (Ehses et al., 2001) and closure of K_{ATP} channels (Drucker, 2007).

Cholecystokinin (CCK) is an incretin hormone that is released from L-cells in the duodenal and jejunal mucosa of the small intestine in response to elevated fat and protein levels (Karhunen et al., 2008). It results from selective processing of the proCCK, a 115 amino acid precursor, to act as a ligand for the CCK receptor 1 (CCK1R) (Karhunen et al., 2008). Binding to CCK1R potentiates insulin and glucagon secretion via coupling to Gq, which activates phospholipase C (PLC) (Winzell and Ahren, 2007).

The pancreatic islets are richly innervated by autonomic nerves, with numerous neurotransmitters and neuropetides stored in vesicles in the nerve terminals (Ahren, 2000). Stimulation of the parasympathetic nerves promotes insulin secretion and it was demonstrated to be essential for the so-called cephalic phase, which takes place before the increase of plasma glucose levels following meal ingestion (Ahren and Holst, 2001). The terminals of the parasympathetic nerves contain acetylcholine (ACh), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and gastrin-releasing peptide (GRP). They induce insulin secretion via their coupling with GPCR (Ahren, 2000). There are three main neurotransmitters stored in the terminals of the sympathetic nerves; noradrenaline, galanin and neuropeptide Y (NPY). Stimulation of the sympathetic nerves blocks glucose-induced insulin secretion (Ahren, 2000).

1.3.3. Insulin action

Insulin exerts a wide range of anabolic effects in multiple tissues including skeletal muscle, adipose tissue and liver. The main action of insulin is to regulate whole body fuel homeostasis. Insulin promotes the uptake and storage of carbohydrates, fat and amino acids and at the same time antagonizing the degradation of these fuel reserves (Zierath et al., 2000). For example, insulin stimulates glucose transport in skeletal muscle cells and promotes glucose storage as glycogen. In liver, insulin

lowers glucose output by antagonizing glycogenolysis and gluconeogenesis, and augments glycogen formation. In adipose tissue, insulin induces glucose uptake and triglyceride formation and exerts an antilipolytic effect (Taniguchi et al., 2006). To perform its pleiotropic actions, insulin binds to cell surface receptor to stimulate multiple signal networks that control several biological functions. When insulin binds to its receptor, the insulin receptor undergoes a conformational change and triggers the tyrosine kinase activity of β subunit of insulin receptor leading to the autophosphorylation and phosphorylation of tyrosine residues on a number of substrates including: insulin receptor substrate 1 and 2 (IRS1 and IRS2). Consequently, these events are linked to the activation of the PI3K/PKB pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–MAPK pathway, which cooperates with the PI3K pathway to control cell growth and differentiation (Taniguchi et al., 2006).

The insulin receptor (IR) is a large tetrameric glycoprotein composed of two extracellular α subunits and two transmembrane β subunits (Vigneri et al., 2010). The human IR is synthesised from a single 22-exon gene that generate by alternative splicing two isoforms (IR-A and IR-B). The α subunit (130 kDa) is derived from the amino-terminal of the proreceptor and constitutes with 192 residues of the β chain the extracellular portion of the IR (Belfiore et al., 2009). The β subunit (95 kDa) contains a 403-residue intracellular domain with intrinsic tyrosine kinase activity. Insulin binds to the α-subunit of insulin receptor which results in conformational changes that bring the β subunits together. The modifications mediate the binding of ATP to the intracellular β subunit which results in the autophosphorylation of different tyrosine residues on the β subunits. This autophosphorylation potentiates the intrinsic activity of the β subunit as a tyrosine kinase, directed against several intracellular substrates, including IR substrates (IRS-1, -2, -3, and -4), IRS-5/DOK4, IRS-6/DOK5, Shc, Gab1, Cbl, associate protein substrate (APS), and the signal regulatory protein family members (Belfiore et al., 2009). Each of these IRS proteins has an N - terminal pleckstrin – homology (PH) domain, a phosphotyrosine - binding (PTB) domain and a COOH - terminal region of variable length that contains multiple tyrosine and serine phosphorylation sites (Virkamaki et al., 1999). The PH domain facilitates the coupling of the IRS to the insulin receptor through its binding to charged headgroups of certain phosphatidylinositides in adjacent membrane

structures. PTB domains recognize the phosphotyrosine in the amino acid sequence asparagine-proline- any amino acid- phosphotyrosine (NPXpY), which containsTyr^{972} in the juxtamembranous domain of the insulin receptor β - subunit, and assists the formation of the IRS – insulin receptor complex (Virkamaki et al., 1999). In addition, the tyrosine phosphorylated sites in the COOH-terminal, after phosphorylation by the IR, couple to other intracellular molecules that encompass Src - homology - 2 domains (SH2 domains). The SH2-containing proteins that couple to phosphorylated IRS proteins act as adaptor molecules, such as the regulatory subunit of PI3 kinase and the adaptor molecule Grb2, or enzymes with kinase or phosphatase activities such as SH2 - domain - containing tyrosine phosphatase 2 (SHP2) and the cytoplasmic tyrosine kinase Fyn (Virkamaki et al., 1999). Binding of SH2-containing signalling molecules to IRS promotes IRS to act as a docking protein so that it transmits insulin signal via the docking, interaction and activation of downstream signal molecules. Therefore, IRS acts as a major site of divergence of insulin signal transduction pathways, for example, leading to activation of PI3 Kinase and Ras/MAP kinase pathways (Figure 1.5) (Belfiore et al., 2009).

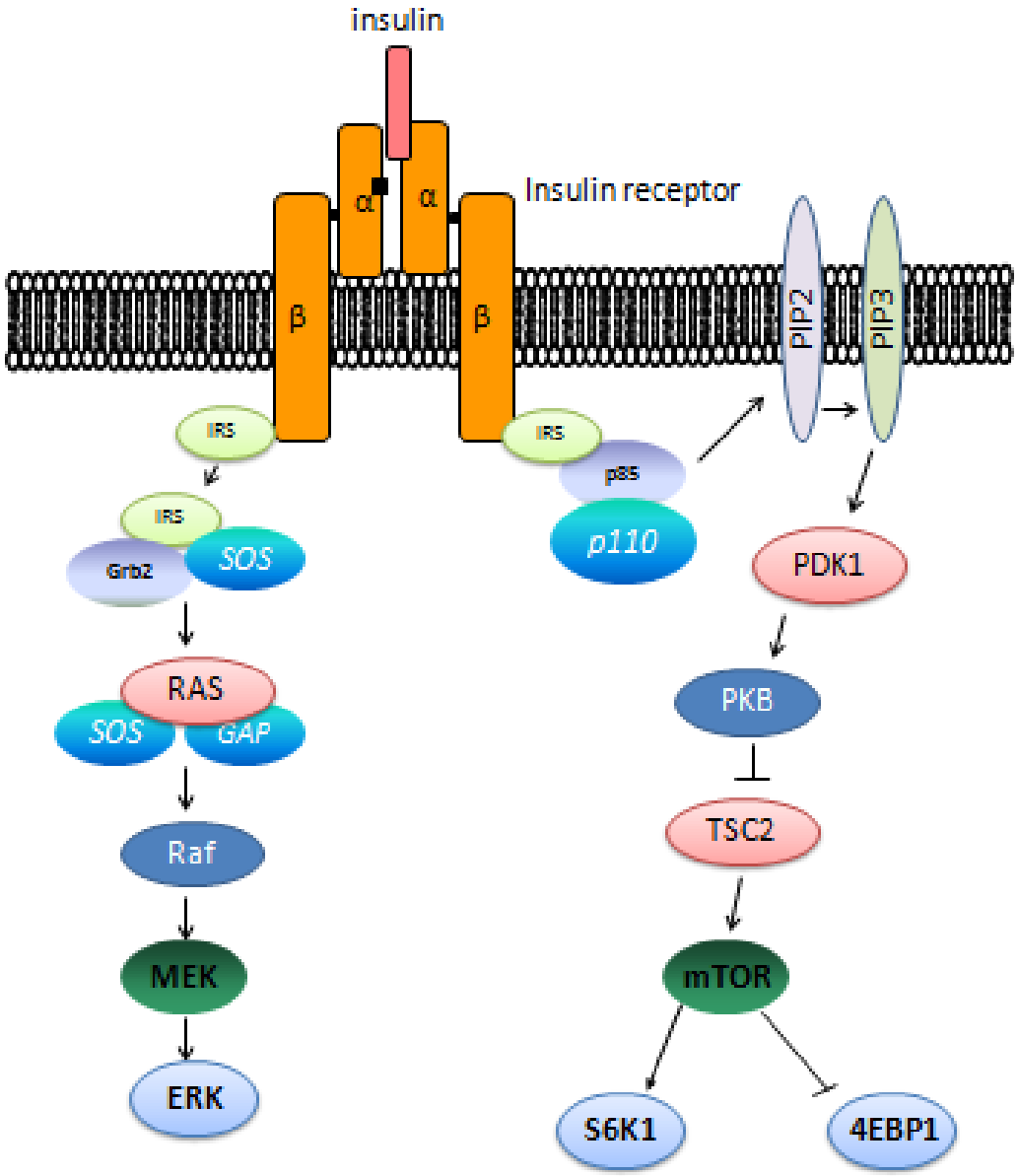


Figure 1.5. The insulin signalling pathway. This figure depicts the insulin signalling pathway, described, in the text in section 1. 2.3.

1.4. Phosphoinositide-3 Kinase (PI3K)

The Phosphoinositide-3-kinases are intracellular lipid kinases that phosphorylate 3-hydroxyl group of phosphatidylinositol (PtdIns) and phosphoinositides (PI). These products of PI3K act as second messengers leading to the activation of many intracellular signalling pathways. Consequently, this results in the mediation of cellular activities such as proliferation, differentiation, cell metabolism, survival and vesicle trafficking (Wymann and Pirola, 1998). They have been divided into three classes (I-III) according to their substrate specificity, structural features (sequence homology) and ways of activation.

1.4.1. Class I Phosphoinositide-3 Kinases

Class I PI3Ks are heterodimeric proteins comprising of a catalytic subunit 110 kDa (called p110) and an adaptor/regulatory subunit. The catalytic subunit processes an adaptor binding domain, a Ras binding domain, a C2 domain, a helical domain and a Kinase domain. *In vitro*, they are capable to utilise PtdIns, PtdIns(4)P, PtdIns(4,5)P_2 as substrates. *In vivo*, however, their preferred substrate appears to be PtdIns(4,5)P_2. Class I PI3Ks are further subdivided into class IA and class IB based on structural and functional difference (Figure 1.6) (Hawkins et al., 2006).

Class IA Phosphoinositide-3 Kinases

In mammals, three isoforms of the p110 catalytic subunit have been identified (p110α, p110β, p110δ). p110α and p110β are expressed in all tissues and are likely to play a major role in insulin signalling. Expression of p110δ is restricted to haematopoietic cells (Katso et al., 2001).

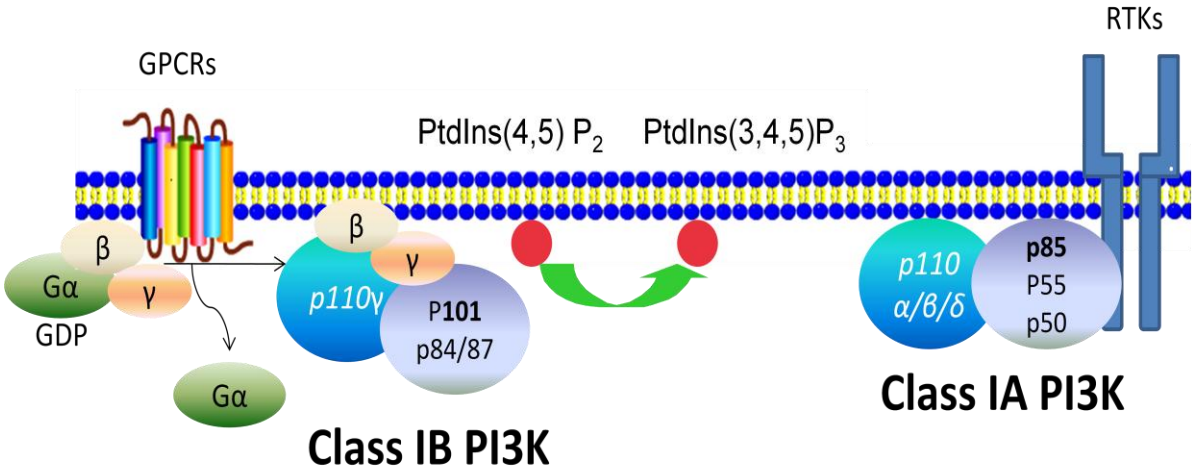


Figure 1.6. Mechanisms of class I phosphatidylinositol-3-kinase activation. Class I PI3K family can be divided into two subtypes: class IA PI3Ks are activated mainly by membrane-bound tyrosine kinase receptor and class IB PI3Ks are exclusively activated by GPCR generally of the G_i subtype. Class IA PI3Ks bind to the adaptor subunit p85, which, in turn, is recruited by its phosphotyrosine binding domain to phosphorylated receptors or adaptor proteins like IRS (insulin receptor substrate). Class IB PI3Ks bind to adaptor/regulators like p101 or p84/87 and to free Gβγ liberated by active GPCRs. All class I PI3Ks phosphorylate PIP2 to produce PIP3, a secondary messenger membrane lipid that functions as a docking site to a large number of proteins containing the pleckstrin homology (PH) domain.

The p110 catalytic subunits are bound to a regulatory subunit, which can be p85α, p55α, p50α, p85β, or p55γ. p85α, p55α, and p50α are derived from the same gene known as PIK3R1 by alternative slicing. PIK3R2 and PIK3R3 encode the p85β and p55γ isoforms of the p85 regulatory subunit, respectively. Regulatory subunits of class IA PI3Ks have two Src homology 2 (SH2) domains that bind with high affinity to phosphorylated tyrosine residues in receptor proteins, as well as in other signalling proteins. The two longer isoforms, p85α, p85β, also encompass an extended N-terminal region containing Src homology 3 (SH3) and a BCR homology (BH) domain lined by two proline-rich (P) regions (Figure 1.7) (Engelman et al., 2006).

The p110 catalytic subunits possess an N-terminal p-85 binding domain that interacts with the p-85 regulatory subunit, a Ras-binding domain that mediates activation by the small GTPase Ras, a C2 domain, a phoshatidylinositol kinase homology (PIK) domain and a C-terminal domain (Figure 1.7) (Engelman et al., 2006).

Activation of class IA PI3Ks can be mediated by receptors with intrinsic tyrosine kinase activity or by non-receptor tyrosine kinases, such as src-family kinases or JAK kinases. Tyrosine kinases activate class IA PI3Ks via at least two mechanisms: tyrosine phosphorylation (pTyr) and increased GTP loading of Ras. Binding of p85 SH2 domains to specific phosphotyrosine residues (within the receptors or other signalling proteins) activates PI3K and recruits the p110 catalytic subunits to the plasma membrane, where the p110 catalytic subunit phosphorylates its lipid substrates; PtdIns(4,5)P_2. On the other hand, some studies have shown that the shorter regulatory subunits have increased potential to recruit and/or activate p110 (Shepherd et al., 1997; Ueki et al., 2000). However, there is no evidence until now for selective binding among any of the catalytic subunits with specific regulatory subunits.

Interestingly, the p110β isoform of class IA PI3K is regulated not only by the p85 regulatory subunit but also by binding to Gβγ subunits of heterotrimeric G proteins. Therefore, the class IA p110β isoform might integrate signals from G-protein-coupled receptors (GPCRs) as well as growth factor receptor tyrosine kinases. It was shown that p110β acts in conjunction to p110γ to provide full PI3K activity downstream of GPCRs (Guillermet-Guibert et al., 2008).

Class IB Phosphoinositide-3 Kinases

The only class IB PI3K identified to date is p110γ catalytic subunit associated with p101or p84 regulatory protein. They do not have p85 binding domain. They are found only in mammals, where they show restricted tissue distribution, being abundant in white blood cells (Brock et al., 2003).

PI3Kγ is activated by G-protein-coupled receptors, where the free Gβγ subunits of heterotrimeric G proteins bind and activate p110γ. The role of p101 in this activation is controversial, with some evidence indicating that p101 plays a role in recruitment and activation of p110γ (Krugmann et al., 1999) whereas other evidence indicates that p110γ is directly stimulated with Gβγ subunits.

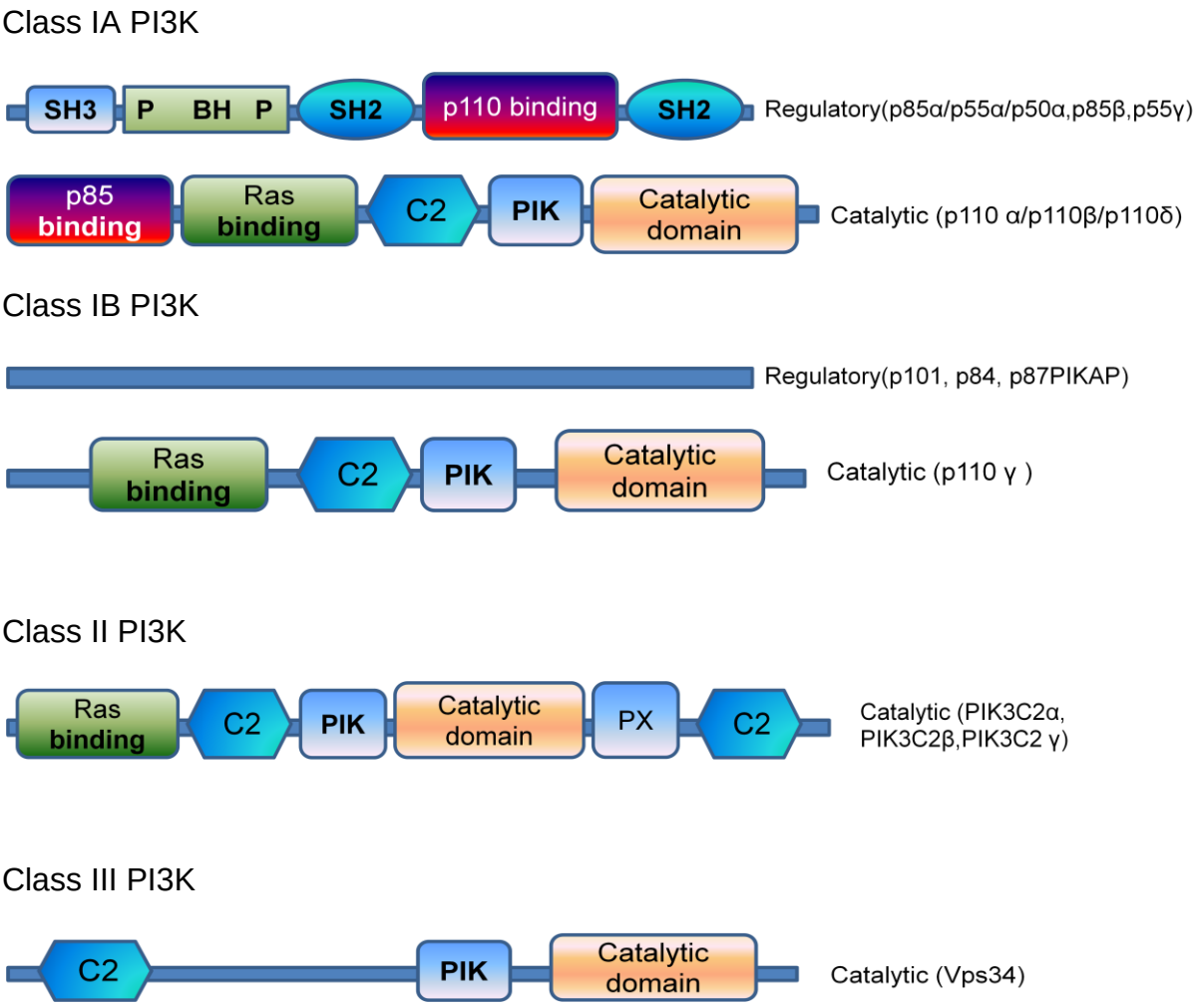


Figure 1.7. Structure of PI3K family members. At their N-terminus, the catalytic domains of PI3K class I have a regulatory subunit binding domain (p85B binds p85/55/50 regulatory subunits; pRegB binds p101 and p84/87^{PIKAP} proteins), followed by a Ras-binding domain (RasB), a C2 domain (protein kinase C homology domain 2) and a PI3K accessory (helical) domain (PI3Ka). PI3Kγ contains interaction sites for βγ subunits of trimeric G proteins in the pRegB and the PI3Kc domains. PI3Ks of the class II do not have a known adaptor subunit-binding site in front of their RasB site, but display two to three proline-rich (P) stretches, as well as a PX (phox) and an additional C2 domain at the C-terminus. The catalytic subunit of the class III enzyme Vps34 is composed only of the C2, PI3Ka and PI3Kc domains. Abbreviations: SH3, Src-homology 3; BH, BCR homology (also known as RhoGAP homology); P, proline-rich motif; SH2, Src-homology 2; PX, Phox homology.

1.4.2. Class II Phosphoinositide-3 Kinases

Class II PI3Ks are monomers that consist of only a single p110-like catalytic subunit. The three isoforms of class II PI3K-PIK3C2α, PIk3C2β, PIk3C2γ-are homologous to the class I p110 subunits. They have an additional Phox homology (PX) and C2 domains at the C terminus (Falasca and Maffucci, 2007).

It was shown that members of this class have a different sensitivity to the classical PI3K inhibitors LY294002 and wortmannin. In fact, the α isoform of Class II PI3K appears to be more resistant (IC50 for wortmannin: 420 nM; IC50 for LY294002: 19 μM compared with the corresponding 5 nM and 0.8 μM for p110α) (Domin et al., 1997).

Class II PI3Ks are capable to phosphorylate PtdIns and PtdIns-4-P *in vitro* and might generate PI-3-P, PI-3,4-P_2 and possibly PIP_3 *in vivo* (Engelman et al., 2006). They can be activated by a number of stimuli including (a) chemokines, such as monocyte chemotactic protein-1 (MCP-1) (Turner et al., 1998), (b) cytokines such as leptin and tumor necrosis factor α (TNFα) (Ktori et al., 2003) and (c) insulin (Falasca et al., 2007). Although class II PI3Ks have been localized to the trans-Golgi network and low-density microsomes (Domin et al., 2000), their mechanism of action is not clear.

1.4.3. Class III Phosphoinositide-3 Kinases

Class III PI3Ks consist of a sole member, Vps34 (vacuolar protein-sorting defective 34). Phosphatidylinositol is the only substrate for Vps34 and therefore its product *in vivo* is PtdIns3P (Backer, 2008). The primary known function of Vps34 is the regulation of vesicular trafficking in the endosomal/lysosomal system (Odorizzi et al., 2000). However, recent studies have demonstrated that Vps34 is implicated in some signalling pathways such as: (a) the regulation of the activity of mTOR in response to amino acid (Nobukuni et al., 2005); (b) trimeric G-protein signalling to mitogen-activated protein kinase in yeast (Slessareva et al., 2006) and ; (c) autophagy, which is a cellular response to nutrient deprivation (Wurmser and Emr, 2002).

Vps34 shows significant homology with the catalytic domain of other PI3Ks especially PI3Kγ but it lacks the Ras-binding domain (Walker et al., 1999). Activation

of Vps34 has been shown to be regulated by another protein kinase, Vps15. For example, Stack et al. demonstrated that both Vps34 and Vps15 co-immunoprecipitate and also reported the inhibition of the production of the Vps34p product, PtdIns3P, in strains expressing kinase-dead mutants of Vps15 (Stack et al., 1993).

1.5. Phosphoinositide-3 Kinases Signalling Pathways

Activation of PI3K in response to hormones and growth factors will result in the generation of phosphorylated phosphoinositdes. Proteins with specific PIP3 binding motifs like specific pleckstrin homology (PH) or FYVE (named after four proteins containing this domain: Fab1, YOTB, Vac1 and EEA1) domains are translocated to the source of PIP3 generation at the plasma or endosomal membranes, respectively. Among the proteins to possess PH domains with PIP3 specificity are two protein-serine kinases, phosphoinositide-dependant kinase 1 (PDK-1) and protein kinase B (PKB; also known as Akt). PDK-1 is a serine/threonine kinase that is constitutively active but only gains access to certain substrates upon binding 3'-phosphoinositides (3'PIs). Phosphorylation by PDK-1 contributes to the activation of many downstream kinases, including PKB and some protein kinase C isoforms (Oudit et al., 2004).

1.6. Protein Kinase B (PKB)

Protein kinase B is a serine/threonine protein kinase that shows a significant homology to PKA and PKC. The PKB family of kinase is comprised of three closely related members: PKBα (Akt1), PKBβ (Akt2) and PKBγ (Akt3). PKB controls a diverse range of vital cellular functions including cell growth, survival, proliferation and metabolism. PKBα knockout mice are smaller in size compared to wild type ones and PKBα-null cells exhibit higher rates of apoptosis, suggesting a critical role of PKBα in cell survival (Chen et al., 2001; Cho et al., 2001). PKBβ knockout mice develop a type 2 diabetes-like phenotype and isolated cells from those mice display impaired glucose utilization, indicating a central role for PKBβ in the maintenance of glucose homeostasis (Garofalo et al., 2003). A role for PKBγ in brain development has been proposed based on the fact that PKBγ knockout mice show impaired brain development (Tschopp et al., 2005). Collectively, these data may suggest that

different cellular processes are under the control of different PKB isoforms, however, analyses of the phenotypic features of double PKB isoform knockout mice demonstrate a kind of overlap or compensation among different isoforms. PKBα/PKBβ double knockout mice display severe growth deficiency and die shortly after birth (Peng et al., 2003). Simultaneous deletion of PKBα and PKBγ are embryonic lethal (Yang et al., 2005). Mice with a functional allele of PKBα ($PKB\alpha^{+/-}$ $PKB\beta^{-/-}PKB\gamma^{-/-}$) are viable despite the decrease of body weight and glucose intolerance (Dummler et al., 2006). Together, these genetic studies provide evidence for overlapping in addition to distinct roles of different PKB isoforms.

PKB is a cytosolic protein, which contains three functional domains: an N-terminal PH domain, a central catalytic kinase domain and a C-terminal regulatory domain (Hanada et al., 2004). The Kinase domain possesses a conserved threonine (Thr^{308} in PKBα, Thr^{309} in PKBβ and Thr^{305} in PKBγ) in the activation loop whose phosphorylation is required for the full activation of PKB. The C-terminal domain contains a conserved serine (Ser^{473} in PKBα, Ser^{474} in PKBβ and Ser^{472} in PKBγ) in the hydrophobic motif. Phosphorylation of this site is essential for the full activation of PKB as it stabilizes the kinase domain in an active conformational status (Sale and Sale, 2008).

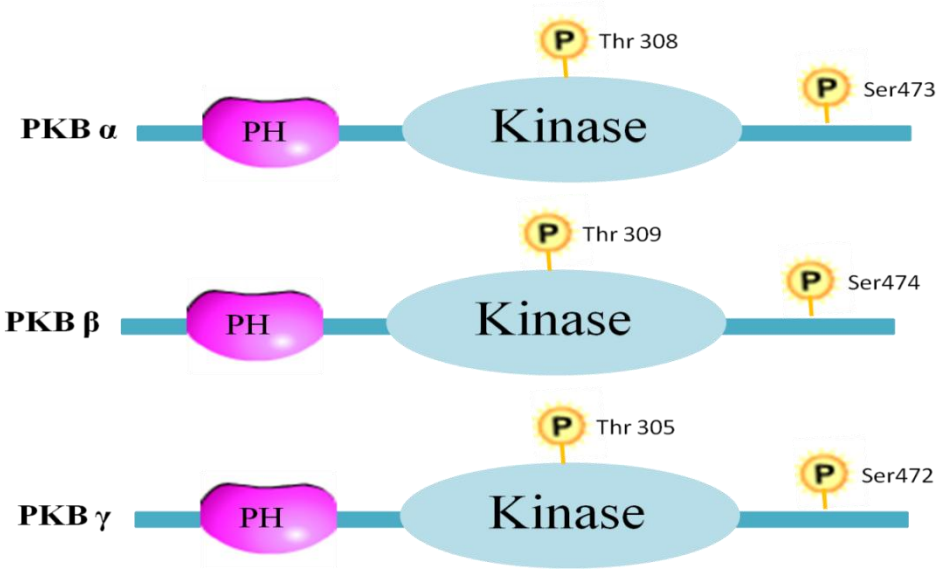


Figure 1.8. Structure of different PKB isoforms. The domains and the phosphorylation sites of PKB isoforms are depicted.

Phosphoinositol-3,4,5-triphosphate and Phosphoinositol-3,4-diphosphate bind to the shallow pocket of the PH domain with the phosphate side chains of the head group forming ionic interactions with specific amino acids in the protein (Thomas et al., 2002). However, this binding does not activate PKB. Alternatively, the interaction between PKB and PtdIns(3,4,5) provokes a conformational change in the structure of PKB that helps its phosphorylation on Thr^{308} and Ser^{473} by PDK-1 and mammalian target of rapamycin complex 2 (mTORC2) (Fayard et al., 2005). After PKB activation, it dissociates from the plasma membrane and can enter the nucleus so it can phosphorylate many cytosolic and nuclear substrates.

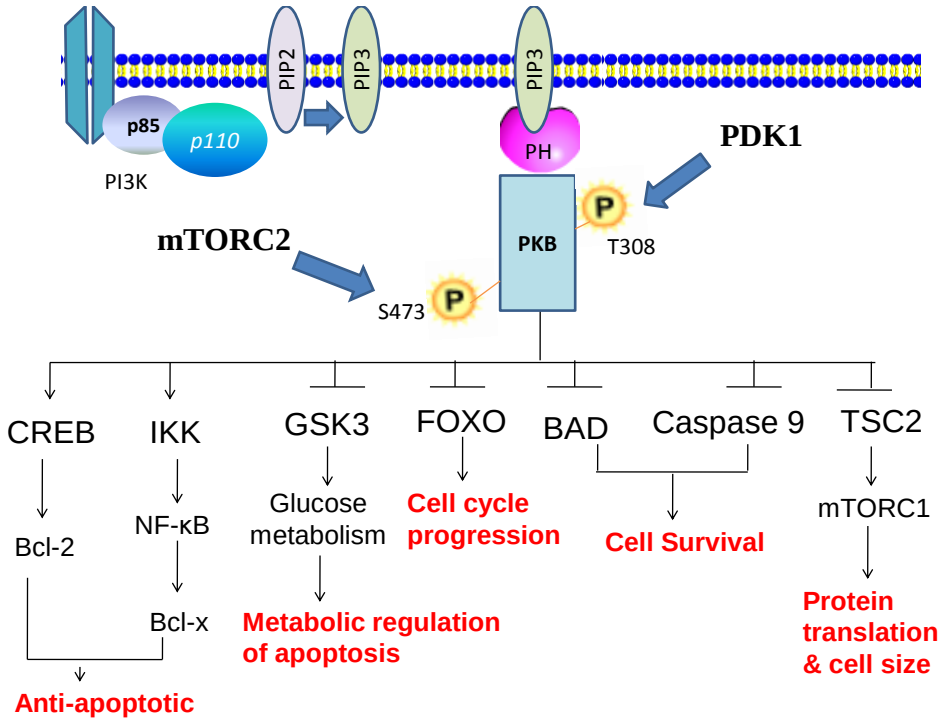


Figure 1.9. Activation and some downstream targets of Protein kinase B. PKB-mediated phosphorylation of these proteins leads to their activation (arrows) or inhibition (blocking arrows). Regulation of these substrates by PKB contributes to activation of the various cellular processes shown (i.e., survival, growth, proliferation and metabolism). See text for detailed descriptions of substrates and functions.

PKB can also be activated by PKA; a mechanism which is independent to the classical PI3K activation. It was reported that cAMP elevating agents like forskolin, 8-bromo-cAMP, chlorophenylthio (CPT)-cAMP and prostaglandin E1 can phosphorylate PKB (Filippa et al., 1999). Moreover, stimulation of PKB by cAMP is insensitive to wortmannin (Sable et al., 1997). Another study showed that activation of Ca^{+2}/calmodulin-dependent protein kinase kinase (CaM-KK) can phosphorylate PKB in the absence of PtdIn(3,4,5) triphosphate (Yano et al., 1998). These findings suggest that PKB can be activated by different mechanisms.

PKB activity is down regulated by c-Jun N-terminal kinase (JNK) activation during stress conditions including oxidative stress, endoplasmic reticulum (ER) stress and exposure to cytokines (Elghazi et al., 2007). In addition, there is some evidence that mTOR/S6K1 via the phosphorylation and suppression of IRS1 and IRS2 inhibits signaling to PKB (Um et al., 2006).

1.6.1. Downstream targets of PKB

PKB plays a crucial role in regulating a variety of cellular responses, including cell growth, cell survival and metabolism. These functions are likely mediated by multiple downstream substrates of PKB as described below.

1.6.1.1. Targets of PKB in promoting cell survival

a) Direct regulation of cell survival

PKB was found by many researchers to promote insulin and growth factor-mediated cell survival and protect against apoptosis. PKB can directly phosphorylate BAD at Ser^{136} which promotes BAD to dissociate from the anti-apoptotic proteins Bcl-2/Bcl-X in the mitochondrial membrane (Datta et al., 1997). PKB can also phosphorylate and inhibit human caspase-9, an important protease in the initiation and progression of apoptosis (Cardone et al., 1998).

(b) Transcriptional control of cell survival

PKB down regulates the transcriptional activity of FoxO proteins (members of forkhead transcription factors family). PKB phosphorylates FOXO1 on Thr^{24}, Ser^{256}, and Ser^{319}, and it phosphorylates FOXO3a and FOXO4 on three equivalent sites as reviewed in (Tran et al., 2003). PKB's phosphorylation of FOXO proteins occurs in the nucleus and upon phosphorylation, FoxO is translocated from the nucleus to the cytoplasm where it is inactive (Woodgett, 2005). Therefore, this inhibits transcriptional induction of pro-apoptotic genes that promote apoptosis, cell-cycle arrest, and metabolic processes.

Another transcription-dependent anti-apoptotic effect of PKB is through the phosphorylation of IκB kinases (IKKs). Transcription factor NF-κB (nuclear factor-κB) is in a complex with its cytosolic inhibitor, IκB. Phosphorylation of NF-κB by IKKs results in the release and degradation of IκB. This permits the translocation of NF-κB to the nucleus and result in transcription of NF-κB-dependent pro-survival genes

such as Bcl-xL and c-Myb (Song et al., 2005). PKB has been reported to activate IKKs, however, the mode of this activation is still poorly understood.

PKB can also promote cell survival through the phosphorylation of MDM2 (or HDM2 in humans), an E3 ubiquitin ligase that triggers p53 degradation, on two sites Ser^{166} and Ser^{186} (Mayo and Donner, 2001). PKB induced phosphorylation of MDM2 promotes the translocation of MDM2 to the nucleus where it down regulates p53 function (Mayo and Donner, 2001).

(c) Metabolic regulation of cell survival

PKB could regulate cell metabolism via phosphorylation of glycogen synthase kinase 3 (GSK-3) on Ser^{21} in the α-isoform and Ser^{9} on the β-isoform. This inhibits its activity and results in the storage of glucose as glycogen (Elghazi et al., 2007). Moreover, it was reported that GSK-3 is implicated in regulation of the cell cycle via regulation of c-Myc stability and levels of cyclin D1 (Chang et al., 2003). Inhibition of GSK 3 activity using lithium chloride was found to protect pancreatic beta cells against apoptosis induced by free fatty acids (Wu and Luo, 2007).

1.6.1.2. Targets of PKB in promoting cell growth

A well established function of PKB is its role to promote cell growth (i.e, an increase in cell mass) (Sale and Sale, 2008). The main mechanism appears to be via the activation of mTORC1, which is regulated by nutrients and growth factor signaling. mTORC1 is a crucial regulator of translation initiation, ribosome biogenesis and plays a conserved role in controlling cell mass (Fingar and Blenis, 2004).

The mechanism by which PKB activates mTOR signalling appears to be through inhibition of TSC2 (Huang and Manning, 2009). PKB has been shown to directly phosphorylate two sites on TSC2 at Ser^{939} and Thr^{1462} (Inoki et al., 2002). TSC2, together with TSC1, forms a complex that acts as a GTPase-activating protein for the Ras-related small G protein Rheb, which activates mTORC1 when in its GTP-bound active form. Therefore, PKB activates mTORC1 indirectly by inhibiting TSC2, thereby allowing Rheb-GTP to activate mTORC1 signalling. A second PKB substrate

was also found to be involved in mTORC1 regulation is the proline-rich Akt substrate of 40 kDa (PRAS40) (Kovacina et al., 2003). It was shown that PKB can directly phosphorylate PRAS40 on Thr^{246} and such phosphorylation is crucial for 14-3-3 binding in nutrient and growth factor-dependent manner (Kovacina et al., 2003). Interestingly, PRAS40 has been shown to associate with mTORC1 and hence can negatively regulate mTORC1 signalling (Sancak et al., 2007). Mutation of PRAS40 at Thr^{246} blocks PKB mediated stimulation of S6K1, indicating that PKB induced phosphorylation of PRAS40 at Thr^{246} stimulates mTORC1 signalling (Sancak et al., 2007). It is not clear the relative importance of TSC2 and PRAS40 for PKB-mediated mTORC1 activation. Activation of mTORC1 elicits a negative feedback loop to limit PKB activity (Huang and Manning, 2009).

1.6.1.3. Targets of PKB in promoting cell proliferation

Activation of PKB can induce cell proliferation via stimulation of several downstream targets impinging on cell cycle regulation. Some reports have shown that PKB can phosphorylate $p27^{KIP1}$ cyclin-dependent kinase inhibitor on Thr^{157} (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002) and this phosphorylation promotes cytosolic sequestration via 14-3-3 binding (Sekimoto et al., 2004). Inhibiting p27 localization to the nucleus attenuates its inhibitory effects on cell cycle. In addition, PKB-dependent phosphorylation of TSC2 and PRAS40 can drive cell proliferation through regulation of mTORC1, a critical regulator of cell proliferation. PKB signalling leads to activation of eIF4E via mTORC1 mediated inhibition of 4EBP1, and eIF4E induces the cap-dependent translation of several target mRNAs, including those encoding cyclin D1 and c-Myc (Mamane et al., 2004).

1.7. Mammalian Target of Rapamycin (mTOR)

mTOR is a serine and threonine protein kinase which integrates diverse signals to regulate fundamental cellular processes such as the initiation of protein translation and cell growth (McDaniel et al., 2002). mTOR was identified shortly after the discovery of the two yeast genes, *TOR1* and *TOR2* (Sabatini et al., 1994). mTOR is a high molecular-weight protein (around 289KDa) that contains 2549 amino acids (Hay and Sonenberg, 2004). mTOR belongs to the phosphatidylinositol kinase-related kinase (PIKK) family in which its catalytic domain is greatly homologous to that of the phosphoinositide 3-kinases (PI3Ks) (Keith and Schreiber, 1995). Like members of PIKK family, mTOR possesses a series of HEAT repeats within its N-terminus (named after Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A, and TOR1) (Andrade and Bork, 1995). The HEAT motif contains some conserved leucines including $Leu^{545}$and Leu^{547} that mediates the nuclear shuttling of mTOR (Bachmann et al., 2006). In addition, mTOR contains a FRAP, ATM and TRRAP (FAT) domain and a FAT C-terminal (FATC) domain (Figure 1.10). Both HEAT and FAT domains act as surface for protein-protein interactions. The FATC domain is crucial for mTOR activity, and the deletion of even a single amino acid from this domain diminishes mTOR activity (Takahashi et al., 2000). Unlike other PIKK family members, mTOR has a FRB (FKBP12/rapamycin binding) domain that lies next to the kinase domain and is the least part required to bind the specific mTOR inhibitor: FKBP12/rapamycin (Chen et al., 1995). mTOR is phosphorylated at multiple sites including Ser^{2448}, Ser^{2481}, Thr^{2446} and Ser^{1261}. Two more phosphorylation sites; Ser^{2159} and Thr^{2164} were recently discovered by Fingar research group as novel mTOR phosphorylation sites that lie at the beginning of the mTOR kinase domain (Ekim et al., 2011). It was reported that Ser^{2481} is a rapamycin-insensitive autophosphorylation site and happens mainly to mTOR in mTORC2 complex (Copp et al., 2009). However, it was recently demonstrated that insulin can induce mTORC1 associated Ser^{2481} autophosphorylation that is abrogated by rapamycin and amino acid withdrawal (Soliman et al., 2009). Ser^{2448} phosphorylation is mediated by p70 ribosomal S6 kinase (S6K) and occurs predominantly to mTOR in mTORC1 complex (Chiang and Abraham, 2005). Thr^{2446} is phosphorylated under conditions of AMPK activation occurring as a result of nutrient deprivation and this phosphorylation is inhibited by insulin stimulation (Cheng et al., 2004). These three

phosphorylation sites lie between the catalytic domain and the FATC domain near the COOH terminus of mTOR. Ser^{1261} is phosphorylated in response to insulin and amino acid stimulation and is essential for mTORC1 activation and promotion of mTOR autophosphorylation at Ser^{2481} (Acosta-Jaquez et al., 2009).

The activity of mTOR is regulated by nutrients, growth factors, and energy metabolism (Fingar and Blenis, 2004). mTOR can also regulate itself via both negative and positive feedback mechanisms (Sarbassov et al., 2005a). Dysregulation of mTOR signalling pathway is associated with many diseases such as cancer, neurodegenerative disorder and diabetes (Goberdhan and Boyd, 2009). mTOR is known to exist in two complexes; mTORC1 and mTORC2. Although both complexes share the same catalytic subunit (the protein kinase of mTOR), they are structurally and functionally distinct protein complexes.

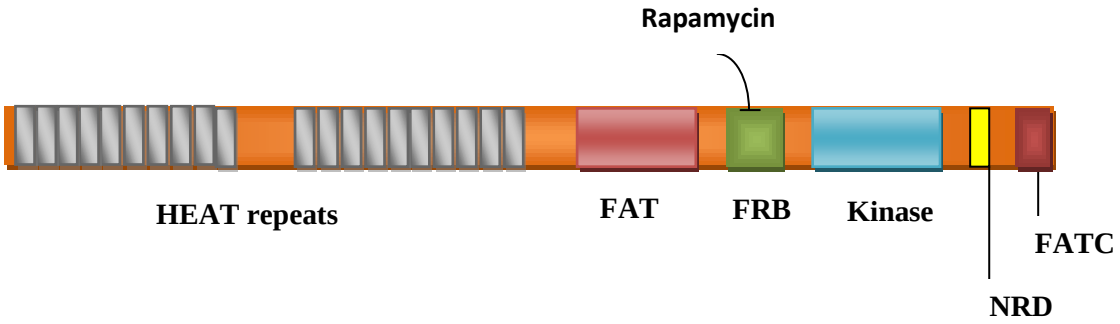


Figure 1.10. The structure of mTOR. mTOR is a large protein kinase containing multiple-functional domains. The kinase domain is located at the C-terminus and is essential for the catalytic activity of mTORC1. The HEAT motif region located at the N-terminus and the FAT domain in the middle are responsible for mediating mTOR interaction with other proteins. The FRB domain is where the FKBP12 and rapamycin complex binds, which is within the region that binds to FKBP38.

1.7.1. mTORC1

In mTORC1, mTOR is present with two highly conserved proteins: raptor (regulatory associated protein of mTOR) and mLST8 (mammalian lethal with sec18 protein 8). Recently, GRp58 was identified as a new mTOR-interacting protein that modulates the assembly and activity of mTORC1 (Ramirez-Rangel et al., 2011). In addition, mTORC1 has two negative regulators, PRAS40 (proline-rich Akt substrate 40 kDa) (Sancak et al., 2007) and DEPTOR (DEPDC6, DEP domain-containing protein 6) (Peterson et al., 2009). Raptor is a polypeptide which contains a highly conserved N-terminal domain followed by three HEAT repeats and seven WD40 repeats near C-terminal (Hara et al., 2002). Raptor is essential for the phosphorylation of mTORC1 downstream targets; eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase (S6K1) (Hara et al., 2002). Knock down of Raptor expression using siRNA not only results in a decrease in S6K1 phosphorylation but in a reduction of insulin-stimulated glucose uptake in 3T3-L1 adipocytes as well (Veilleux et al., 2010). A recent study has demonstrated that mTORC1 activity can be regulated by the phosphorylation status of raptor (Wang et al., 2009). Phosphorylation of raptor on Ser^{863} mediated by mTOR is crucial for insulin stimulated mTORC1 activity toward S6K1 at Thr^{389} (Wang et al., 2009). In addition, the p90 ribosomal S6 kinases (RSKs) mediate the phosphorylation of raptor on three serine residues: Ser^{719}, Ser^{721} and Ser^{722} which are essential for the mTORC1 activity induced by the Ras/MAPK pathway. Recently, it has been shown that ERK can induce the phosphorylation of raptor at three sites; Ser^{8}, Ser^{696} and Ser^{863} to positively regulate mTORC1 activity (Carriere et al., 2010).

mLST8 (also known as GβL) is a 36KDa protein which contains seven WD40 repeats greatly homologous to β subunits of heterotrimeric G proteins (Kim et al., 2003). It was identified as a positive regulatory subunit of the mTOR signalling complex through interaction with the mTOR kinase domain (Kim et al., 2003). Furthermore, it was speculated that mLST8 is vital for a nutrient- and rapamycin-sensitive interaction between raptor and mTOR (Kim et al., 2003) . In contrast, another study has revealed that knock down of mLST8 using siRNA has no effect on S6K1 and 4EBP1 phosphorylation and that the interaction between raptor and mTOR remains intact while loss of mLST8 impairs the rictor-mTOR association and

inhibits the phosphorylation of PKB at Ser^{473} (Guertin et al., 2006). Clearly, more studies are required to elucidate the role of mLST8 in mTOR signalling.

PRAS40, a 40 kDa proline-rich Akt substrate, has been recognized as a negative regulator of mTOR signalling pathway (Sancak et al., 2007). PRAS40 was first known as a key substrate of PKB and a major 14-3-3 binding protein (Kovacina et al., 2003). Activation of PKB by insulin results in the phosphorylation of PRAS40 at Thr^{246} (Kovacina et al., 2003) . Consequent reports revealed that PRAS40 can interact with mTORC1 via raptor and this association results in reduction of mTORC1 activity (Sancak et al., 2007; Wang et al., 2007). Furthermore, PRAS40 has been implicated as a direct physiological substrate of mTORC1 as mTORC1 was shown to phosphorylate PRAS40 at three serine residues; Ser^{183} (Oshiro et al., 2007), Ser^{212} and Ser^{221} (Wang et al., 2008a).

DEPTOR is another negative regulator of mTORC1 and is a binding partner for both mTORC1 and mTORC2 (Peterson et al., 2009). It was shown that knock down of DEPTOR results in an increase of S6K1 and PKB phosphorylation at Ser^{473} as well as an increase of cell size and protection against apoptosis induced by serum deprivation (Peterson et al., 2009). On the other hand, over-expression of DEPTOR reduces the phosphorylation of S6K1 while the phosphorylation of PKB is not affected (Peterson et al., 2009).

1.7.2. mTORC2

mTORC2 includes mTOR and mLST8, but instead of raptor, mTORC2 contains two special subunits, rictor (rapamycin independent companion of mTOR) and mSin1 (mammalian stress-activated protein kinase (SAPK)-interacting protein 1). Moreover, protor (protein observed with rictor), DEPTOR, PRR5 (proline-rich protein 5) and Hsp70 are other novel components of mTORC2 (Martin et al., 2008; Pearce et al., 2007; Woo et al., 2007).

Rictor was first identified by Sabatini's group as a unique partner to mTORC2 (Sarbassov et al., 2004). FKBP12-rapamycin cannot bind to the rictor containing mTOR complex thus mTORC2 is insensitive to acute rapamycin exposure. On the

other hand, prolonged rapamycin treatment interferes with the assembly of mTORC2 (Sarbassov et al., 2006). It was shown that the rictor-mTOR complex does not modulate the phosphorylation of S6K1 and 4EBP1 but regulates the phosphorylation of PKB (Sarbassov et al., 2005b), PKCα (Facchinetti et al., 2008) and serum- and glucocorticoid-induced protein kinase 1 (SGK1) (Garcia-Martinez and Alessi, 2008).

mSin1 is another fundamental component of mTORC2 as it is essential for mTORC2 integrity and the phosphorylation of PKB at Ser^{473} (Frias et al., 2006). Loss of mSin1 abolishes the phosphorylation of PKB at Ser^{473} and PKCε without affecting the phosphorylation of S6K1 (Cameron et al., 2011).

1.8. Upstream regulators of mTOR

1.8.1. TSC1 and TSC2

Tuberous sclerosis (TSC) is a dominant genetic disorder characterised by the formation of benign tumours known as hamartomas in different tissues and organs. TSC1 (also called hamartin) is a 130-kDa protein and is encoded by the tuberous sclerosis complex 1 gene. TSC2 (also called tuberin) is a 198 kDa protein and is encoded by the tuberous sclerosis complex 2 (Cheadle et al., 2000). TSC1 and TSC2 interact through their N terminal to form a heterodimer that suppresses cell growth and proliferation. Both TSC1 and TSC2 proteins are upstream regulators of mTOR. Over-expression of TSC1 and TSC2 inhibits the phosphorylation of downstream targets of mTOR: S6K1 and 4EBP1 (Tee et al., 2002), whereas S6K1 is highly phosphorylated in TSC2 null cells (Goncharova et al., 2002).

TSC2 has a GAP (GTPase-activating protein) domain that induces the intrinsic GTPase activity of the small G-protein Rheb (Ras homolog enriched in brain), thus stimulates the conversion of Rheb into its GDP- bound inactive state. Rheb is a potential activator of mTORC1 which may be mediated via direct binding to mTOR (Bhaskar and Hay, 2007). TSC1-TSC2 complex senses signals from many signalling pathways through distinct phosphorylation events to regulate mTORC1 (Huang and Manning, 2008). These signaling pathways include: PKB pathway, ERK–RSK (extracellular-signal-regulated kinase–p90 ribosomal protein S6 kinase), IKKβ

(inhibitory κB kinase β), AMPK (AMP-dependent protein kinase) pathway and CDKs (cyclin-dependent kinases).

In response to growth factors, PKB phosphorylates TSC2 directly on several phosphorylation sites including Ser^{939}, Ser^{981}, Ser^{1130}, Ser^{1132} and Thr^{1462} (Cai et al., 2006; Inoki et al., 2002). Phosphorylation at these sites decreases the GAP activity of TSC2 toward Rheb and destabilizes TSC2 protein. This destabilization is obtained by disrupting complex formation between TSC1 and TSC2 (Inoki et al., 2002). Indirectly, PKB can regulate TSC2 activity through activation of transcription factor FOXO1 (Cao et al., 2006). Phosphorylation of FOXO1 results in its physical association to TSC2 through its C terminal domain in the cytoplasm. Such interaction inhibits the GAP activity towards Rheb, and in turn leads to the activation of p70S6K1; downstream target of mTORC1 (Cao et al., 2006).

In addition to PKB pathway, activation of ERK and its downstream target RSK can also induce mTORC1 activation mediated by the phosphorylation of the TSC1–TSC2 complex. It has been demonstrated that ERK phosphorylates TSC2 at two sites; Ser^{664} and Ser^{540} (Ma et al., 2005). Activation of RSK induces the phosphorylation of TSC2 at Ser^{1798} located at C terminal, leading to the activation of S6K1 (Roux et al., 2004) .

Most cytokines senses to mTORC1 complex via the activation of PKB or ERK signalling. Nevertheless, the pro-inflammatory cytokine tumor necrosis factor α (TNFα) has been demonstrated to activate mTORC1 signalling pathway via IKKβ (inhibitory κB kinase β)-mediated phosphorylation of TSC1 (Lee et al., 2007). IKKβ interacts physically with TSC1 and phosphorylates TSC1 at two sites Ser^{487} and Ser^{511}.

AMPK is a regulator of cellular energy status and its α-subunit is phosphorylated on Thr^{172} by its upstream kinase LKB1 in response to an increase in the cellular AMP/ATP ratio (Hardie, 2005) . Upon activation, AMPK phosphorylates TSC2 on at least two residues Ser^{1387} and Thr^{1271} (Hardie, 2005) . However, it is still unclear how the phosphorylation of these sites activates TSC1/TSC2 complex. Interestingly, AMPK-dependent phosphorylation of TSC2 on Ser^{1387} prepares TSC2 for

subsequent phosphorylation by GSK3β (glycogen synthase kinase 3β) (Inoki et al., 2006). It looks that the effect of GSK3β on TSC/mTOR signalling is specific to the Wnt pathway, rather than downstream of PKB, as Wnt signalling inhibits GSK3β-mediated phosphorylation of TSC2. Under conditions of energy stress, GSK3β phosphorylates TSC2 on Ser^{1371}, Ser^{1375}, Ser^{1379}, and Ser^{1383} and this contributes to TSC1–TSC2 complex activation and subsequent mTORC1 inhibition (Inoki et al., 2006).

A study has proposed a role for CDKs in the regulation of the TSC1–TSC2 complex. TSC1 is phosphorylated on at least three sites; Thr^{417}, Ser^{584} and Thr^{1047} by CDK1–cyclin B complexes (Zacharek et al., 2005). Experiments over-expressing of CDK6–cyclin D complexes have revealed that cyclin D is associated with the TSC1-TSC2 complex and promotes the phosphorylation of both TSC1 and TSC2 to regulate mTORC1 activity (Zacharek et al., 2005).

TSC2 was demonstrated to be phosphorylated by the p38-activated kinase MK2 (also known as MAPKAPK2) which is activated by p38 MAPK (mitogen-activated protein kinase) on Ser^{1210} (Li et al., 2003). Phosphorylation on this site creates a 14-3-3 binding site on TSC2 and thus modulates the activity of TSC2 (Li et al., 2003).

1.8.2. Rheb

Rheb is a member of the Ras subfamily of small GTPases that is highly conserved from yeast to human. Rheb has an intrinsic GTPase activity and can exit in two forms; GDP-bound form and a GTP-bound form (Aspuria and Tamanoi, 2004). Several studies (Garami et al., 2003; Inoki et al., 2003) have shown that TSC1/TSC2 complex acts as a GAP for Rheb and can bind GTP-bound Rheb to stimulate GTP hydrolysis. It was shown that over-expression of TSC1/TSC2 reduces the GTP/GDP ratio of Rheb, suggesting that TSC2 is a Rheb GAP (Inoki et al., 2003). In addition, over-expression of Rheb induces the activation of mTORC1 as evidenced by the phosphorylation of S6K and mobility shift of 4EBP1 (Inoki et al., 2003). It is not fully understood the mechanisms by which Rheb signals to mTOR. It has been suggested that Rheb might interact directly with the catalytic domain of mTOR to promote its signalling function (Long et al., 2005b). Moreover, Rheb was shown to bind mLST8

and raptor as well (Long et al., 2005b). A recent paper has demonstrated that Rheb is phosphorylated at Ser^{130} by p38 MAPK signalling pathway under conditions of energy stress to inhibit the phosphorylation of downstream targets of mTORC1 (Zheng et al., 2011).

1.8.3. FKBP38

FKBP38 is a member of a family of FK506-binding protein (FKBP) which belongs to cis-trans peptidyl-prolyl isomerases (Marks, 1996). FKBP38 acts as an endogenous inhibitor to mTORC1 as its over-expression suppresses the activity of mTORC1 (Bai et al., 2007). Knocking down the expression of FKBP38 using siRNA increases the phosphorylation of S6K and 4EBP1. It was also demonstrated in this study that FKBP38 is associated with the components of mTORC1 and this association is regulated by Rheb in a GTP-dependent manner (Bai et al., 2007). Rheb interacts with FKBP38 strongly when loaded with GTP and loosely when loaded with GDP. Binding of Rheb with FKBP38 prevents its association with mTOR (Bai et al., 2007). The binding of Rheb with FKBP38 in cells is regulated by nutrients availability. In cells deprived of serum or amino acids, Rheb shows a weak association with FKBP38, which is strongly induced upon re-addition of the omitted elements. The increased binding of Rheb with FKBP38 is associated with a decrease in the interaction of FKBP38 with mTOR (Bai et al., 2007). Clearly, it was suggested that nutrients regulate the association of FKBP38 with mTOR through Rheb, most probably by controlling its nucleotide-binding states. However, some reports (Uhlenbrock et al., 2009; Wang et al., 2008b) have provided evidence that Rheb and FKBP38 do not physically interact. It was shown that FKBP38 can be associated with mTOR, however this interaction is not affected by either amino acid starvation or insulin treatment (Wang et al., 2008b). Therefore, these findings argued against a role of FKBP38 in Rheb-dependent mTORC1 activation.

1.8.4. TCTP

The translationally controlled tumour protein (TCTP) is a well conserved protein widely expressed in all eukaryotes (Bommer and Thiele, 2004). It is also known as histamine-releasing factor (HRF) and is a key protein in vital cellular functions

including cell growth and division, cell cycle progression and protection against apoptosis (Bommer and Thiele, 2004). TCTP was first identified as a component of the TSC-Rheb pathway in Drosophila (Hsu et al., 2007). Down-regulation of TCTP decreases cell size, cell number and organ size. Interestingly, TCTP can bind Rheb and shows a guanine nucleotide exchange activity toward Rheb. Thus, TCTP acts as downstream of Rheb and upstream of mTORC1 (Hsu et al., 2007). Similarly, it was shown that human TCTP enhances the GDP release of Rheb and activates mTORC1 as indicated by an increase in the phosphorylation of S6K (Dong et al., 2009). In contrast, Wang et al failed to demonstrate that TCTP is able to interact with Rheb and is ineffective in regulating mTORC1 activity (Wang et al., 2008b).

1.8.5. PRAS40

PRAS40 was first identified as downstream target of PKB and later was recognized as a part of mTORC1 complex. PRAS40 has been shown to bind raptor and suppress mTORC1 kinase activity both *in vitro* and in cells (Sancak et al., 2007). Moreover, the interaction of PRAS40 with mTORC1 increases under conditions that inhibit mTOR signalling, such as nutrient or serum deprivation or mitochondrial metabolic inhibition (Vander Haar et al., 2007). PRAS40 has a TOS motif responsible for its binding to raptor which is similar to that found in mTORC1 substrates. Initially, a TOR signalling (TOS) motif was identified in the N terminus of all known S6 kinases and in the extreme C terminus of the 4EBPs (Schalm and Blenis, 2002; Schalm et al., 2003). The TOS motif of mTORC1 substrates is also essential for raptor to bind S6K1 and 4EBP1 and the consequent mTOR-mediated phosphorylation of S6K1 and 4EBP1 (Wang et al., 2007). Therefore, it has been proposed that PRAS40 inhibits mTORC1-directed phosphorylation of S6K and 4E-BP1 by competing with these proteins for raptor binding (Wang et al., 2007).

It has been demonstrated that insulin mediates the phosphorylation of PRAS40 at Thr^{246} by PKB. The PKB-induced phosphorylation creates a docking site for 14-3-3, which binds to PRAS40 and interferes with its interaction with mTORC1 (Kovacina et al., 2003; Sancak et al., 2007). In addition to PKB-TSC2-Rheb signalling axis, this PKB-PRAS40 pathway counts for the second mechanism of how PKB activates mTORC1.

1.8.6. IKK

Inhibitor of nuclear factor κ B (NFκB) kinase (IKK) complex is a high molecular weight complex that contains two protein kinases acting as catalytic subunits: IKKα and IKKβ, in addition to a regulatory subunit IKKγ (Hacker and Karin, 2006). IKK is activated by proinflammatory cytokines including TNFα which was found also to activate mTORC1 (Lee et al., 2008). Upon TNFα stimulation, IKKβ binds to TSC1 and results in its phosphorylation at Ser^{487} and Ser^{511} (Lee et al., 2008). Phosphorylation at these sites prevents the formation of TSC1/TSC2 complex rendering TSC2 to mediate mTORC1 activation (Lee et al., 2008). A study by Dan et al has shown that the interaction between IKK and mTORC1 is mediated by PKB (Dan and Baldwin, 2008). Moreover, over-expression of IKK augments the activity of mTORC1 so IKK is the positive regulator of mTORC1 (Dan and Baldwin, 2008).

1.8.7. Calcium and class III PI3K

Class III PI3K (also known as hVps34) has been implicated in regulation of amino acid sensing to mTORC1. Amino acid-induced activation of mTORC1 is sensitive to PI3K inhibitors, however knock down of class I PI3K by siRNA blocks insulin-induced mTORC1 activation and has no effect on amino acid-stimulated mTORC1 activation (Nobukuni et al., 2005) . This suggests the role of another class of PI3K that mediates amino acid sensing to mTORC1. George Thomas's group has found that siRNA knocked down of hVps34 suppresses the amino acid-stimulated activation of mTORC1 (Nobukuni et al., 2005). Later, they have also shown that amino acid induced mTORC1 activation is associated with an increase in intracellular calcium (Gulati et al., 2008). In addition, treatment with thapsigargin (an endoplasmic reticulum calcium- ATPase inhibitor) that increases intracellular calcium activates mTORC1 in the absence of amino acids. On the other hand, calcium chelators prevent amino-acid stimulated mTORC1 activation (Gulati et al., 2008). Collectively, these results suggest the intracellular calcium is essential and enough for mTORC1 activation. hVps34 encloses a Ca^{+2}/CaM binding domain and it was shown that binding of CaM in presence of calcium to this domain is essential for amino acid to stimulate mTORC1 (Gulati et al., 2008).

1.8.8. MAP4K3

MAP4K3 is a conserved Serine/Threonine kinase that was identified to regulate amino acid signals to mTORC1. It was demonstrated that knockdown of MAP4K3 using siRNA inhibits amino acid-induced phosphorylation of S6K1 and rpS6 with a marked decrease in cell size (Findlay et al., 2007). In contrast, over-expression of MAP4K3 induces the phosphorylation of S6K1 and 4EBP1 in a rapamycin-sensitive manner. In addition, it was found that amino acids and not insulin stimulate the activity of MAP4K3 (Findlay et al., 2007). Amino acids were shown to regulate the phosphorylation of MAP4K3 at Ser^{170} and that the withdrawal of amino acids promotes the dephosphorylation of MAP3K4 in an Okadaic acid (an inhibitor of PP2A)-sensitive manner (Yan et al., 2010). It was further demonstrated that a specific PP2A-type phosphatase, $PP2A_{T61\varepsilon}$, is involved in regulation of mTORC1 via dephosphorylation of MAP4K3 at Ser^{170} (Yan et al., 2010).

1.8.9. RalA

Ral is a member of the Ras family of small GTPases that comprises of two proteins; RalA and RalB (van Dam and Robinson, 2006). Although showing high structure homology, RalA and RalB have distinct functions. RalA is involved in cell migration, membrane dynamics and proliferation while RalB contributes to apoptotic signalling (Maehama et al., 2008). RalA has also been found to regulate nutrient signalling to mTORC1. Decrease the expression of RalA using siRNA suppresses phosphorylation of downstream targets of mTORC1 which can be stimulated by amino acids or glucose. Similarly, knock down of RalGDS, the upstream activator and guanine exchange factor of RalA inhibits the phosphorylation of S6K suggesting a role of the RalGDS/RalA pathway in the activation of mTORC1. Although it is still poorly understood how Ra1A regulates mTORC1 in response to nutritional status, there is some evidence that Rheb is involved by acting as upstream of RalA because inhibition of mTORC1 by siRNA knocked down of Rheb expression is partially reversed by expression of a constitutively active RalA mutant (Maehama et al., 2008). Therefore, mTOR is regulated at multiple levels including direct association with its regulators, phosphorylation, and which consecutively are mediated by a range of signalling pathways that are responsive to nutritional and energy status.

1.9. Downstream targets of mTOR

1.9.1. Eukaryotic Initiation Factor 4E binding Proteins (4EBPs)

Eukaryotic Initiation Factor 4E binding Proteins (4EBPs) belong to a family of translational repressor proteins that are characterised by their low molecular weight. They can be classified into three members: 4EBP1, 4EBP2, and 4EBP3, each encoded by a separate gene (Hay and Sonenberg, 2004). 4EBP1 in its hypophosphorylated status inhibits the initiation of translation (rate limiting step in cap-dependent translation) through its interaction with eIF4E (Richter and Sonenberg, 2005). By interaction with eIF4E, it prevents its binding to other partners of initiation factor complex including eIF4G. Thus, it blocks the assembly of eIF4F complex, a multi-subunit complex composed of the cap-binding protein, eIF4E; the scaffold protein, eIF4G; and the ATP-dependent DEAD-box helicase, eIF4A, which is crucial for normal cap-dependent translation (Richter and Sonenberg, 2005). 4EBP1 is known to be phosphorylated at seven sites Thr ^{37}, Thr ^{46}, Ser ^{65}, Thr^{70}, Ser ^{83}, Ser^{101}, and Ser^{112} (Hay and Sonenberg, 2004). Of these phosphorylation sites, the first four listed are essential for the release of 4E-BP1 (Hay and Sonenberg, 2004). Phosphorylation of 4EBP1 in HEK293 cells was reported to proceed in a particular order, with the phosphorylation of Thr^{37} and Thr^{46} occurring prior to the phosphorylation of Thr^{70} and finally Ser^{65}. Phosphorylation at two residues (Thr^{70} and Ser^{65}) is critical to regulate the association of 4EBP1 and eIF4E, while phosphorylation at Thr^{70} promotes the release of 4EBP1 from eIF4E, phosphorylation at Ser^{65} prevents rebinding (Fadden et al., 1997; Gingras et al., 1999). Phosphorylation at Thr^{70} and Ser^{101} is essential for Ser^{65} phosphorylation (Proud, 2004). Insulin in the presence of amino acids was demonstrated to phosphorylate 4EBP1 at Ser^{65} and Thr^{70} in a rapamycin dependent manner. These data suggests that mTOR plays a fundamental role in the phosphorylation of 4EBP1 at these sites. In presence of amino acids, phosphorylation at Thr^{37} and Thr^{46} is detected and is further augmented by insulin. On the other hand, neither insulin nor rapamycin affect the phosphorylation of 4EBP1 at Ser^{101} and Ser^{112} (Wang et al., 2003). The phosphorylation of 4EBP1 at Ser^{83} appears to be of minor importance in the control of 4EBP1 *in vivo*.

1.9.2. S6 kinase

S6K is a member of the AGC family of serine / threonine kinases. Mammalian cells express two highly homologous forms of S6 kinase: S6K1 and S6K2, each encoded by a distinct gene (Shima et al., 1998). The structure of S6K contains four domains: the N-terminal domain containing the TOS motif, the catalytic domain containing the activation loop, a linker domain containing two important regulatory phosphorylation sites (the TM and HM sites) and the autoinhibitory pseudosubstrate domain (Shima et al., 1998).The catalytic subunits of both S6K1 and S6K2 are highly conserved while the main difference lies in the extreme N- and C- terminal domains leading to distinct regulation of two kinases as well as diverse downstream targets. For example, the C-terminal of S6K1 contains PDZ binding domain that binds to neurabin and thus permits recruitment of F-actin to nerve terminals (Burnett et al., 1998b) while the C-terminal of S6K2 has a proline-rich domain that mediates the binding of S6K2 to proteins containing SH3 domain (Gout et al., 1998). S6K1 is ubiquitously expressed and plays a major role in the control of cell growth. Activation of S6K1 involves its phosphorylation at least eight sites in a hierarchical manner by a wide variety of extracellular signals (Weng et al., 1998). Of these sites, Thr^{229}, Ser^{371} and Thr^{389} are crucial for full activation of S6K1 (Zhou and Huang, 2010). Thr^{229} is situated in the activation loop (“T loop”) and can be phosphorylated by the phosphoinositide dependent kinase, PDK-1 (Mora et al., 2004). S6K activation is sensitive to rapamycin suggesting that mTORC1 plays an essential role in the regulation of S6K. mTOR can activate S6K1 through phosphorylation of Ser^{371} and Thr^{389} (Burnett et al., 1998a). Activation of S6K1 may also be mediated independently of mTOR by other signalling pathways such as RAFT1, a member of the ataxia telangiectasia mutated (ATM)-related family of kinases, which involves the phosphorylation of sites (mainly Thr^{389}) on the C-terminal autoinhibitory domain of S6K1 (Burnett et al., 1998a). A model of how mTOR regulates the activity of S6K1 was proposed by (Holz et al., 2005). S6K1 in its inactive form is bound to eIF3 (eukaryotic initiation factor 3). Upon hormone or mitogenic stimulation, mTORC1 interacts with eIF3 and consequently phosphorylates S6K1 at Thr^{389}. The activation of S6K1 by mTORC1 results in its dissociation from the eIF3- mTORC1 so S6K1 is accessible to phosphorylate its downstream targets (Holz et al., 2005) .

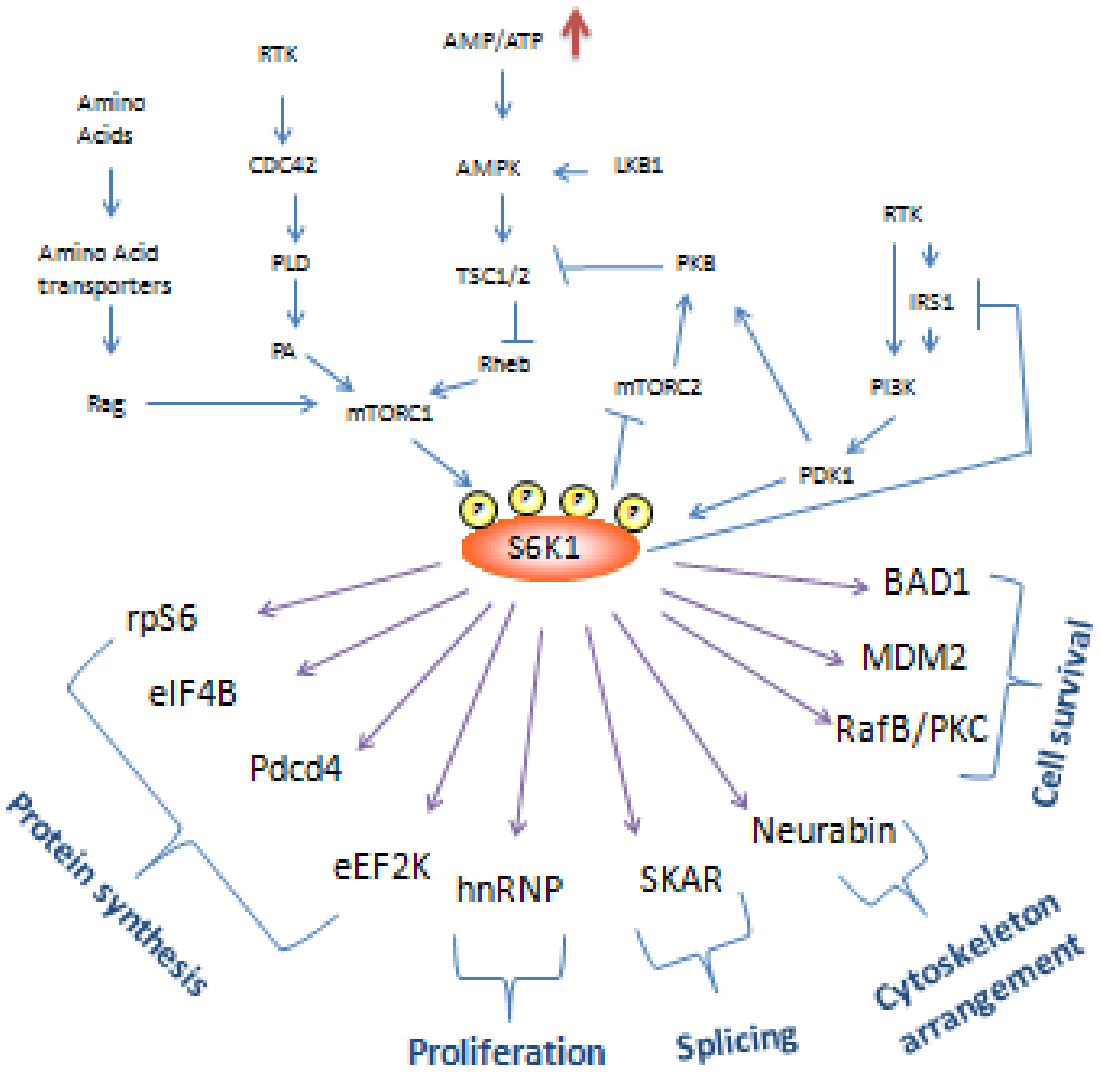


Figure 1.11 Major upstream regulators and downstream effectors of S6K signalling.

1.9.2.1. S6 kinase function

The physiological functions of S6K have been demonstrated by studies performed in *C. elegans*, *drosophila* and mice where S6K gene was knocked down. Deletion of *Drosophila* S6K1 (dS6K1) causes the death of most flies at the larval stage. In addition, those flies that succeed to survive have a very short life span and their females are sterile (Montagne et al., 1999). Those $S6K1^{-/-}$ flies show a reduction in body size compared to wild type and this reduction in body mass is due to a decrease in individual cell size rather than cell number, proposing that S6K acts rather as a regulator of cell growth than proliferation (Montagne et al., 1999). In $S6K1^{-/-}$ mice, S6K2 is upregulated as a compensatory mechanism and their body size is smaller than the corresponding wild type (Pende et al., 2004). On the other hand, $S6K2^{-/-}$ mice have a normal body size compared to wild type littermates (Pende et al., 2004). Those mice lacking S6K1 gene show hypoinsulinemia that result in development of glucose intolerance. In addition, there is a reduction of pancreatic β- cell mass that results in a decrease of insulin production (Pende et al., 2000). Therefore, S6K1 plays an important role in cell growth that consequently has impact on cellular function. Additional functions of S6K are mediated through its downstream effectors as described below.

1.9.2.2. S6 kinase substrates

Ribosomal protein S6 (rpS6)

In eukaryotic cells, ribosomes are composed of two subunits: the 40S (small) and 60S (large) subunits. The 40S subunit contains a single molecule of RNA, known as 18S rRNA, and 33 proteins, while the 60S subunit has three RNA molecules, known as 5S, 5.8S and 28S rRNA, and 46–47 proteins (Geoffrey M. Cooper, 2007). Of all of the ribosomal proteins, rpS6 is the most extensively studied as it was the first ribosomal protein discovered to undergo inducible phosphorylation. rpS6 is phosphorylated at five sites; Ser^{235}, Ser^{236}, Ser^{240}, Ser^{244} and Ser^{247}, all are located at the C-terminus (Krieg et al., 1988). It has been suggested that phosphorylation of rpS6 occurs in an ordered manner in which Ser^{236} is the primary phosphorylation site (Flotow and Thomas, 1992). Studies performed on S6K knock-out mice revealed that both S6K1 and S6K2 are essential for full phosphorylation of rpS6, although data

suggests S6K2 to play a predominant role in rpS6 phosphorylation. It was demonstrated that tissues isolated from S6K1$^{-/-}$ mice exhibit minimal defects in the phosphorylation of rpS6, in contrast deletion of S6K2 reduced rpS6 phosphorylation detected in both cytosol and nucleus (Pende et al., 2004). Furthermore, phosphorylation of rpS6 at Ser^{235} and Ser^{236} can still be detected in cells isolated from S6K1$^{-/-}$ S6K2$^{-/-}$ double knock-out mice. This phosphorylation is rapamycin resistant and it has been suggested the phosphorylation of these two sites are mediated through mitogen-activated protein kinase (MAPK)-dependent kinase (Pende et al., 2004) and PKA (Moore et al., 2009).

Based on the features of rpS6 of being found to interact with tRNA, initiation factors and mRNA, one might expect that rpS6 plays a role in regulation in translation initiation that results in up-regulation of 5' TOP mRNAs (oligopyrimidine tract at the 5' terminus). However, a study performed in mice deficient in ribosomal protein S6 phosphorylation (rpS6$^{P-/-}$ mice) has demonstrated that translation of 5' TOP mRNAs in those mice is not affected (Ruvinsky et al., 2005). On the other hand, rpS6$^{P-/-}$ mice display a reduced β-cell size and a decrease in insulin production that results in impaired glucose tolerance, similar to mice lacking S6K1$^{-/-}$ (Pende et al., 2000).

Eukaryotic Elongation Factor 2(eEF2)

Eukaryotic Elongation Factor 2(eEF2) plays an important regulatory role in the translocation step of elongation thereby regulating protein synthesis. Phosphorylation of eEF2 at Thr^{56} inhibits its activity by preventing its binding to the ribosome (Kaul et al., 2011). Growth factors induce the dephosphorylation of eEF2 which is sensitive to rapamycin. These effects are mediated via a kinase known as Eukaryotic Elongation Factor 2 kinase (eEF2K). The phosphorylation of eEF2K is promoted by S6K and p90RSK in response to activation of the Ras MAPK pathway. In contrast, rapamycin inhibits the phosphorylation of eEF2k on at least three sites: Ser^{78}, Ser^{359}, and Ser^{366} (Kaul et al., 2011).

Eukaryotic Translation Initiation Factor 4B (eIF4B)

eIF4B is a crucial component of the cap binding complex that is required for promoting ribosome binding to mRNA (Raught et al., 2004). It was demonstrated that serum stimulates the phosphorylation of eIF4B at Ser^{422} and this phosphorylation is inhibited by rapamycin. Further experiments showed that this is mediated by S6K1 (Raught et al., 2004) suggesting a role of S6K in the initiation of translation. Therefore, signalling of mitogens and nutrients to mTOR regulates cap-dependent translation via both 4EBP1 and S6K1 that controls eIF4E and eIF4B, respectively.

S6K1 Aly/REF-like target (SKAR)

S6K1 Aly/REF-like target (SKAR) is a RNA binding protein that was found to bind to S6K1 through its catalytic and/or linker domains upon insulin treatment (Richardson et al., 2004). SKAR is phosphorylated at multiple sites, of those phosphorylation at Ser^{383} and Ser^{385} is mediated by S6K1 following stimulation with insulin and such phosphorylation is sensitive to rapamycin (Richardson et al., 2004). Furthermore, it was demonstrated in this study that SKAR knocked down using siRNA results in a reduction of cell size and the authors postulated that SKAR plays a regulatory role in mRNA processing through coupling, transcription, splicing, and/or export, ultimately leading to control cell growth (Richardson et al., 2004). Another study by Blenis' group has revealed that activation of S6K1 results in its recruitment to the exon junction complex (EJC) on newly spliced mRNA through binding to SKAR. Such interaction promotes S6K1 to be located in proximity of several substrates, an effect that may drive the initiation or pioneer round of protein synthesis (Ma et al., 2008).

Heterogenous ribonucleoproteins (hnRNPs)

Heterogeneous ribonucleoproteins (hnRNPs) are a family of chromatin-associated RNA- binding proteins, which bound RNA polymerase II transcripts to form hnRNP particles. It was revealed recently that hnRNP-F is essential for cell proliferation driven by S6K2 (Goh et al., 2010). This is evidenced by the decrease in cell proliferation upon the knockdown of hnPNP-F using siRNA. In contrast, over-expression of hnRNP-F results in a marked increase in proliferation in rapamycin-sensitive manner (Goh et al., 2010).

BAD

BAD is a BH3 domain-containing protein that can be phosphorylated in response to growth factors at Ser^{112} and Ser^{136}, located within 14-3-3 consensus binding sites. Phosphorylation of BAD renders 14-3-3-mediated sequestration in the cytosol, allowing the dissociation of BCL-X_L or BCL-2 to promote survival. BAD can be phosphorylated by several kinases including PKB, Rsk1 and S6K. S6K1 induces the phosphorylation of BAD at Ser^{136} in response to IGF treatment and this phosphoryaltion is sensitive to rapamycin (Harada et al., 2001). In addition, it was demonstrated in $S6K1^{-/-}$ ES cells that IGF induced phosphorylation of BAD at Ser^{136} is lost (Harada et al., 2001). This suggests an additional role of S6K in promoting cell survival.

The murine double minute 2 (MDM2)

The murine double minute 2 (MDM2) is an oncoprotein that is up-regulated in many types of cancer (Meek and Hupp, 2009). MDM2 is a well known substrate of PKB (Meek and Hupp, 2009) which mediates MDM2 translocation to nucleus, thus inhibits MDM2 auto-ubiquitination and protects MDM2 from proteasome-dependent degradation. However, a study by Fang et al has demonstrated that MDM2 can be phosphorylated on Ser^{166} independently of PKB but by S6K1, which results in improved rates of cell survival (Fang et al., 2006). The phosphorylation of MDM2 is inhibited upon knocking down of S6K1 using siRNA. In contrast, over-expression of S6K1 increases the levels of MDM2 (Fang et al., 2006).

B-Raf and PKC ε

PKC*ε* and B-Raf were demonstrated to form a signalling complex with S6K2 upon stimulation with fibroblast growth factor-2 (FGF-2) (Pardo et al., 2006). The formation of this complex is associated with an increase in antiapoptotic proteins, XIAP and Bcl-X_L. Knocking down of any of these components forming this complex inhibits FGF-2 mediated antiapoptotic effects. Over-expression of S6K2 but not S6K1 enhances XIAP and Bcl-X_L levels, and S6K2 activation alone is enough to promote FGF-2-induced prosurvival effects (Pardo et al., 2006). S6K2 could not be substituted by S6K1 in the formation of this signalling complex containing PKCε and B-Raf and in mediating the downstream survival signal.

Insulin Receptor Substrate Proteins (IRS Proteins)

Insulin Receptor Substrate Proteins can be phosphorylated by a number of kinases to interfere with elements along insulin signalling pathway. As stated before, $S6K1^{-/-}$ mice exhibit defects in insulin secretion and show insulin resistance (Pende et al., 2000), suggesting that S6K1 has an impact on insulin signalling. Meanwhile, it was revealed that $TSC2^{-/-}$ MEF cells display a high basal activity of S6K activity with concomitant reduction of PI3K signalling to PKB (Harrington et al., 2004). It was further demonstrated that S6K phosphorylates IRS1 at Ser^{302} and prevents its association with the insulin receptor. This phosphorylation is sensitive to rapamycin and to the decrease of S6k1 expression by RNAi (Harrington et al., 2004). In pancreatic β-cell line, chronic exposure to high glucose levels reduces IRS2 expression following the activation of mTORC1 (Briaud et al., 2005). Over-expression of mTOR causes an increase of the phosphorylation of IRS2 as detected by gel mobility shift, leading to its proteasomal degradation. However, expression of a kinase-dead mTOR reduces the Ser/Thr phosphorylation of IRS2 and protects against its proteasomal degradation (Briaud et al., 2005). In addition to down regulating IRS1 levels by S6K, recent studies have pointed to the effect of S6K1 on PKB activation through the phosphorylation of mTORC2 component, rictor, at Thr^{1135} (Dibble et al., 2009; Julien et al., 2009; Treins et al., 2009).

1.9.3. YY1

The activity of mTOR is essential to control mitochondrial oxidative function. Inhibition of mTORC1 by rapamycin or knocking down of one of mTORC1 components, mTOR and raptor, reduces mitochondrial gene targets that is regulated by Peroxisome-proliferator-activated receptor coactivator (PGC)-1α and decreases the oxygen consumption and mitochondrial DNA content (Cunningham et al., 2007). Constitutive active mTORC1 in $TSC2^{-/-}$ cells exhibits an increase in the expression of mitochondrial genes. Further investigations have identified YY1, a transcription factor, as a substrate of mTORC1 (Cunningham et al., 2007). It was shown that mTOR and raptor are able to interact with YY1 to control the function of PGC-1α and in turn, the expression of mitochondrial genes (Cunningham et al., 2007).

1.9.4. Signal transducer and activator of transcription 3 (STAT3)

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which transmits signals from stimulated cytokines and certain receptor tyrosine kinases such as growth factor-receptors located in the plasma membrane, to the nucleus to modulate transcription of genes involved in vital cell function such as cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses (Johnston and Grandis, 2011). STAT3 is phosphorylated on Tyr^{705} in response to cytokine stimulation, however for full transcriptional activity of STAT3, it is further phosphorylated at Ser^{727} (Johnston and Grandis, 2011). It was revealed that mTOR is the kinase responsible for phosphorylation of STAT3 at Ser^{727} and hence the full activation of STAT3 (Yokogami et al., 2000). Thus, it implicates a role of mTOR in regulation of transcription mediated by STAT3.

1.9.5. Serum and Glucocorticoid-inducible kinase (SGK1)

The serum- and glucocorticoid-inducible kinase SGK1 plays an important role in regulation of several cell functions including cell metabolism, transport, transcription and apoptosis. SGK1 is regulated by a wide variety of signalling pathways (reviewed in (Lang et al., 2009)). Several studies have pointed to the role of mTORC1 and/or mTORC2 in regulation of SGK1. It was demonstrated that mTOR phosphorylates

SGK1 at Ser^{422} which consequently leads to the phosphorylation of cyclin-dependent kinase inhibitor, p27, at Thr^{157} in a rapamycin dependent manner. This suggests that SGK1 is a potential substrate of mTORC1 (Hong et al., 2008). Interestingly, the authors further showed that knocking down of rictor or raptor suppresses the phosphorylation of both SGK1 and p27 proposing that both mTORC1 and mTORC2 are involved in regulating SGK1 (Hong et al., 2008). In contrast, a several reports have shown that SGK1 is an mTORC2 substrate (Garcia-Martinez and Alessi, 2008; Lu et al., 2011; Lu et al., 2010).

1.10. Amino Acid Transporters

Amino acid transporters are active transport systems found at the surface of the cells and are able to transport amino acids against a concentration gradient as generally the intracellular amino acid concentration are higher or equal to the extracellular amino acid concentration (McGivan and Pastor-Anglada, 1994). Table 1.3 summarizes the classification of amino acid transporters according to the specificity of their substrates, transport mechanism and their distribution. The transport systems A, ASC and L are expressed in all cell types (McGivan and Pastor-Anglada, 1994).

Amino acid transporters fall in two major categories; those that couple amino acid flux with Na^+ influx such as the System A transporters of the Slc38 gene family and those acting as amino acid exchanger coupling the cellular uptake of essential amino acids with the efflux of cytoplasmic amino acids such as the LAT1 and LAT2 System L transporters of the Slc7 gene family. In the first case, the sodium gradient is maintained via the activity of Na^+, K^+ ATPase which utilises the energy produced from a single ATP to drive three sodium ions out of the cell and two potassium ions into the cell (Lingrel and Kuntzweiler, 1994). Thus, the sodium dependent active amino acid transporters use this sodium gradient to accumulate amino acids across the plasma membrane via secondary active transport (Figure 1.12).

Amino acid exchangers such as System L, ASC and y+L function independently of Na+ or other ions and act as tertiary active transporters (Figure 1.12). They regulate the uptake of extracellular amino acids against a concentration gradient using intracellular short-chain neutral amino acids (including glutamine) supplied by secondary active transporters as an efflux substrates. Thus, the operation of this kind of systems is highly dependent on the activity of secondary active transporters (Hyde et al., 2003).

System L operates as an obligatory 1:1 amino acid exchanger that uses intracellular amino acids such as L-glutamine as an efflux substrate to regulate the uptake of extracellular essential branched-chain and aromatic neutral amino acids. Therefore, they can modify the overall intracellular amino acid concentration without any change

in the total amino acid concentration on either side of the cell membrane (Verrey, 2003).

Several reports have documented the coupling between sodium-dependent transporters and sodium-independent exchangers (Meier et al., 2002; Nicklin et al., 2009; Ramadan et al., 2007). For example, it has been shown that there is a cooperation between ASCT2 also known as Solute carrier family 1 member 5 (SLC1A5) and LAT1 also known as Solute carrier family 7 member 5 (SLC7A5) in the regulation of mTORC1, autophagy and cell growth (Nicklin et al., 2009). First, ASCT2 is responsible for the accumulation of intracellular L-glutamine and LAT1 utilises the transmembrane gradient of L-glutamine to drive branched chain and essential amino acids (mainly L-Leucine) influx. Thus L-glutamine is considered as a rate limiting step for essential amino acid mediated activation of mTORC1.

Another example was presented by Ramadan et al who observed a functional combination of aromatic amino acid transporter TAT1 (SLC16A10) and neutral amino acid exchanger LAT2 (SLC7A8). TAT1 supplies LAT2 with the efflux substrates to control the intracellular concentration of neutral amino acids (Ramadan et al., 2007).

In this way sodium dependent transport systems such as System A can control indirectly the intracellular concentration of branched chain and essential amino acid which it does not transport itself. This was evidenced by prolonged exposure of L6 cells to α-methylaminoisobutyric acid (Me-AIB), a synthetic non metabolisable substrate. selective for System A, results in depletion of intracellular levels of System L substrates (Bevington et al., 2002).

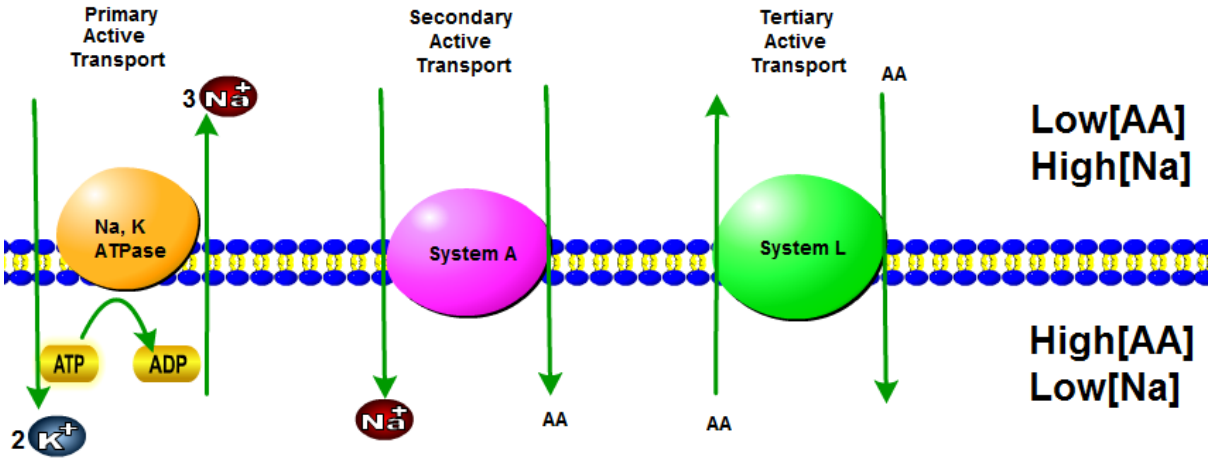
Table 1.3 Amino acid transporters

(ai) Neutral-amino acid transporters: sodium-dependent				
System	**Protein**	**Gene**	**Amino acid substrates (one letter code)**	**Distribution**
A	SNAT1	SLC38A1	G, A, S, C, Q, N, H, M, T, MeAIB, P, Y, V	
	SNAT2	SLC38A2	G, P, A, S, C, Q, N, H, M, MeAIB	widespread
	SNAT4	SLC38A4	G, P, A, S, C, N, M, H, K, R	
ASC	ASCT1	SLC1A4	A, S, C	widespread
	ASCT2	SLC1A5	A, S, C, T, Q	
BETA	GAT1	SLC6A1	GABA	
	GAT2	SLC6A13	GABA, Betaine, P, β-A	widespread
	GAT3	SLC6A11	GABA, Betaine, taurine	
	BGT1	SLC6A12	GABA, Betaine	
	TAUT	SLC6A6	Taurine	
Gly	GLYT1	SLC6A9	G, sarcosine	Liver, erythrocyte, brain
	GLYT2	SLC6A5	G, sarcosine	
IMINO	-	-	P	-
N	SN1	SLC38A3	Q, N, H	Liver
	SN2	SLC38A5	Q, N, H, S, G	
N^m	-	-	Q, N, H	
N^b	-	-	Q, N, H	
PHE	-	-	F, M	
PROT	PROT	SLC6A7	P	
(aii) Neutral-amino acid transporters: sodium independent				
Asc	Asc1	SLC7A10	G, A, S, C, T	
	Asc2		G, A, S, T	

imino	PAT1/LYAAT1	SLC36A1	P, G, A, β-A, GABA Me-AIB	
	PAT2/LYAAT2	SLC36A2	P, G, A, β-A, GABA Me-AIB	
L	LAT1	SLC7A5	H, M, L, I, V, F, Y, W, Q	
	LAT2	SLC7A8	A, S, C, T, N, Q, H, M, L, I, V, F, Y, W	Widespread
T	TAT1	SLC16A10	F, Y, W	
(bi) Anionic-amino-acid transporters: sodium-dependent				
X^-_{AG}	EAAT1	SLC1A3	E,D	
	EAAT2	SLC1A2	E, D	
	EAAT3	SLC1A1	E, D, C	Widespread
	EAAT4	SLC1A6	E, D	
	EAAT5	SLC1A7	E, D	
(bii) Anionic-amino-acid transporters: sodium-independent				
x^{-c}	xCT	SLC7A11	E,Ci	Hepatocytes, fibroblasts
-	XAT2	-	D, E	
(ci) Cationic-amino-acid transporters: sodium-dependent				
$B^{o,+}$	ATB(o,+)	SLC6A14	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W	Blastocysts, Xenopus oocytes, Tibroblasts
y^+L	y+LAT1	SLC7A7	K, R, Q, H, M, L	
	y+LAT2	SLC7A6	K, R, Q, H, M, L, A, C	
(cii) Cationic-amino-acid transporters: sodium-independent				
$b^{o,+}$	b(o,+)AT	SLC7A9	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W, Ci	Blastocysts, Xenopus oocytes, Tibroblasts
y^+	Cat-1	SLC7A1	R, K, H	Widespread
	Cat-2	SLC7A2	R, K, H	
	Cat-3	SLC7A3	R,K	
	Cat-4	SLC7A4	Unknown	

Reproduced from (McGivan and Pastor-Anglada, 1994) and (Hyde et al., 2003)

Figure 1.12 Examples of Primary, Secondary and Tertiary active transport



1.10.1. The sodium-coupled neutral amino acid transporters (SNAT)

The sodium-coupled neutral amino acid transporters (SNAT) of the SLC38 gene family are subdivided to System A and System N amino acid transporters. Classically, System A includes three members; SNAT1=SLC38A1, SNAT2=SLC38A2, SNAT4=SLC38A4 and System N includes two members; SNAT3=SLC38A3, SNAT5 =SLC38A5 (Mackenzie and Erickson, 2004). Six additional orphan transporters termed SNAT6 (SLC38A6) to SNAT11 (SLC38A11) have been recently identified (Sundberg et al., 2008). Although the physiological role of orphan or unidentified members of the SLC38 is not clear, they show broad expression profile within different tissues (Sundberg et al., 2008).

1.10.1.1. Regulation of sodium-coupled neutral amino acid transporters

System A transporters regulates Na^+- dependent transport of small, aliphatic amino acid including alanine, asparagine, cysteine, L-glutamine, glycine, methionine and serine as well as Me-AIB (Mackenzie and Erickson, 2004). The activity of System A transporters is pH sensitive. Inhibition of System A activity was observed when the extracellular pH is lowered below 7.4 (Bevington et al., 2002; Chaudhry et al., 2002). On the other hand, increases in extracellular pH above 7.4 raise System A activity (Munoz et al., 1992). The sensitivity of System A transport activity to changes in pH was explained by the protonation of histidine residues in the system A carrier (Munoz

et al., 1992). Furthermore, changes in the concentration of extracellular amino acids can regulate the activity of System A, a phenomenon termed adaptive regulation (Ling et al., 2001). Another phenomenon that also characterises System A transporters is trans-inhibition in which the amino acid uptake is inhibited by intracellular accumulation of their substrate (Bracy et al., 1986).

System N transporters have a limited number of substrates compared to System A as they favours the influx of glutamine, histidine and asparagine (Mackenzie and Erickson, 2004). Like System A, they are Na^+ dependent, pH sensitive (Baird et al., 2006) and subject to adaptive regulation (Kilberg et al., 1980). They differ from System A in that the uptake of their substrates is coupled to Na^+-H^+ exchange; i.e the influx of one Na^+ and one amino acid in exchange for efflux of one proton (Broer et al., 2002) . MeAIB is a poor substrate of System N transporters (Umapathy et al., 2005).

1.10.1.2. Distribution of SNAT

System A subfamily

SNAT1 (formerly known as ATA1, GlnT, SA2, SAT1, or mouse NAT2) is highly expressed in the brain particularly in ependyma glutamatergic and GABAergic neurons, retina, heart, placenta and adrenal gland. Its preferred substrates are L-glutamine, alanine, asparagine, cysteine, histidine, and serine (Mackenzie and Erickson, 2004). Its functional role is not clear but it may be implicated in neuronal excitability due to its predominant expression in the central nervous system.

SNAT2 has a broad expression profile including brain, spinal cord (neurons), placenta, adrenal glands, testis, thymus, muscle, liver, intestine, kidney, lung, adipose, spleen and skin. The preferred SNAT2 substrates are alanine, asparagine, cysteine, glutamine, glycine, histidine, methionine, proline and serine (Mackenzie and Erickson, 2004).

SNAT4 is predominantly expressed in liver but also detected in skeletal muscle and kidney. It favours the influx of alanine, asparagines, cysteine, glycine, serine and

threonine. It is worth mentioning that L-glutamine is not among the favoured substrates of this transporter (Mackenzie and Erickson, 2004).

System N subfamily

SNAT3 (also known as SN1) is abundant in astrocytes in the brain as well as retina and can be also found in liver, kidney and adipose tissue. It can transport only glutamine, asparagine and histidine (Mackenzie and Erickson, 2004).

SNAT5 (also referred to as SN2) mediates the transport of glutamine, asparagines, histidine, and serine and in some species, it can also participate in glycine and alanine influx. It is expressed in stomach, brain, liver, lung, small intestine, spleen, colon and kidney (Mackenzie and Erickson, 2004).

In pancreatic islets of Langerhans, the relative expression of System A transporter especially SNAT2 is predominant to N transporters (Gammelsaeter et al., 2009). This was confirmed by work in our lab as it was demonstrated that rat pancreatic islets express the five isoforms of SNATs with SNAT2 as the major abundant isoform while in mouse pancreatic islets only SNAT2 was detected (Archana Nair and Terry Herbert, unpublished data).

1.10.2. SNAT2

SNAT2 is the most abundant expressed System A in mammalian cells. SNAT2 transports its substrates in symport with Na^+ with a 1:1 stiochiometry and uses the electrochemical gradient of Na^+ to accumulate amino acids to the cytosol. The accumulated intracellular amino acids can be the driving force for transmembrane transport of essential amino acids such as leucine by System L which in turn may play role in protein synthesis, metabolism and cell growth (Hundal and Taylor, 2009).

1.10.2.1. Regulation of SNAT2

The activity of SNAT2 is subject to strict regulatory control at both transcriptional and post-translational level. An increase in SNAT2 activity is observed following amino

acid deprivation, experimental diabetes and treatment of cyclic cAMP agonists in certain cell types including hepatocytes and skeletal muscle cells (McGivan and Pastor-Anglada, 1994).

An important feature of SNAT2 transporters is that its expression and hence its activity is increased in response to amino acid withdrawal, a process referred to as “adaptive regulation”. It occurs in two phases: a) acute phase involves the translocation of SNAT2 from the intracellular compartments to the cell membrane when increased uptake of amino acids is required, b) chronic phase requires increase synthesis of SNAT2 transporters (Jones et al., 2006). The induction of the SNAT2 mRNA requires eukaryotic initiation factor 2 α (eIF2α) phosphorylation (Gaccioli et al., 2006) as well as activation of the extracellular signal-regulated protein kinase (ERK) 1/2 pathway (Franchi-Gazzola et al., 1999). The phosphorylation of eIF2α, mediated via the activation of general control non-derepressible -2 (GCN2), increases the translation of mRNA encoding activating transcription factor 4 (ATF4) and hence increase transcription of ATF4 dependent genes, of which SNAT2 is one. It was also found that SNAT2 5’untranslated region (5’UTR) contains an internal ribosome entry sequence (IRES) that can be constitutively active under amino acid depleted conditions. Therefore, despite any global decrease in protein synthesis under amino acid starvation status, the SNAT2 mRNA can be efficiently translated via a cap-independent mechanism (Gaccioli et al., 2006).

SNAT2 activity can be also modulated by hormones such as insulin and growth factors. However, this increase in SNAT2 activity is not dependent on the increase of gene expression. SNAT2 carriers are constitutively recycled at the cell membrane thus its regulation relies on the level of exocytosis and endocytosis. It was demonstrated that insulin-induced stimulation of SNAT2 activity is via an increase in the recruitment of SNAT2 carrier from intracellular compartment to the plasma membrane. This was evidenced using chloroquine (CQ), a reagent that disrupts endosomal function through impairing the trafficking of endosomal transferrin receptors to the cell membranes. CQ inhibits the recruitment of SNAT2 carriers from their internal compartment to the cell surface as well as the rise in SNAT2 activity following insulin treatment. Insulin signalling to PKB was not affected by CQ

treatment suggesting that the inhibitory effect exerted by CQ on insulin-stimulated SNAT2 exocytosis was due to the inhibition occurring at the intracellular compartment (Hyde et al., 2002). The molecular mechanism that explains how insulin signalling increases SNAT2 activity is not fully understood. However, there is some evidence that it involves the PI3K and PKB signalling pathways. The stimulatory effects of insulin on SNAT2 activity can be blocked by an inhibitor of PKB (AKTi) (Green et al., 2008) and the expression of a constitutively active form of PKB mimics the insulin effect on SNAT2 activity (Hajduch et al., 1998).

In addition to insulin, there are other hormones and growth factors that can modulate SNAT2 activity. Short-term treatment with glucagon and EGF can up-regulate SNAT2 activity in liver cells by causing membrane hyperpolorization. Catecholamines and glucocorticoids in liver parenchymal cells, and adrenaline in rat hepatocytes, also increase SNAT2 activity (McGivan and Pastor-Anglada, 1994). The stimulatory effect exerted by adrenaline was inhibited by actinomycin D and cycloheximide suggesting that it is mediated via an increase in transcription and translation of SNAT2 genes (McGivan and Pastor-Anglada, 1994).

1.10.2.2. Physiological function of SNAT2

1.10.2.2.1. Regulation of cell volume

SNAT2 activity affects the intracellular concentration of amino acids which constitute a major portion of cell organic osmolytes. Modification of SNAT2 activity influences the composition of intracellular amino acid pool and in turn the cell volume (Franchi-Gazzola et al., 2006). Up-regulation of SNAT2 under hypertonic conditions will lead to accumulative transport of amino acids which result in recovery of the cell volume. The mechanism of this response is similar to the chronic adaptive regulation, i.e it relies on the synthesis of new SNAT2 carriers (Franchi-Gazzola et al., 2006). Key evidence for the involvement of SNAT2 in cell volume recovery is obtained using RNA interference experiments to suppress the expression of transporters. As expected, in silenced cells the basal activity of SNAT2 is inhibited and the intracellular concentration of amino acid pool is considerably reduced. Most importantly, the up-regulation of SNAT2 in response to hypertonic stress is inhibited

in cells transfected with SNAT2 siRNA and hence the volume recovery is delayed (Bevilacqua et al., 2005).

Experimental evidence, provided for muscle and liver cells, indicated that an increase of extracellular concentration of amino acids will lead to cell swelling. This cellular response to increased amino acid loading is functionally important as it drives protein and glycogen synthesis and prevents proteolysis (Hyde et al., 2003). On the other hand, amino acid starvation causes remarkable cell shrinkage due to efflux of amino acids from the cell. Interestingly, the cell responds by up-regulation of SNAT2 via an increase in its synthesis (Gaccioli et al., 2006).

Upon exposure to hypotonic environment, cell swelling occurs as a result of the osmolic movement of water into the cell. Adaptation involves down regulation of SNAT2 leading to decrease of cell volume (Franchi-Gazzola et al., 2006).

1.10.2.2.2. Regulation of insulin secretion

Stimulation of insulin secretion is a major role of a number of amino acids including glutamine; the major substrate of SNAT2 transporter (Newsholme et al., 2007). Alone glutamine does not stimulate insulin secretion but it was reported to enhance glucose-stimulated insulin secretion (Newsholme et al., 2003). Glutamine may potentiate glucose-induced insulin release as a result of being metabolized into glutamate. Glutamine metabolism may initially elevate the cellular content of ATP, leading to closure of the ATP-sensitive K^+ (K_{ATP}) channels, depolorization of the plasma membrane, activation of the voltage-gated calcium channels, Ca^{+2} influx and insulin exocytosis. Moreover, Glutamine is co-transported with Na^+ via SNAT2 carrier so it may cause membrane depolorization occurring due to Na^+ transport and in turn, enhance insulin secretion by activating voltage gated Ca^{+2} channels (Newsholme et al., 2007). In islets, glutamine can be metabolized to γ-amino butyric acid (GABA) which is considered as an important modulator of insulin secretion in pancreatic β-cells (Newsholme et al., 2003).

An evidence for the role of glutamine in enhancing amino acid insulin secretion was demonstrated by Li et al. It was shown in this study that glutamine can be generated

endogenously in the β-cells when glucose is metabolised to provide glutamate and ATP to act as substrates for glutamine synthase to produce glutamine (Li et al., 2004). Treatment with methionine sulfoximine, a potent glutamine synthetase inhibitor, blocks insulin release in response to a glucose ramp. Such effect is reversed by glutamine or its non-metabolizable analogue 6-diazo-5-oxo-L-norleucine (DON) (Li et al., 2004). Therefore, the authors postulated that glutamine plays an important role in amino acid and glucose stimulated insulin secretion.

Indirectly, the activity of SNAT2 can regulate insulin release by maintaining the intracellular pool of branched amino acids including Leucine through coupling with L-system (maintain tertiary active transport system). Leucine can also induce insulin secretion by 2 main mechanisms; first, its metabolism to α-ketoisocaproate (KIC) to provide intermediates for tricarboxylic acid cycle (TCA) with the concomitant release of ATP that can regulate K_{ATP} channels, induce membrane depolorization and result in an increase of free cytosolic Ca^{+2}, which then triggers insulin secretory granules exocytosis, Second, leucine can enhance glutaminolysis by allosterically activating glutamate dehydrogenase, a key enzyme to control oxidation of glutamate (Yang et al., 2010).

Moreover, glutamine can induce the secretion of GLP-1 from intestinal L-cells which is driven by membrane depolorization associated with the inward current generated by Na^{+}-coupled glutamine uptake via SNAT2 transporter and elevation of cAMP (Tolhurst et al., 2011).

1.10.2.2.3. Regulation of gene expression

It is well documented that amino acids can regulate gene expression in various tissues and cells. Glutamine, the most abundant free amino acid and main substrate of SNAT2, can modulate the expression of several genes related to vital cell functions (Curi et al., 2005a). It was reported that glutamine can regulate the expression of AP-1 and c-Jun, transcription factors involved in cell division, in IEC-6 (epithelial cells from rat small intestine) and IPEC-J2 (porcine intestine epithelial cell line) cells via the activation of Erk and JNK (Rhoads et al., 1997). Another study performed in neonatal rat cardiomyocytes has demonstrated the stimulation effect of

glutamine on expression of adenylosuccinate synthase (ADSS-1) which can regulate cell proliferation via activation of protein kinase A and mTOR signalling pathways (Xia et al., 2003). Furthermore, glutamine can also induce cardiomyocyte growth and maturation governed by an increase in mRNA levels of contractile proteins including α-myosin heavy chain (α-MHC) and cardiac α-actin (Xia et al., 2003).

In pancreatic β-cell line, chronic treatment with 10 mM glutamine modulates the expression of a number of genes involved in β-cell signal transduction, metabolism, apoptosis and insulin secretion (Curi et al., 2005b). For example, they found that glutamine up-regulate the calcineurin catalytic and regulatory subunit mRNA expression. They also reported a significant glutamine-dependent upregulation of PDX-1 and acetyl-CoA carboxylase at the mRNA level (Curi et al., 2005b). They postulated that glutamine's role in β-cell gene expression may be required for the optimum ability of β-cell to respond to nutrient availability, metabolism, hormonal stimuli of insulin secretion, and regulators of functional integrity.

1.10.2.2.4. Regulation of nutrient signalling

It is now established that amino acids can regulate several signalling pathways including GCN2 and mTOR pathways to modulate vital cell functions. The activity of these amino acids-regulated kinases is driven by the intracellular concentrations of amino acids. The level and composition of intracellular amino acid pool is governed by: a) the activity of amino acid transporter, b) protein synthesis/breakdown and aminoacyl tRNA production, c) amino acid synthesis and degradation (Hundal and Taylor, 2009). SNAT2 may function as a dual transporter/receptor (transceptor) since they can modulate nutrient signalling not only by controlling the intracellular pool but also via their ability to sense changes in the availability of extracellular amino acids (Hyde et al., 2007) Figure 1.13.

A decrease of the intracellular concentration of amino acids results in elevation of uncharged tRNAs and these activate GCN2. GCN2 activation increases the phosphorylation of eIF2α resulting in a reduction of the rate of general translational initiation to repress the global protein synthesis while up-regulating the expression of

a subset of protein including the transcription factor ATF4 that up-regulates genes for amino acids biosynthesis and transport (Kilberg et al., 2005; Zhang et al., 2002).

While a reduction in intracellular concentration of amino acids has a positive effect on GCN2 signalling, a diminished availability of amino acids via amino acid withdrawal or inhibition of transporter activity reduces mTOR signalling. The effect of amino acid starvation on mTORC1 activation was first shown by Hara eta al (Hara et al., 1998). In this study, 2 hours removal of amino acids resulted in inhibition of downstream targets of mTOR; S6K1 and 4EBP1 whereas the re-addition of amino acids restored the activity of mTOR (Hara et al., 1998). A study by Hundal group has pointed to the correlation between SNAT2 activity and mTORC1 activity in L6 muscle cells (Hyde et al., 2005). They demonstrated that inhibition of SNAT2 activity by Ceramide, a product of sphingolipid metabolism and cytokine signalling, is associated with a reduction in mTOR signalling pathway as well as impairment of protein synthesis (Hyde et al., 2005). Another study, also in L6 muscle cells, by the Bevington group demonstrated that the inhibition of SNAT2 with MeAIB or by silencing SNAT2 expression impairs the amino acid- dependent signalling through mTORC1 (Evans et al., 2007).

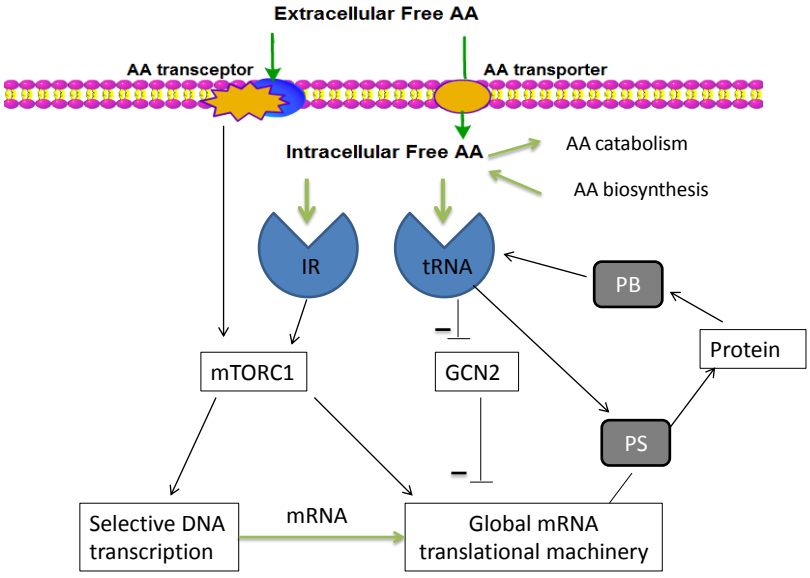


Figure 1.13. Interaction of intracellular free amino acids with major nutrient signalling pathways. IR, intracellular receptor; PB, protein breakdown; PS, protein synthesis.

SNAT2 acts as secondary active transporter, coupling the uptake of amino acids to the influx of Na^+ against its concentration gradient which generates a change in the voltage across the plasma membrane and consequently can activate calcium channels. Both membrane depolorization and the elevation of intracellular calcium may trigger the activation of a number of signalling pathways. For example, treatment of Hela cells with amino acids elevates the intracellular calcium that promotes the association of calmodulin (CaM) to hvps34 (class III PI3K). Such interaction will result in the activation of mTORC1 (Gulati et al., 2008).

It was shown by a few studies a coupling mechanism involving the cooperation between SNAT2 and LAT1 and their role in activating mTORC1 via an increase in the intracellular concentration of leucine (Nicklin et al., 2009; Xu et al., 2001). SNAT2 transports glutamine into the cell and the LAT1 exports this glutamine and increases the influx of leucine. Thus, both SNAT2 and LAT1 activities are correlated with positive mTORC1 activation. This is evident by using inhibitors of SNAT2 and LAT1 which reduce mTORC1 and protein synthesis. In Hela cells, both glutamine starvation and 2-aminobicyclo-(2,2,1)heptanecarboxylic acid (BCH), a potent inhibitor of L-system, reduce mTORC1 activation and consequently impair cell growth and activate autophagy (Nicklin et al., 2009). In pancreatic beta cells, Xu et al demonstrated that the role of Leucine in activation of growth-related protein synthesis is via activation of mTOR signaling pathway (Xu et al., 2001) .

It has been highlighted the importance of AMPK in regulation of mTORC1 activity in pancreatic beta cells. Glutamine was found to cause a marked reduction in AMPK activity via its metabolism, the subsequent elevation of ATP to AMP ratio and phosphorylation of LBK1, a regulator of AMPK activity. This glutamine induced-reduction of AMPK was associated with the activation of mTORC1 (Gleason et al., 2007).

1.11. Thesis Aims

Glucagon like peptide 1 is an incretin hormone secreted in response to nutrient intake from endocrine L-cells. The main action of GLP-1 is its effect to augment glucose-stimulated insulin secretion. GLP-1 also stimulates β-cell proliferation, growth and differentiation and inhibits apoptosis. Many kinases have been shown to be activated in response to GLP-1 including PI3K, PKB, extracellular regulated kinase (Erk) and protein kinase Cζ. Amino acids play a key role as nutritional signals that regulate multiple cellular processes. Two protein kinases that are activated by GLP-1 and amino acids in β-cells, mTOR and PKB, have been the focus of this study. Activation of PKB and mTORC1 has been shown by several studies to stimulate β-cell proliferation and protect cells against cytokine induced apoptosis (Kwon et al., 2004a; Li et al., 2005; McDaniel et al., 2002).

In view of the probable functional importance of GLP-1 and amino acids to pancreatic beta cells, their activation of PKB/mTOR signalling pathway, the principal aim of the work described in this thesis was to study the underlying mechanisms involved in the activation of mTOR/PKB signalling by GLP-1 and amino acids in pancreatic β-cells (clonal pancreatic β-cell lines or rat islets of Langerhans).

CHAPTER 2

Chapter 2: Materials and Methods

2.1 General Reagents and Materials.

Foetal calf serum was purchased from Invitrogen (Carlsbad, CA). [32P]γATP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Collogenase was purchased from (Serva, Heidelberg, Germany). Antibodies were obtained either from Cell Signaling Technology (Beverly, MA, USA) or Santa Cruz Biotechnology, Santa Cruz, CA). Anti-rabbit Ig HRP-linked antibody was purchased from New England Biolabs (Hitchin, UK). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Tissue culture plates and flasks were obtained from Nunc or TPP. Tissue culture pipettes were purchased from Greiner or Corning. Disposable plastics (1.5ml eppendorff tubes, non-filtered pipette tips) were purchased from Sarstedt. Filtered tips were purchased from Axygen. LY294002, wortmannin, rapamycin, AG1478 and forskolin were all purchased from Calbiochem.

Recombinant adenoviruses encoding MKP3 (Rolfe et al., 2005), (constitutively active AMPK, dominant negative AMPK) (Woods et al., 2000) and constitutively active PKB (Hajduch et al., 1998) 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 Cell culture

2.2.1 Maintenance of Cell lines

Rat insulinoma cell lines (INS1E cells) which are a sub-clone of parental INS1 cells (Asfari et al., 1992) were kindly provided by Prof. Pierre Maechler, Central Medical University, Switzerland. INS1E were used between passages 65 and 90 at ~80% confluence. Cells were maintained in a humidified incubator (95% air, 5 % CO_2) at 37ºC in Roswell Park Memorial Institute medium (RPMI 1640) (sigma) supplemented with 11 mM glucose, 2 mM L-glutamine, 5% (v/v) heat inactivated foetal calf serum (FCS), 1 mM sodium pyruvate, 10mM HEPES, 50 µM β-mercaptoethanol, 100 µg/ml streptomycin, 100 units/ml penicillin sulphate and 100 units/ml Neomycin.

Mouse Insulinoma 6 (MIN6) cells which respond to physiological changes in glucose concentrations (Miyazaki et al., 1990) were kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University Medical School, Japan. MIN6 cells were used between passages 30 and 45 at ~80% confluence. MIN6 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 25 mM glucose supplemented with 15% (v/v) heat-inactivated FCS, 100 µg/ml streptomycin, 100 units/ml penicillin sulphate, 100 units/ml Neomycin, 75 mM 2-mercaptoethanol and 40 mM sodium bicarbonate, equilibrated with 5% CO_2 and 95% air at 37 °C.

2.2.2 Cell Splitting

Cells were passaged by trypsinization and subcultured once a week. Upon reaching 80% confluence, media was removed and cells were washed with 1x phosphate buffered saline (PBS) and then 1 ml of 0.5% trypsin/EDTA (Gibco BRL) was added for 3-5 minutes at 37ºC. Cells were split 1:3 for maintenance or as required for experiments. The media was changed every 2-3 days.

2.2.3 Islet Isolation

Male Wistar Albino rats weighing 200g-250g were used for isolation of pancreatic islets. Pancreases were distended by intraductal injection of 6 ml of collagenase solution (1 mg/ml in RPMI 1640 medium) into the common bile duct after occlusion of the distal end, close to the duodenum. Digestion was performed in water bath at 37 °C for 17 minutes. Subsequently, the digestion was stopped by addition of cold RPMI 1640 and the suspension was washed twice from collagenase (RPMI, 1200rpm, 3 min, and 4 °C). After washing, digested tissue was resuspended in RPMI 1640 containing 5% foetal calf serum and filtered through a stainless-steel sieve (0.5 mm mesh pore size), then centrifuged for 3 minutes at 4 °C at 1200rpm. The digest was then resuspended in 10 ml of Histopaque 1077 (sigma) and over-layered with 10ml of RPMI. Islet separation was done by centrifugation for 20 minutes, 2800rpm, 4 °C without brake and very low acceleration. The islets floating on the gradient were collected and washed once in RPMI containing 5% foetal calf serum. To ensure 100 % purity of the preparation, islets were handpicked and counted under an inverted microscope. The islets were dispersed as described later or cultured overnight in Petri´s dishes at 37 °C in humidified 5 % CO_2 in CMRL 1066 medium containing 5.5

mM glucose, 1mM glutamine and 1% bovine serum albumin (100 µg/ml streptomycin, 100 units/ml penicillin sulphate and 100 units/ml Neomycin).

2.2.4 Dispersion of islets

Cells were dispersed from the isolated islets by a modification of the method of (Jonkers and Henquin, 2001). Briefly, the islets were resuspended in calcium free Krebs buffer (Table 2.1). They were then shaken gently for 10 minutes. After centrifugation, this solution was replaced by culture medium, and the islets were disrupted by gentle pipetting in order to disperse them into single cells. Clusters and isolated cells were then cultured in 24 well plate pre-coated with poly-D-Lysine at 37 °C in humidified 5 % CO_2 in RPMI-1640 medium supplemented with 10 % of foetal calf serum and antibiotics (100 µg/ml streptomycin, 100 units/ml penicillin sulphate and 100 units/ml Neomycin).

Table 2.1. Calcium free Krebs Buffer

Reagent	Final concentration
NaCl	138 mM
KCl	5.6 mM
$MgCl_2$	12 mM
HEPES	5 mM
EGTA	1 mM
BSA	0.02%

2.3 Experimentation

2.3.1 Treatment of cell lines

For experiments in chapter 3 and 4

Approximately 16 hours prior to experiments, cells received fresh serum free medium (CMRL 1066 medium) that contained low (5.5mM) glucose. 1 hour prior to treatment, the medium was removed and the cells were washed twice with glucose-free KRB (Krebs–Ringer bicarbonate buffer; 115 mM NaCl, 5 mM KCl, 10 mM

$NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 20 mM HEPES, pH 7.4, supplemented with amino acids (Sigma) and glutamine, to minimize endogenous insulin secretion. The cells were then incubated for a further one hour at 37 °C in KRB (control) or KRB containing different treatments as indicated in figure legends. Inhibitors were added 30 minutes before addition of treatments.

In the calcium-free experiments, the cells were incubated with KRB for one hour then the media was removed, replaced with a nominal calcium KRB buffer containing 100nM calcium and stimulated in the same buffer for another hour.

In experiments when it is required to treat the cells 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.

For SNAT2 Transport Studies in chapter 5

Intact or dispersed islets were cultured overnight in serum- free media (CMRL 1066 medium) containing 1mM glutamine and 1% bovine serum albumin. Cells were incubated in the appropriate test medium from basal medium comprising of Earle's Balanced Salt solution (EBSS) containing 20mM glucose, 1X MEM essential amino acids solution (50X stock of 12 MEM amino acids; L-Arg; L-Cys; L-His; L-Iso; L-Leu: L-Lys; L-Met; L-Phe; L-Thr; L-Typ; L-Tyr; L-Val.), 1% v/v penicillin-streptomycin (100X stock). Varying amounts of $NaHCO_3$ were added to adjust the pH at 7.4. In experiments where the major System L amino acids L-Leu, L-Ile, L-Val, L-Phe, L-Tyr and L-Trp were removed, the following mixture of amino acids (table 2.2) was used instead of 1X MEM essential amino acids solution:

Table 2.2 Stock Concentrates of Amino Acid mixture without system L amino acids

Amino acid	For 50 ml of 50 x AA concentrate(g)
L-Arginine.HCl	0.315
L-Cystine	0.06
L-Glutamine	NONE
L-Histidine.HCl.H2O	0.105
L-Lysine.HCl	0.1825
L-Methionine	0.0375
L-Threonine	0.12

2.3.2 Infection of cell lines with adenoviruses

MIN6 cells or INS1E cells were grown in 4 cm diameter dish at ≈70 % confluency. The growth medium was removed and replaced with 400µl of DMEM (in case of MIN6 cells) or RPMI (in case of INS1E cells) without foetal calf serum and presence of antibiotics. High titre viral stock was then added and the cells were incubated for an hour at 37 °C in humidified 5 % CO_2. 1.2 ml of full medium (containing foetal calf serum and other supplements) was added to the cells. Cells were incubated for a further 48 hours prior to experimentation.

2.3.3 Infection of intact islets of Langerhans with adenoviruses

After isolation of islets as described before, islets were picked in eppendorf tubes containing RPMI-1640 with 10% foetal calf serum and antibiotics. High titre viral stock was then added and the tubes were left for an hour at 37ºC in the incubator with lids open in order to allow air exchange. The tubes were tapped every 10-20 minutes to mix the islets so that they do not become hypoxic at the bottom of the tube. After one hour, islets were then transferred into sterile suspension dishes and cultured at 37 °C in humidified 5 % CO_2 in RPMI-1640 medium supplemented with

10 % of foetal calf serum and antibiotics (100 µg/ml streptomycin, 100 units/ml penicillin sulphate and 100 units/ml Neomycin) for 48 hours prior to experimentation.

2.3.4 Cell Lysis

After treatment, the cells were scrapped off the culture dishes and lysed by the addition of ice-cold lysis buffer (1 % Triton X-100, 10mM β-glycerophosphate, pH 7.4, 1mM EDTA pH 8, 10mM EGTA, 50mM Tris base HCL pH7.5, 0.1% β-mercaptoethanol, 50 mM Na fluoride, 1mM sodium orthovanadate and protease inhibitors; Benzamidine, PMSF, Pepstatin A, leupeptin). Protein lysates were precleared by centrifugation at 14000 rpm and 4°C for 10 minutes and total protein content was determined using Bradford assay (Biorad).

2.4 Bradford assay

2.4.1 Principle

The Bradford protein assay, a spectroscopic analytical procedure, is based on absorbance shift in the Coomassie Brilliant Blue G-250 dye (Bradford, 1976). Under acidic conditions, the red form of the dye is converted to its bluer form to bind to the protein being assayed. First, the red form of coomassie dye donates its free electron to the ionisable groups of the protein leading to a disruption of the protein state which consequently causes the exposure of the hydrophobic groups which bind non-covalently with non-polar part of the dye via van der Waals forces. This binding is further strengthened by an ionic interaction which stabilizes the blue form of the Coomassie dye. The amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading. The bound form of the dye has an absorption spectrum at 595 nm. The unbound forms are green or red. The binding of the dye to the protein stabilizes the blue form. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the concentration of protein present in the sample.

Protein

Red <=> Green <=> Blue <=> Blue-Protein

(470nm) (650nm) (595nm) (595nm)

H^+ H^+

2.4.2 Procedure

Standard solutions were prepared using different concentrations (0, 1.25, 2.5,5, 10 mg/ml) of bovine serum albumin (BSA) and used to construct a standard curve. 2 µl of sample or 10 µl of BSA was incubated with 1 ml of Bradford reagent for 5 minutes. The colour intensity is stable for 30 minutes. The absorbance readings at 595nm were used to assess the protein content of the sample using BSA standard curve.

2.5 RNA Techniques

2.5.1 Preparation of siRNA for silencing of SNAT2

SNAT2-silencing siRNAs was prepared previously by Dr A. Bevington with the siRNA design tool: http://www.ambion.com/techlib/misc/siRNA_finder.html
Then, BLAST searching identified 3 potential target sequences for siRNA silencing within rat SNAT2 mRNA as particularly strong candidates for silencing. These sequences (see Table 2.3) were ordered and purchased from Eurogentec:
http://www.eurogentec.com/product/research-custom-sirna.html

The synthesised oligonucleotides were supplied as pairs of single-stranded siRNAs (forward and reverse strands) which were dissolved in sterile diethylpyrocarbonate (DEPC) - treated water to give 50µM stock solutions.

Table 2.3 Target sequences for silencing rat SNAT2

Candidate Sequences	Directed against the following position in the SNAT2 gene sequence:
5'- AACAUUGGGACAUAAGGCAU	Base position 404 onwards
5'- AACUGACAUUCUCCUCCUCGU	Base position 1095 onwards
5'- AACCCAUGAGAUCCGUGCAAA	Base position 1388 onwards

The siRNA that had the forward sequence 5'-CUGACAUUCUCCUCCUCGUdTdT directed against base position 1095 onward in the gene sequence was identified by Dr Kate Evans as the most effective silencing of SNAT2 transporter activity (14C-MeAIB uptake into myoblasts). Thus, this sequence was used for my experiments. Other sequences for silencing rat IGF receptor, rat insulin receptor and rat SNAT2 were commercially available and were purchased from Dharmacon as shown in Table 2.4.

Table 2.4. Target sequences of different siRNA

	Target sequence
Rat IGF-1R On-Targetplus SMARTpool siRNA (J-091936-10-0010, NM_052807 Dharmacon)	CCGGAUAACUGCCCCGAUA
Rat insulin receptor On-TARGETplus SMARTpool siRNA (J-080102-10-0010, NM_017071, Dharmacon)	CCGUAACGUUCCGGAUGA
Rat SLC38A2 On-TARGETplus SMARTpool siRNA(J-093251-11-0010, Dharmacon)	GGGAUCAUGUAGACGCAAA
scrambled control siRNA	5'-CGCUCUACUCUACUUGUCCdTdT

2.5.2 Small Interfering RNA Transfection of INS1E cells and Islet Cells.

siRNA transfection was performed using Lipofectamine^{TM} 2000 protocol according to the manufacturer's instructions. Briefly, INS1E cells at 30-50% confluency or dispersed islets were seeded into 24 well plates one day prior to transfection in 500µl of growth medium without antibiotics. Oligomer-Lipofectamine complexes were prepared by combining the mixture of (1) 100nM of siRNA in 50µl Opti-MEM and (2) 1 µl of lipofectamine 2000 in 50µl Opti-MEM. Oligomer- Lipofectamine mixture was added drop wise to the wells to be transfected. The plate was rocked back and forth to insure proper mixing. After 4-6 hours, the transfection medium was replaced with culture medium and cells were incubated at 37°C in a CO_2 for 72 hours before experiment.

2.5.3 SNAT2 mRNA determination

2.5.3.1 RNA isolation

At 72 hours after transfection, dispersed islets grown on 24 well plates were washed with ice-cold PBS and processed for RNA extraction using Trizol reagent. After adding 100µl of Trizol reagent to each well, the cells were transferred to 1.5 ml eppendorff tubes. The samples were incubated at room temperature for 2 minutes to allow complete dissociation of nucleoprotein complexes. The procedure was then continued by an addition of 20µl 1-bromo-3-chloropropane. Tubes were vortexed and incubated at room temperature for 2 minutes and centrifuged at 11800 rpm for 15 minutes at 4°C. The RNA was precipitated from the colourless upper aqueous phase in a fresh tube by addition of 50 µl isopropyl alcohol, incubation for 2 hours at -80°C and centrifuge at 14000 rpm for 10 minutes at 4 °C. The RNA pellet was washed with 500 µl of 70% (v/v) ethanol and collected by centrifuge at 14000 rpm for 10 minutes at 4 °C. After a brief air dry and dissolution in 10 µl of RNAase-free water, the RNA was quantified by measuring absorption at 260nm.

2.5.3.2 RT- PCR

Following the manufacturer's instructions, 3µg of total RNA from each extraction was used as template for reverse-transcription with SuperScript™ II Reverse Transcriptase (Invitrogen) using oligo dT in a total reaction volume of 20µl. PCRs were then performed in a 50 l volume containing 0.5 units of Taq DNA polymerase (Promega) using gene-specific primers: rat SNAT2, (693-bp product): forward 5'-TACGAACAGTTGGGACATAAGG-3' and reverse 5'-AGTTCCCACGATCGCAGAGTAG-3'; rat Actin, forward 5'-CCCGCGAGTACAACCTTCT-3' and reverse 5'-CGTCATCCATGGCGAACT -3'. Amplification was performed using the following thermal cycling conditions: an initial denaturation step at 94 °C for 3 minutes followed by a number of cycles (as indicated in figure legends) at 94 °C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension step at 72 °C for 5 minutes. RT-PCR products were resolved on 1.5% w/v agarose and visualised by ultraviolet fluorescence.

2.5.3.3 TAE-Agarose gel electrophoresis

Reagents

1X TAE (Tris-Acetate EDTA)

40mM tris-acetate

1mM EDTA pH 8

6X loading buffer

50% v/v glycerol

0.1M EDTA pH 8

1%w/v SDS

1mg/ml bromophenol blue

1mg/ ml xylene cyanol

1.5 %Agarose (Melford) was dissolved in 1X TAE by heating in the microwave for approximately 2 minutes. Once cooled to about 50-60°C, 0.1µg/ml ethidium bromide was added and the gel was allowed to set. RT-PCR products were prepared by addition of loading buffer and were loaded alongside with 100bp DNA ladder (New England Biolabs). Agarose gels were run horizontally immerced in 1X TAE at 80 volts.

2.6 PI3K kinase assay

Activity of PI3K kinases was assessed by a modification of the method from Howells et al (Hawkins et al., 1997; Howells et al., 2005). PI3K activity was measured in INS1E cells as follows. Cells were treated as stated in the figure legend. After one hour incubation with different treatments, cells were lysed in PBS extraction buffer containing (137mM NaCl, 2.7mM KCL, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM NaF,20mM Tris-base, 0.5mM Na_3VO_4, 0.2mM PMSF, 10% glycerol (v/v), 1% Igepal (v/v), 10mM benzamidine, 10mg/ml aprotinin, 10µg/ml Pepstatin and 10µg/ml leupeptin). The protein content in the lysates was determined using Bradford Protein assay to check that that protein content is 0.5-1 mg protein/ml. 800 µl of the lysate supernatant was incubated with 8µl 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, 4^{o}C. Immunoprecipitates were washed three times with PBS extraction buffer then washed twice with 100mM Tris·HCl (pH 8), 0.5M LiCl and once with 0.15M NaCl, 10mM Tris-HCL (pH 7.6), 100µM and 1mM EDTA followed by another was with 20mM HEPES, 1mM DTT, 5mM $MgCl_2$ (pH 7.6). PI3K activity associated with phosphotyrosine was determined by resuspending the immunoprecipitates in 40µl of kinase assay buffer containing 30mM Beta Glycerophosphate, 5mM sodium pyrophosphate, 30mM NaCl and 1mM DTT (pH7.2) then 20 µl of phosphatidylinositol/cholate substrate was then added. The mix was incubated at 37°C for 5 minutes before addition of 40 µl of ATP mix (3µM Na_2ATP, 7.5 mM $MgCl_2$, and 0.37 MBq γ^{32}P-ATP) and additional incubation for 5 minutes at 37°C. The reaction was stopped by the addition of 450 µL chloroform/methanol (1:2 v/v). This reaction mixture was stored at -20° C till next day. A 150 µL chloroform and 150 µl of 0.1M HCl were added to reaction mixture then another 150 µL chloroform and 150 µl of 0.1M HCl were added to the synthetic phase, which was then dried using eppendorf concentrator 5301(eppendorff, Germany). The dried lipid was resuspended in 25 µl of chloroform: methanol: HCl (200:100:1 by volume) and applied to an oxalate treated silica gel thin-layer chromatography (TLC) plate (VWR). The lipid extract tube was washed out with a further 25ul of Chloroform: Methanol: 0.1M HCl 200:100:1, and this was then spotted onto the TLC plate in the same

position as the original sample. Samples on the TLC plate were then resolved in a chromatography tank containing a pre-equilibrated mixture of 42.9ml methanol, 30ml chloroform, 7.65ml ammonia (29.1%, 15.15M) and 9.45ml H_2O until the solvent front was approximately 1cm from the top of the TLC plate. The plates were then air dried and placed face down on a scientific imaging film (Kodak) in a developing cassette and exposed at -80°C for 72 hours. The ^{32}P-labelled PI(3)P which had migrated on the TLC plate with an Rf value of approximately 0.85 was visualised by autoradiography.

2.7 Amino Acid HPLC Analysis

Intact islets were treated as indicated in figure legends. After 30 minutes of incubation with different treatments, islets were rapidly chilled on ice, rinsed three times with ice-cold 0.9% (wt/vol) NaCl to remove extracellular amino acids, and deproteinized by adding 50 µl of 0.3 M perchloric acid. The resulting lysate was transferred to microcentrifuge tubes on ice, followed by incubation for 30 minutes to allow as much protein as possible to precipitate. Precipitated protein was sedimented (10 min, 4°C, 14000rpm) and retained for total protein assay. Supernatant was filtered through a 0.45µm microfilter and was used for determination of amino acids on an Agilent 1100 high-performance liquid chromatograph with Zorbax Eclipse AAA column (4.6 x 75 mm, 3.5 µm) at 40°C with *o*-phthalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate precolumn derivatization and ultraviolet and fluorimetric postcolumn detection (Evans et al., 2007).

2.8 cAMP assay

INS1E cells were grown to 80% confluency in 24 well plates. Cells were then washed twice and incubated at 37 °C for 1 hour in 450µl Krebs–Ringer bicarbonate buffer; 115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 20 mM HEPES, pH 7.4 containing 1mM glucose, 0.125x amino acid, glutamine, 0.5% bovine serum albumin and 1mM IBMX. After 1 hour, 50 µl of KRB buffer alone or different agonists as indicated in figure legends were added for a further one hour. Reactions were terminated by aspiration of buffer and addition of 400µl ice-cold 0.5M

trichloroacetic acid. Samples were collected to a 1.5ml microfuge tube containing 50µl of 10mM EDTA. 500µl of 50:50 (v/v) mixture of 1,1-trichloro-trifluroethane and tri-n-octyl-amine was then added to each tube. The reaction mixture was then vortexed, left for 15 minutes at room temperature and centrifuged 13000rpm for 2 minutes. 200 µl of the upper phase were transferred to fresh tubes containing 50µl of 60mM $NaHCO_3$. Samples were stored at 4°C prior to assay. The amount of cAMP were determined using a method previously described by Brown et al(Brown et al., 1971). Briefly, standard concentrations of cAMP (0-10 pmol) were diluted in blank buffer (50mM Tris-HCl, 4mM EDTA, pH7.5). In fresh 1.5ml microfuge tubes, 50µl of either standard or sample was mixed with 100µl [3H]-cAMP (34Ci.mmol-1). Reactions were started by addition of 150µl cAMP binding protein and samples were incubated in ice for 90 minutes. Reactions were stopped by adding 250µl ice-cold charcoal (0.25g/ml) and BSA (0.1g/ml) diluted in assay buffer (50mM Tris-HCl, 4mM EDTA, pH 7.5). The reaction mixture were incubated in ice for 12 minutes, vortexed and centrifuged at 14000 rpm for 4 minutes at 4°C. 400µl of the supernatant was transferred to a scintillation tube and mixed with 4.2ml of scintillation liquid. Radioactivity was measured using liquid scintillation counting.

2.9 Insulin secretion assay

The culture media from different experiments were collected and centrifuged at 3000 rpm for 10 min at 4ºC. The supernatant was stored at -20°C until assaying the insulin content by radioimmunoassay. The basis of this technique resides in the ability of insulin to react strongly with insulin-binding antibodies present in guinea pig antibovine insulin serum and by doing so, to inhibit competitively the binding of insulin-^{125}I to antibody (Yalow and Berson, 1960).

2.9.1 Reagents

Borate Buffer:

Reagents	Amount (g/2 litres)	Final concentration
Boric acid	15.6	133.4 mM
EDTA	7.4	10mM
NaOH	5.4	67.5mM
BSA	2	0.1%

Boric acid, EDTA and NAOH were dissolved in 1.8 litres dH_2O then the pH of the solution was adjusted to 8 by 10mM HCL. Final volume was made up to 2 litres with dH_2O before adding BSA.

Polyethylene glycol-based precipitating reagent:
To make 600ml of precipitant, 600mg of γ globulin was dissolved in 300 ml PBS then 300 ml of 30% PEG was added and finally, 300µl of Tween 20 was added.

2.9.2 Procedure

On the first day of the assay, 100 µl of the supernatants diluted with borate buffer were added into a tube then 100 µl of anti-insulin antibody was added. Next, 100 µl (approximately 12,000 cpm) of I^{125} radiolabeled insulin was added to each tube. Samples were vortexed and incubated for 48 hours at 4°C. Standard curve was created using solutions of 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5.0, and 10.0 ng/ml of insulin standard prepared in borate buffer. All standards and controls were run in triplicate and samples were run in duplicate. After 48 hours, 1 ml of a polyethylene glycol-based second antibody precipitating reagent was added. The samples were vortexed, and centrifuged at 3000 rpm for 20 minutes at 4°C. The supernatants were aspirated and tubes were then counted with a gamma counter.

2.10 Protein techniques

2.10.1 SDS- Polyacrylamide Gel Electrphoresis

2.10.1.1 Principle

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is based on the migration of charged molecules in polyacrylamide gel matrix in response to an electric field. By this technique, it is likely to separate a complex mixture of proteins based on their molecular weight and electrical properties as they migrate through a polyacrylamide gel matrix. A polyacrylamide gel is usually used as the solid gel using a recipe giving the desired pore size. A common variant is to use a denaturing gel which contains a detergent, sodium dodecylsulphate.

Heating the protein sample in a boiling water bath in the presence of reducing agent will result in the dissociation of inter- and intra-chain disulfide bonds causing the linearization of proteins. In addition, proteins are coated with a negative charge in the presence of the anionic surfactant SDS. Afterwards, they are separated according to their molecular weight and resolved as discrete bands as they migrate in an electric field through the sieving action of the acrylamide gel matrix. Following electrophoretic separation, protein electroblotting is used for transfer the separated protein bands onto a PVDF membrane for immunoblot assay.

2.10.1.2 Western Blotting solutions:

4x SDS gel-loading buffer, 100 ml
0.25M Tris-HCl, pH 6.8
4% w/v SDS, 4g
40% v/v Glycerol, 40ml
10% v/v β-mercaptoethanol, 10ml
20μg/ml Bromophenol blue

10X Tris-Glycine buffer, 1000 ml
Tris base, 30g
Glycine, 144g

10xPhosphate-buffered saline (PBS), pH 7.4, 1000 ml (adjust to pH 7.4 with HCl)

NaCl, 100 g

KCl, 3 g

Na_2HPO_4, 14 g

KH_2PO_4, 3 g

PBS-Tween (PBST)

1X PBS

0.1% v/v Tween-20

Semi-Dry Transfer Buffer

1X Tris-Glycine buffer

0.01% w/v SDS

20% v/v Methanol

SDS-PAGE Running Buffer

1X Tris-Glycine buffer

0.1% w/v SDS

Stripping buffer, 500ml

10X Tris-Glycine buffer, 50 ml

Methanol, 100ml

10% SDS, 1 ml

2.10.1.3 Casting the gels

The polyacrylamide gel is polymerized in *situ* between two glass plates. The gel is made to a recipe giving suitable porosity so that as the SDS-proteins migrate in the electric field towards the anode, they are mainly separated by molecular sieving; small ones moving fastest.

In practical terms the resolving gel (see Table 2.5) was poured between two glass plates and over-layed with H_2O. Once the resolving gel had set, the H_2O was removed and the stacking gel was poured on top and well combs were immediately inserted in the stacking gel to produce defined sample loading wells.

Table 2.5. Solutions for preparing 2 ATTO systems mingel (12-well)

Solution	Resolving Gel		Stacking Gel
	7.5%	**12.5%**	
40% Acrylamide	2.925 ml	4.725 ml	1.24 ml
2% Bis-acrylamide	1.56 ml	2.52 ml	0.65 ml
1.5M Tris-HCl, pH8.8	3.75 ml	3.75 ml	-
1.0M Tris-HCl, pH6.8	-	-	1.25 ml
Water	6.54 ml	3.78 ml	6.7 ml
10% SDS	0.15 ml	0.15 ml	0.1 ml
NNN'N' tetramethylethylenediamine (TEMED)	7.5 µl	7.5 µl	10 µl
10% Ammonium persulphate (APS)	110 µl	110 µl	75 µl

2.10.1.4 Preparation of samples for SDS-PAGE

The cell lysates (25-50µg of protein per sample) were mixed with (4X) Laemmli SDS sample buffer to give a 1X final concentration. Then, samples were boiled for 3 minutes at 100ºC and then briefly centrifuged on a microcentrifuge at 13,000 rpm.

2.10.1.5 Running the gels

The plates with the gel between them were held vertically with the top and bottom edges of the gel exposed to tanks of the SDS-buffer solution. The comb was removed and the samples were applied with a pipette into the wells under the buffer along with molecular weight markers, Broad Range Markers (NEB). Electrophoresis was performed at 180 volts until the Bromophenol Blue dye front reached the bottom of the gel (approximately 80 min). Gels for S6K1 mobility shift assays were run for an extended period (approximately 2 hours) to allow separation of the phospho-activated and non-activated isoforms of the protein to be more easily visualized.

2.10.2 Western Blotting

The gels were transferred on to polyvinylidine fluoride (PVDF) microporous membranes (Millipore) (15 volts for 38 minutes) using a Semi-Dry Transfer cell (Bio-Rad) and a transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, 0.2% SDS.

2.10.2.1 Blocking the membrane and immunostaining

The resulting blot was then blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for one hour at room temperature to block non-specific binding sites. Afterwards, the blot was washed three times for 10 minutes with 1xPBS containing 0.1% Tween 20 and next incubated with a designed primary antibody in PBS containing 5% bovine serum albumin and 0.1% Tween 20 overnight at 4ºC.

Membranes were then washed with 1xPBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature.

Following three 10-min washes in 1xPBS containing 0.1% Tween 20, membranes were treated with an enhanced chemiluminescence (ECL) reagents (Amersham Bioscience) for 1 minute and exposed to x-ray film at room temperature. Intensities of immunoblot bands were quantified using the SynGene GeneGnome System with gene tools software.

Antibodies

Antibody	Source	Primary Dilution	Secondary antibody
Anti-phospho Akt/PKB (Ser473)	New England Biolabs	1/1000	Rabbit
Anti-phospho Akt/PKB (Thr308)	New England Biolabs	1/500	Rabbit

Anti-Akt/PKB	New England Biolabs	1/1000	Rabbit
Anti-phospho ribosomal protein S6 (Ser240 /244)	New England Biolabs	1/1000	Rabbit
Anti-ribosomal protein S6	Santa Cruz	1/1000	Mouse
Anti-phospho GSK-3 α/β (ser21/9)	New England Biolabs	1/1000	Rabbit
Anti-phospho Fox01 (ser319)/Fox04(ser262)	New England Biolabs	1/1000	Rabbit
Anti-GADPH	Santa Cruz	1/1000	Rabbit
Anti-phospho AMPK (Thr172) antibody	New England Biolabs	1/1000	Rabbit
Anti-AMPK antibody	New England Biolabs	1/1000	Rabbit
Anti-4EBP1	Provided by Ann Willis, University of Nottingham	1/1000	Rabbit
Anti-Erk2 antisera	New England Biolabs	1/1000	Rabbit
Anti-phospho p70S6Kinase (Thr^{389})	New England Biolabs	1/1000	Rabbit
Anti-insulin receptor β	Santa Cruz	1/1000	Rabbit
Anti- Insulin growth factor-I receptor β	Santa Cruz	1/1000	Rabbit
Anti-phospho-PRAS40 (Thr246)	New England Biolabs	1/1000	Rabbit

2.11 eIF4E-containing 5’ mRNA cap complex analysis.

30μl of 7- methyl GTP Sepharose 4B beads (GE Healthcare, Piscataway, NJ, #27-5025-01)) per sample were washed twice in 1 ml of lysis buffer, centrifuged at 800 x g for 1 minute and the supernatant discarded. 100μl of cell lysate was added to the beads to allow binding for one hour at 4°C. The Pelleted beads were washed 3 times in the lysis buffer before proteins were released from the beads by boiling the samples for 3 minutes in 2x Laemmli buffer. Total eluate was resolved by 12.5% SDS-PAGE gels. Levels of eIF4E, eIF4G and 4EBP1 levels contained in the eluates were analyzed by western analysis.

2.12 Statistical Analysis

Results are expressed as means ± SEM. Data were analyzed by one-way analysis of variance, ANOVA, followed by Bonferroni correction for all pair-wise comparisons. Significance was assigned at $P< 0.05$.

CHAPTER 3

Chapter 3: Regulation of Mammalian Target of Rapamycin by Glucagon Like Peptide-1 in Pancreatic β-cells

3.1 Introduction

3.1.1 mTOR signalling in pancreatic β-cells

A large number of evidence has revealed that mTOR/S6K plays a central role in the control of insulin secretion and glucose homeostasis through regulation of β-cell size. *Rip-Tsc1*cKO mice, in which TSC1 gene is deleted, display an increase in β-cell mass, cell size and insulin content, and these effects are reversed by rapamycin (Mori et al., 2009). Similar results are obtained from mice with conditional deletion of *Tsc2* in pancreatic β-cells ($\beta Tsc2^{-/-}$). These mice are characterized by hypoglycaemia, hyperinsulinaemia and improved glucose tolerance that results from expansion of β-cell mass mediated via an increase in proliferation and cell size (Rachdi et al., 2008). Similar manifestations were reported in young mice deficient in TSC2, however older $\beta TSC2^{-/-}$ mice display progressive hyperglycaemia and hypoinsulinaemia concomitant by a decrease in islet mass due predominantly to a decrease in the number of β-cells (Shigeyama et al., 2008). Furthermore, transgenic over-expression of Rheb exhibits increased release of insulin and enhanced glucose tolerance that result from β-cell expansion associated with an increase in β-cell size (Hamada et al., 2009). Collectively, these data suggest that constitutive activation of mTORC1 has a positive impact on insulin secretion and β-cell mass. On the other hand, mice treated with rapamycin or knockouts of the mTORC1 target S6K1 ($S6K1^{-/-}$) display a reduction in β-cell mass and a decrease in glucose-stimulated insulin secretion, that leads to hypoinsulinaemia and glucose intolerance (Fraenkel et al., 2008; Pende et al., 2000). It was demonstrated in INS1E cells that over-expression of either a rapamycin resistance S6K1 or increased expression of eIF4E increase β-cell survival in conditions where AMPK is activated by exposure of cells to low concentrations of glucose and this effect is augmented when both constructs were co-expressed together (Cai et al., 2008).

Numerous studies have documented the role of nutrients including glucose and amino acids to modulate pancreatic β-cell function and insulin secretion. It was shown that glucose endorses the ability of IGF to phosphorylate S6K1. Furthermore,

it was demonstrated that glucose mediates β-cell proliferation via activation of mTOR/S6K1 (Briaud et al., 2003). In rat islets, glucose also stimulates the phosphorylation of 4EBP1 in a time and concentration dependent manner (Xu et al., 1998b). Blocking insulin signalling, by either wortmannin (PI3K inhibitor) or incubation of islets at 24°C to inhibit insulin secretion, suppresses glucose-induced phosphorylation of 4EBP1 (Xu et al., 1998b). Therefore, the authors postulate that glucose induced mTORC1 stimulation is mediated by the autocrine effect of insulin. They also demonstrated that essential amino acids are crucial for either glucose or exogenous insulin to activate mTORC1 and essential amino acids alone can stimulate the phosphorylation of 4EBP1 in a rapamycin dependent manner (Xu et al., 1998b). Additional study by the McDaniel group showed that MDL-12330A, an adenylyl cyclase inhibitor, blocks glucose stimulated phosphorylation of S6K1 in a dose dependent manner and without affecting glucose stimulated insulin secretion (Kwon et al., 2004a). This suggests an additional mechanism of glucose stimulated activation of mTORC1 independent of insulin secretion. This was further supported by the use of either forskolin, an artificial activator of adenylyl cyclase or exentide, GLP-1 receptor agonist, which was found to induce S6K1 phosphorylation in a rapamycin sensitive manner. It has been shown that cAMP can metabolize Ca^{+2} from intracellular stores in MIN6 cells, a pancreatic β-cell line (Tsuboi et al., 2003). Chelation of Ca^{+2} by BAPTA suppresses exentide stimulated S6K1 phosphorylation (Kwon et al., 2004a). Collectively, the authors suggest that metabolism of amino acids and glucose results in the production of ATP. ATP inhibits K_{ATP} channel leading to depolorization of cell membrane and activation of Ca^{+2} channel and influx of Ca^{+2} to stimulate Ca^{+2}-induced Ca^{+2} release from intracellular stores. This rise in intracellular Ca^{+2} leads to upregulation of mitochondrial dehydrogenase with enhanced production of ATP to act as a fuel for mTOR (Kwon et al., 2004a).

Another possible mechanism for activation of mTOR by cAMP is via AMPK inhibition. It was demonstrated in INS1 cells that glucose dependent insulinotropic polypeptide (GIP) increases the production of cAMP and inhibits AMPK activity (Hurley et al., 2006). In addition, agents that elevate cAMP such as forskolin and IBMX can also inhibit AMPK activity evident by their ability to reduce the phosphorylation at Thr^{172} (Hurley et al., 2006). Inhibition of AMPK by cAMP was shown to be mediated by the

inhibition of CaMKKα and/ or CaMKKβ, which are AMPK upstream kinases, yet it is LKB1 independent (Hurley et al., 2006).

3.1.2 Aims

Hormones and growth factors signal to mTORC1/S6K1 to regulate cell growth and proliferation. In pancreatic β-cells, it was revealed that the phosphorylation and the activation of S6K of ribosomal protein S6 plays an important regulatory role in the control of cell size. In addition, GLP-1 was demonstrated to activate mTOR and stimulate β-cell growth and proliferation. Nevertheless, the mechanism by which GLP-1 stimulates the phosphorylation of rpS6 in pancreatic β-cells is not clear yet. This study aims to determine the signalling pathway that is responsible to mediate GLP-1 phosphorylation of rpS6.

3.2 Results

3.2.1 GLP-1 stimulates glucose-dependent phosphorylation of rpS6 in pancreatic β-cells

I initially characterized the temporal dynamics of the activation of rpS6 in response to glucose and GLP1 in INS1E cells, a pancreatic β-cell line that is sensitive to known modulators of insulin secretion and responds to glucose at physiologically relevant glucose concentrations (Asfari et al., 1992). I also looked at insulin signalling to rpS6 since both glucose and GLP-1 treatment lead to insulin secretion and it was reported that glucose induces the phosphorylation of 4EBP1 via the autocrine effect of insulin (Xu et al., 1998b), so may be the effects of glucose and GLP-1 on rpS6 are mediated via the autocrine effect of insulin. INS1E cells were serum starved for overnight in CMRL-1066 containing glutamine. One hour prior to treatment, medium was removed, washed twice and incubated with modified KRB containing 0.25x amino acids. Cells were then treated with 7.8 mM glucose, 7.8 mM glucose plus 10 nM GLP-1 or 100 nM insulin for the specific times as indicated in figure 3.1. Glucose induced the phosphorylation of rpS6 at Ser240/244, a downstream target of S6K and a marker of mTOR activation, after 30 minutes. Phosphorylation of rpS6 at Ser240/244 gradually increased after 20 minutes of GLP-1 treatment reaching maximal levels by 60 minutes (Figure 3.1B). On the other hand, I did not observe any phosphorylation of rpS6 in INS1E cells treated with insulin (Figure 3.1C). Glucose and GLP-1 also stimulated the phosphorylation of potential activators of mTOR pathway including PKB and GSK3 (Figure 3.1B) and Erk1/2 (Figure 3.2A). GLP-1 induced phosphorylation of p70S6K1 at Thr389 paralleled the phosphorylation of rpS6 at Ser 240/244 (Figure 3.2A). Similar results were observed in rat islets of Langerhans in which Exendin, a GLP-1 receptor agonist, augmented glucose induced phosphorylation of rpS6 at Ser240/244, PKB at Ser473 and PRAS40 at Thr246 (Figure 3.2B). Increased phosphorylation of PKB at both sites; Ser473 and Thr308 was evident by 20 minutes following addition of these agents and sustained for 1 hour (figure 3.1 A, 1B and 1C). There was also an increase in the phosphorylation of FoxO1 and GSK-3 which matched the phosphorylation of PKB in cells treated with glucose or glucose plus GLP-1. FoxO1 and GSK-3 were also phosphorylated in response to insulin treatment. However, GSK-3 was rapidly dephosphorylated after 20 minutes in cells treated with insulin. Therefore, other

kinases may phosphorylate GSK-3. Indeed, PKB, PKA, $p90^{RSK}$/MAPKAP kinase-1 and p70 ribosomal S6 kinase have all been shown to phosphorylate GSK-3 (Forde and Dale, 2007).

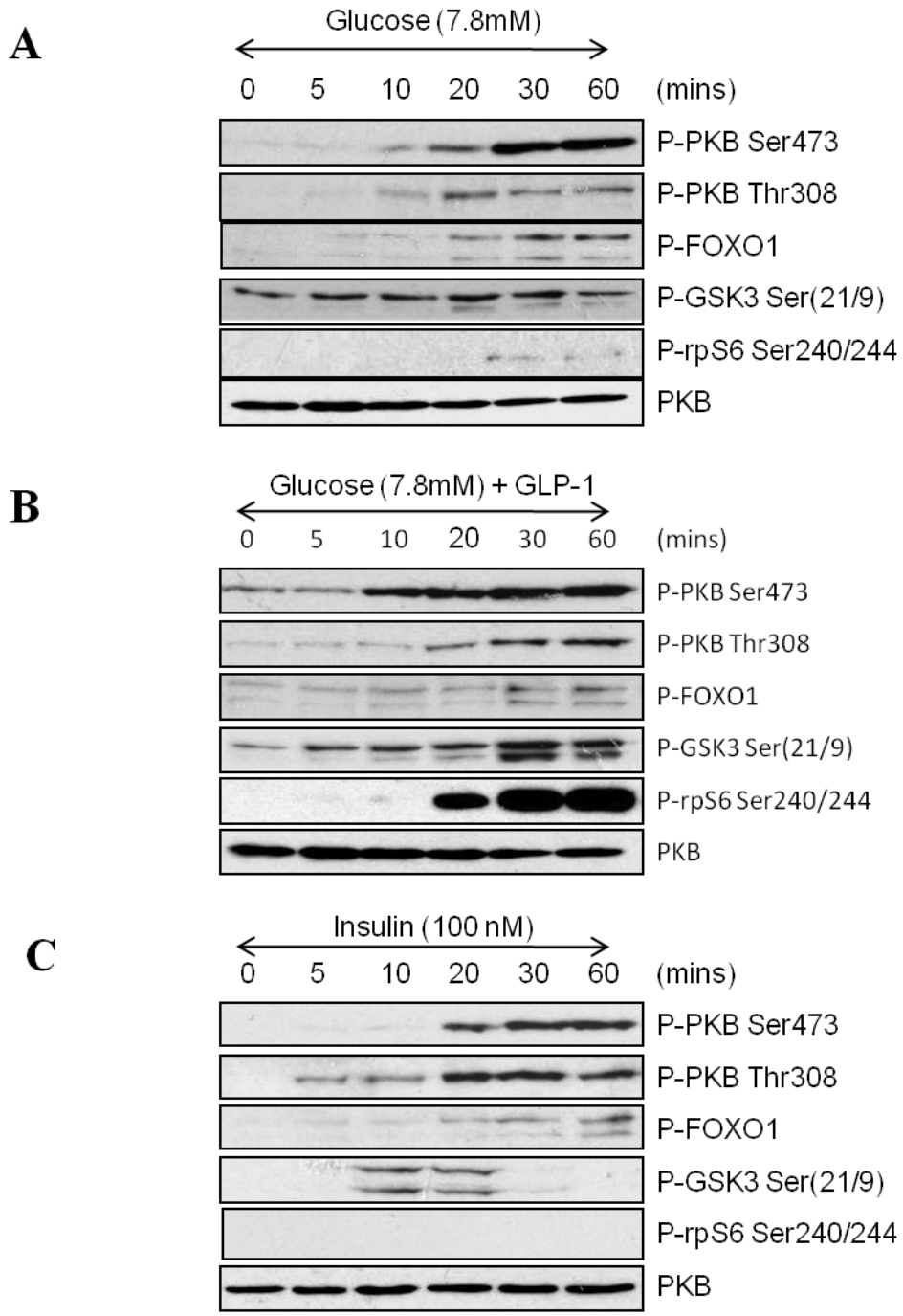


Figure 3.1. Time course of important modulators of the PKB/mTOR signalling pathway in response to glucose, glucose plus GLP-1 and insulin treatments in INS1E cells. INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to treatment, the medium was removed and replaced with KRB containing 0.25x amino acids. The cells were then treated with (A) glucose (7.8 mM) or (B) glucose (7.8mM) plus GLP-1 (10nm) or (C) insulin (100 nM), Cells were lysed after 0, 5, 10, 20, 30 and 60 minutes of treatments. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB (Ser473 or Thr308), phospho-GSK-3 Ser21/9, phospho-FOXO1. PKB is the loading control. Results are representative of three experiments.

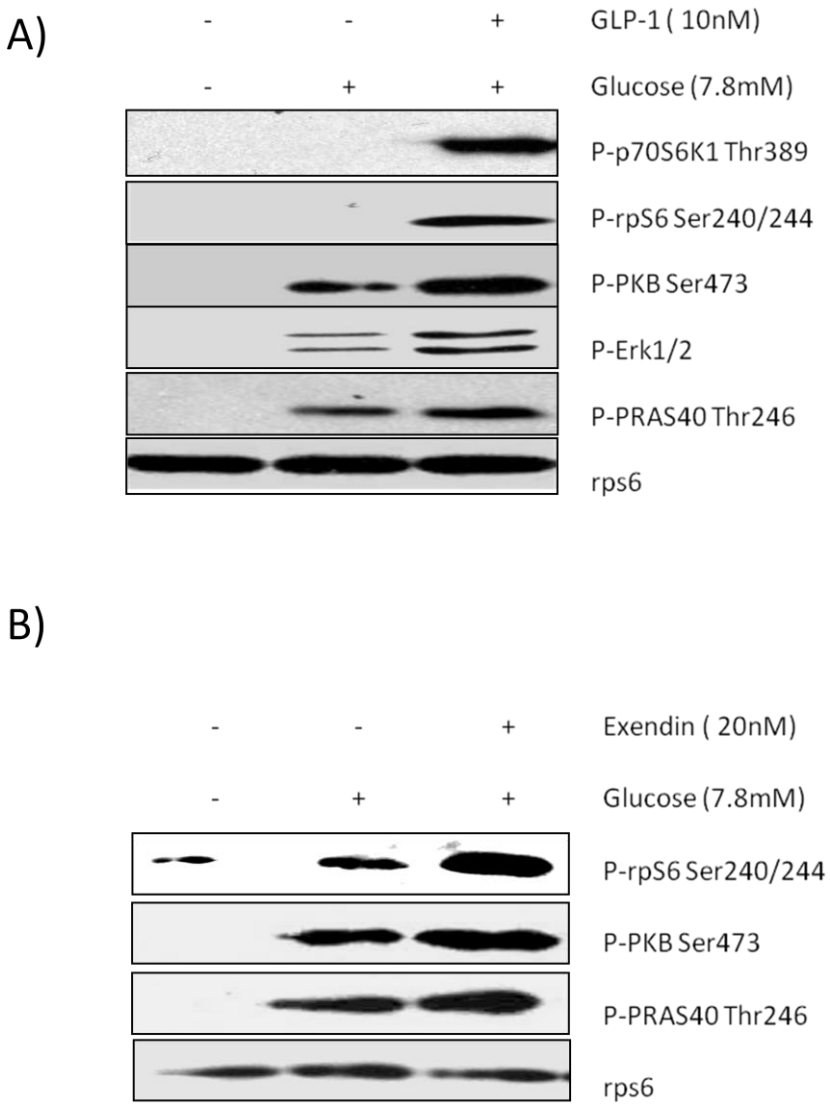


Figure 3.2. Glucose and glucose plus GLP-1 activate PKB/mTOR signalling pathway in INS1E and rat Islets of Langerhans. (A) INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to treatment, the medium was removed and replaced with KRB containing 0.25x amino acids and glutamine. Cells were then treated with glucose (7.8 mM) or glucose (7.8mM) plus GLP-1 (10nm), Cells were lysed after 60 minutes of treatments. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-S6K1 Thr389, phospho-rpS6 Ser 240/244, phospho-PKB Ser473, phospho- PRAS40 Thr246, phospho-Erk1/2 and rpS6. (B) Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, the medium was removed and replaced with KRB containing 0.125x amino acids. Cells were treated with glucose (7.8mM) or glucose (7.8mM) plus Exendin (20nM). Results are representative of three experiments.

3.2.2 Role of cAMP in GLP-1 induced rpS6 phosphorylation

Binding of GLP-1 to its receptor leads to the activation of adenylate cyclase and the generation of cAMP. To evaluate the role of cAMP in regulation of mTOR signalling in pancreatic beta cells, I used forskolin, an activator of adenylyl cyclase, to mimic the effect of GLP-1 on mTOR activation. I first characterised the effect of cAMP on mTOR signalling. INS1E cells were treated with increasing concentrations of forskolin which led to a dose-dependent phosphorylation of PKB and rpS6 (Figure 3.3A). The addition of IBMX, a non specific inhibitor to all phosphodiesterase isoforms, inhibited forskolin stimulated PKB activation but augmented the forskolin effect on the phosphorylation of rpS6 which appeared to parallel the phosphorylation of Erk1/2. The effect of forskolin in the presence or absence of IBMX on insulin secretion was also determined. Increasing concentrations of forskolin stimulated the secretion of insulin in a dose-dependent manner, an effect that was significantly potentiated in the presence of IBMX (Figure 3.3 B). These results indicate that cAMP can stimulate rpS6 phosphorylation via a mechanism which is independent of PKB activation but possibly dependent on insulin secretion.

To investigate whether phosphorylation of Erk mediates cAMP activation of mTOR, INS1E cells were incubated with MEK inhibitor PD184352 (1.25 μM) before treatment with glucose (7.8mM) or glucose (7.8mM) plus GLP-1 (10nM) or forskolin (10μM) plus IBMX (1μM). PD184352 reduced the phosphorylation of both rpS6 and PKB induced by glucose, glucose plus GLP-1 and forskolin. As a control, I assessed the effect of PD184352 on the phosphorylation of Erk. As shown in figure 3.4, PD184352 effectively blocked Erk phosphorylation induced by glucose, glucose plus GLP-1 and forskolin. However, it has been recently shown that PD184352 activates AMP-activated protein kinase (AMPK) (Moore et al., 2009) and increased AMPK activity was found to block glucose-stimulated mTORC1 signalling, which in turn activates S6K in β-cells (Gleason et al., 2007). Therefore, I used recombinant adenovirus encoding mitogen-activated protein kinase phosphatase 3 (MKP3) to inhibit Erk signalling. As seen in figure 3.5B, overexpression of MKP3 effectively blocked glucose plus GLP-1 and forskolin-induced Erk phosphorylation. Yet, overexpression of MKP3 had no effect on glucose plus GLP-1or forskolin-induced rpS6 phosphorylation. Therefore, it can be concluded that the phosphorylation of

rpS6 induced by glucose or glucose plus GLP-1 is not dependent on the activation of Erk.

To determine whether the release of cAMP and the activation of rpS6 by GLP-1 is dependent on glucose effect on membrane depolarization and under conditions that prevent insulin secretion, INS1E cells were incubated in KRB containing 2.5mM of calcium and 0.25x amino acid or K50 KRB to depolarize the cell membrane and contains 100nM of calcium (nominal calcium) that prevents the influx of extracellular Ca^{+2} across the plasma membrane as there would be equal concentrations of the intracellular and the extracellular calcium to counteract the effect of secretion. The intracellular levels of cAMP were detected using radioimmunoassay with Dr Yan Huang help in performing this assay in INS1E cells treated with GLP-1(10nM) or glucose (7.8mM) plus GLP-1 and under conditions that mimics the depolarization of cell membrane without influx or release of calcium. GLP-1 solely increased the intracellular levels of cAMP independent to glucose and membrane depolarization (Figure 3.6A). However, in absence of glucose GLP-1 was not able to phosphorylate rpS6 at Ser240/244 (Figure 3.6B). Further, depolarizing the membrane without in/efflux of calcium was not sufficient to mediate GLP-1 phosphorylation of rpS6 (Figure 3.6B). Thus, GLP-1 requires glucose to stimulate the phosphorylation of rpS6 and the released cAMP in response to GLP-1 is not sufficient to activate rpS6 and suggesting the importance of calcium influx in the activation of rpS6.

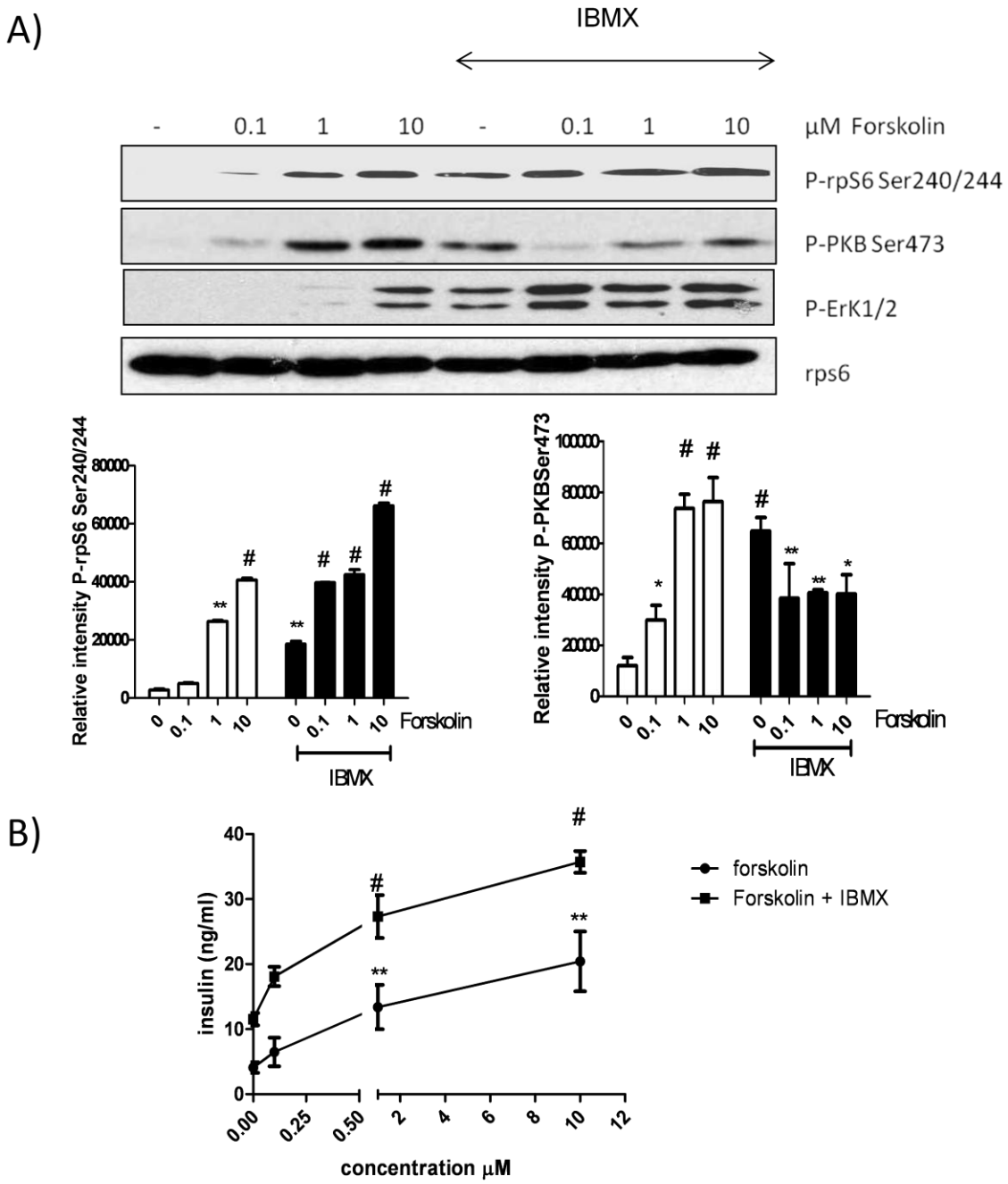


Figure 3.3. Role of cAMP in PKB/mTOR signalling pathway. INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to treatment, the medium was removed and replaced with KRB containing 0.25x amino acids. Cells were treated with different concentrations of forskolin in the presence or absence of IBMX (1µM). Lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser473, or phospho-Erk1/2. The lower panel shows quantified data of phosphorylated rpS6 Ser 240/244 and phosphorylated PKB Ser473. (B) Secretion of insulin by INS1E cells exposed to different concentrations of forskolin or forskolin plus IBMX (1µM), as indicated. The results are mean ± S.E.M for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and #, $P < 0.001$, compared with control.

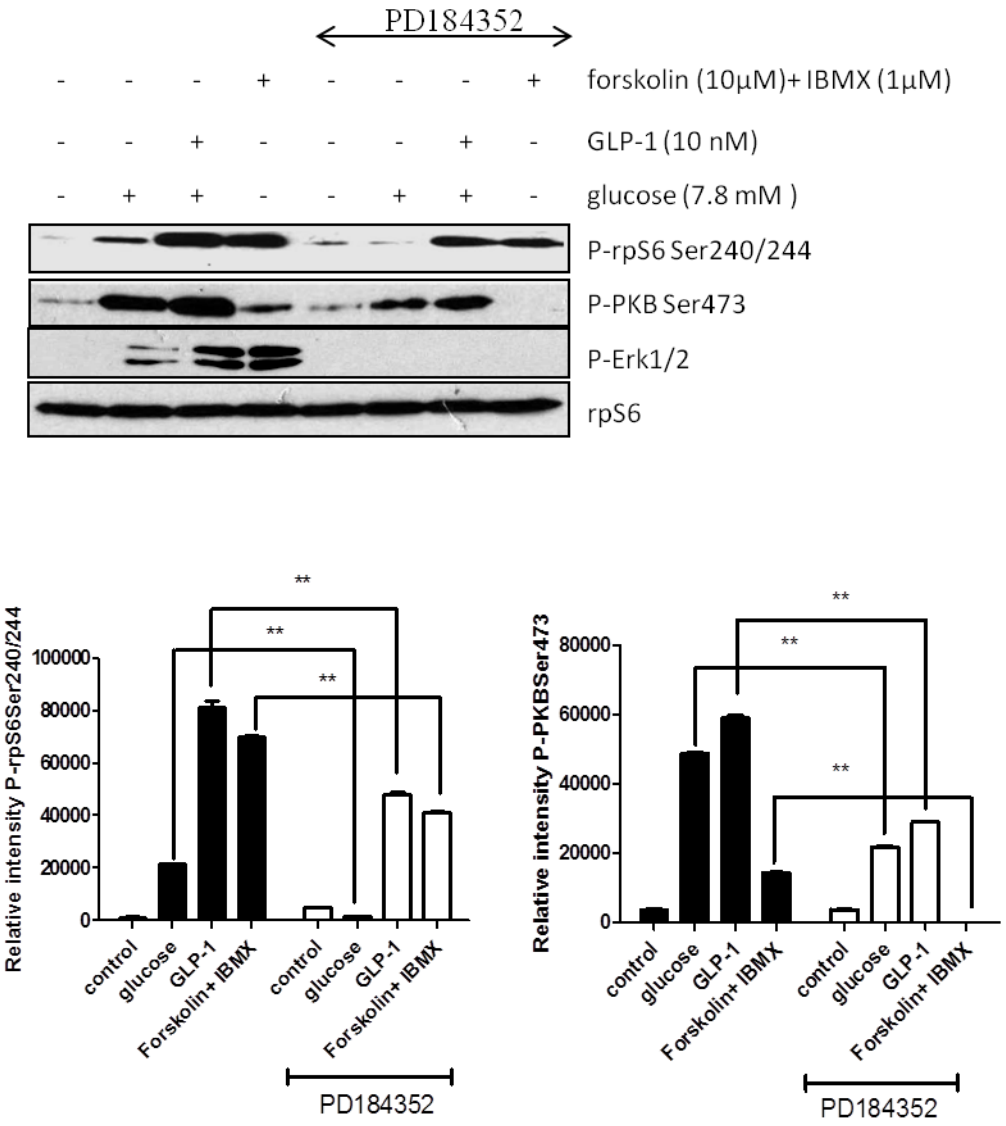


Figure 3.4. Effect of PD184352 on glucose plus GLP-1 induced phosphorylation of rpS6 and PKB. INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, cells were incubated with KRB containing 0.25x amino acids and then incubated for 30 minutes with PD184352 (1.25 µM) before treatment with glucose (7.8 mM) or glucose (7.8 mM) plus GLP-1 (10nM) or forskolin (10µM) plus IBMX (1µM). Whole-cell lysates were then analysed by Western blotting using specific antibodies. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244 and phosphorylated PKB Ser473. The results are mean + S.E.M for three independent experiments. *, P< 0.05 and **, P < 0.01;compared with their respective treatments in absence of PD184352.

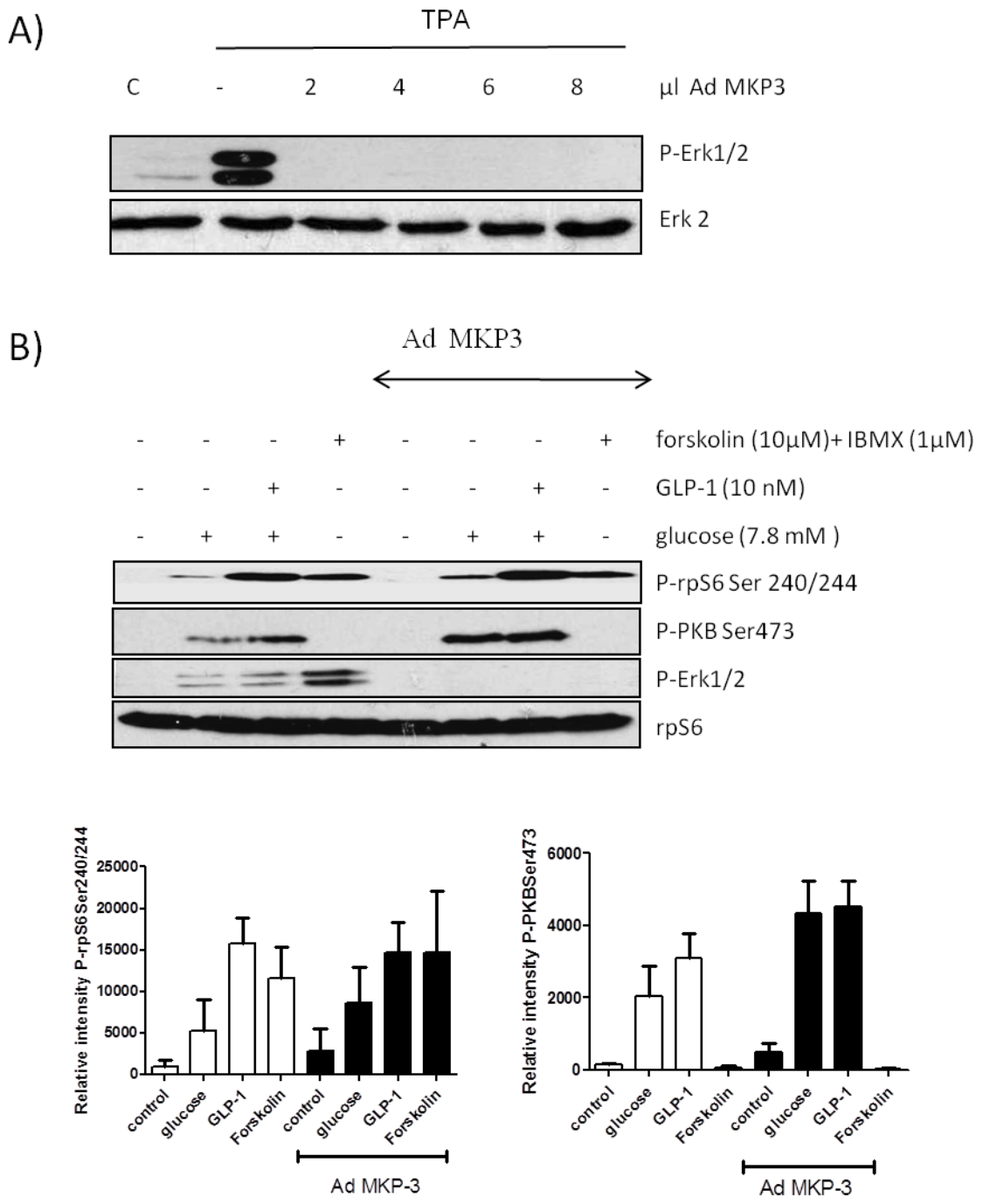


Figure 3.5. Phosphorylation of rpS6 is independent to Erk activation. A) Titration of Ad MKP3 virus in INS1E cells.(B) INS1E cells were uninfected or infected with the recombinant adenovirus expressing MKP3 (AdMKP3) for 48 h. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. 1h prior to stimulation, cells were incubated with KRB containing 0.25x amino acids and then incubated for 1 h with glucose (7.8 mM) or glucose (7.8 mM) plus GLP-1 (10nM) or forskolin (10µM) plus IBMX (1µM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser473 or phospho-Erk1/2. The lower panel shows quantified data of phosphorylated rpS6 Ser 240/244 and phosphorylated PKB Ser473. The results are mean ± S.E.M for three independent experiments.

3.2.3 Role of calcium in GLP-1 induced activation of mTOR signalling pathway

Glucose metabolism increases the ATP/ADP ratio that in turn induces Ca^{+2} influx through voltage gated Ca^{+2} channel and causes Ca^{+2}-induced Ca^{+2} release from intracellular stores. This effect is augmented by GLP-1. To further investigate the role of calcium in GLP-1 stimulated mTOR activation, INS1E cells were treated with the calcium intracellular chelator, BAPTA-AM which resulted in inhibition of GLP-1 stimulated rpS6 and PKB phosphorylation. In contrast, BAPTA did not affect forskolin (an activator of adenylyl cyclase) stimulated rpS6 phosphorylation (figure 3.7).

Likewise, INS1E cells were incubated in KRB containing 2.5mM of calcium and 0.25x amino acid or KRB containing 100nM of calcium (nominal calcium) that prevents the influx of extracellular Ca^{+2} across the plasma membrane as there would be equal concentrations of the intracellular and the extracellular calcium in presence or absence of thapsigargin which depletes Ca^{+2} stores. Then, cells were treated for an hour with 7.8mM glucose or 7.8mM glucose plus GLP-1. GLP-1 induced activation of rpS6, S6K1 and PKB was reduced when cells were pre-incubated in KRB containing nominal calcium or nominal calcium plus thapsigargin (Figure 3.8A). Similar results were confirmed in another pancreatic β-cell line, MIN6 cells (Figure 3.8B). While the pre-treatment with thapsigargin alone did not affect either glucose or GLP-1 induced activation of rpS6 and PKB in MIN6 cells, it reduced the phosphorylation of PKB and rpS6 in INS1E cells.

To confirm the results in primary cells, pancreatic rat islets were incubated in KRB containing 2.5mM of calcium and 0.125x amino acid or KRB containing 100nM of calcium in presence of thapsigargin. Then, cells were treated for 1h with 7.8mM glucose or 7.8mM glucose plus Exendin (20nM). Exendin induced activation of rpS6 and PKB was reduced significantly when cells were pre-incubated in KRB containing nominal calcium plus thapsigargin (Figure 3.9B). In contrast, incubation of islets in KRB containing nominal calcium plus thapsigargin had no effect on the phosphorylation of rpS6 and mobility shift of 4EBP1 induced by treatment with forskolin and IBMX (Figure 3.9C). I also measured insulin secreted in the media upon treatment the rat islets with glucose, Exendin, forskolin and IBMX for one hour following the incubation of the islets in either KRB containing 2.5mM of calcium and 0.125x amino acid or KRB containing 100nM of calcium in presence of thapsigargin.

A significant reduction of released insulin in response to glucose or exendin was observed when the islets were incubated in KRB containing 100nM of calcium in presence of thapsigargin (the left panel in figure 3.9).

Collectively, this data points to the importance of extracellular influx of Ca^{+2} to mediate the GLP-1 stimulation to PKB/mTOR. On the other hand, rises in intracellular Ca^{+2} is not required for forskolin induced rpS6 phosphorylation.

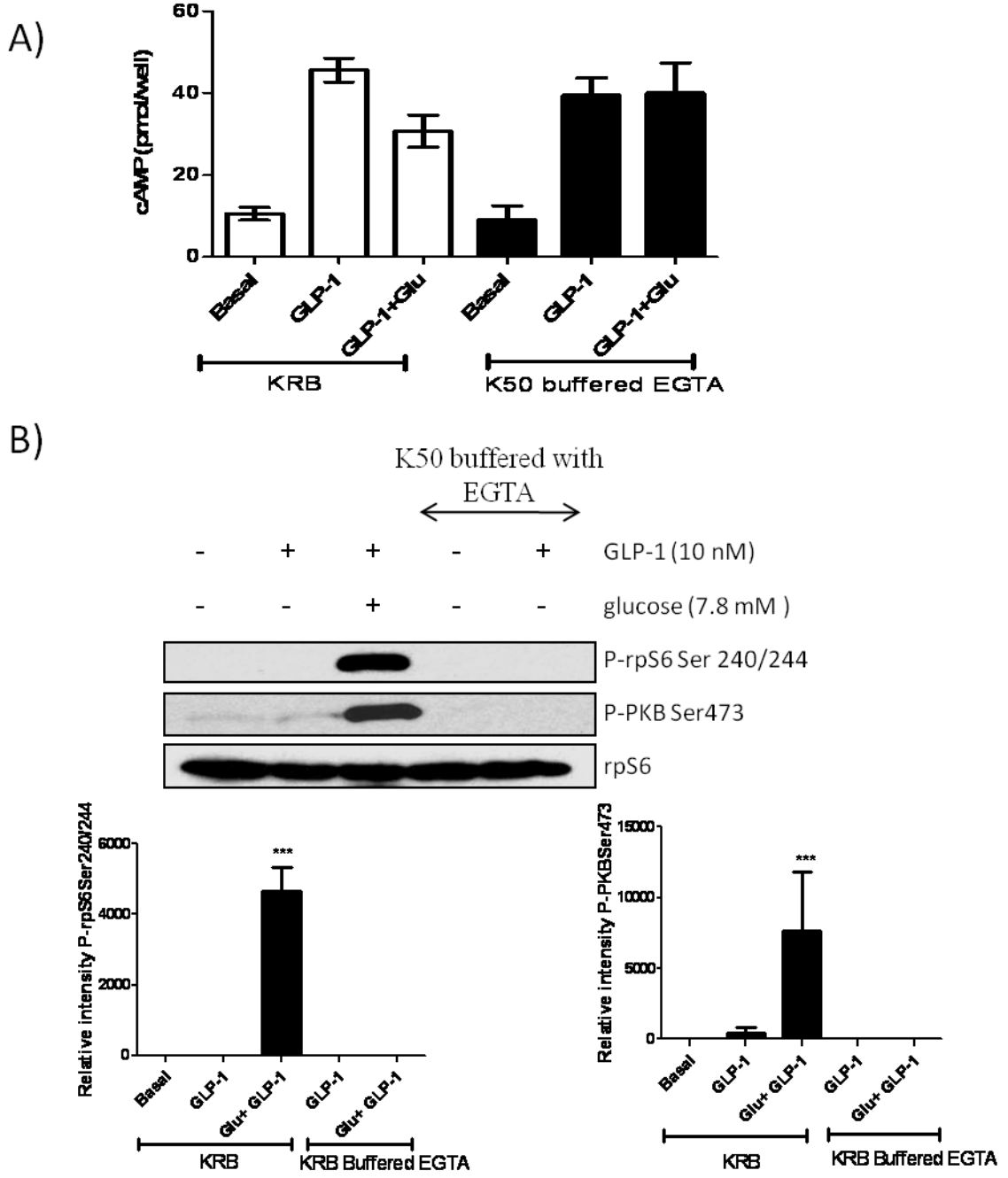


Figure 3.6. GLP-1 can produce cAMP independent to glucose or Calcium influx however this cAMP is not sufficient to activate PKB/mTOR pathway. INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to treatment, the medium was removed and replaced with KRB containing 0.25x amino acids. Cells were then treated for one hour with 10 nM GLP-1 or 7.8 mM glucose plus GLP-1(10nM) in presence or absence of KRB buffered with EGTA to contain 100nM calcium. (A) Total intracellular cAMP was measured as described in the material and methods (B) Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244 and phospho-PKB Ser473. The lower panels show quantified data of phosphorylated rpS6 Ser 240/244 and phosphorylated PKB Ser473. The results are mean $\pm$ S.E.M for three independent experiments. ** P< 0.01, ***P < 0.001, compared with control.

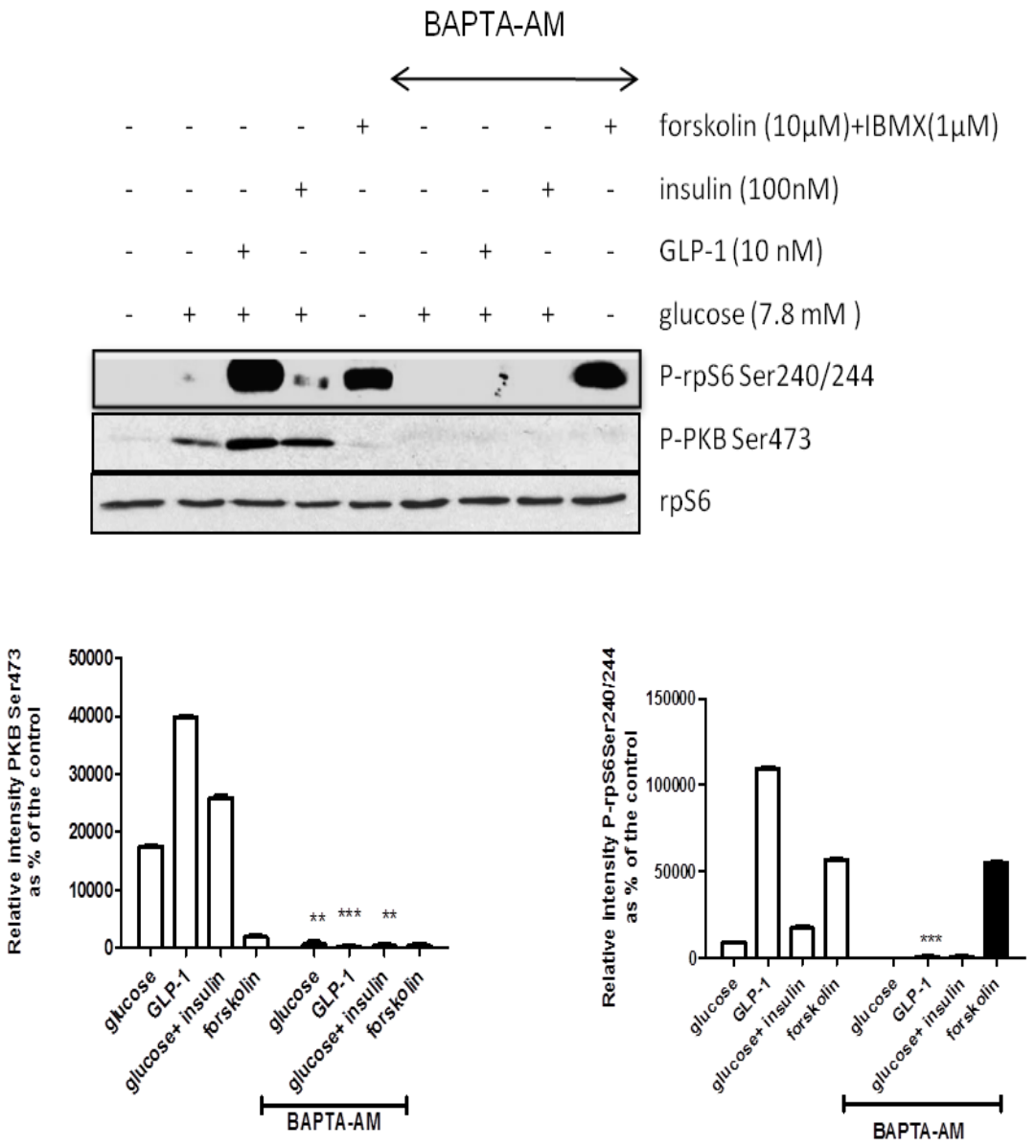


Figure 3.7. The release of intracellular calcium may be involved in GLP-1 induced phosphorylation of rpS6 Ser 240/244 and PKB in INS1E cells. INS1E cells were serum starved overnight and then incubated for 1 hour in modified KRB containing 0.25x amino acids. Cells were treated for a further one hour with 7.8 mM glucose, 7.8 mM glucose plus 10nM GLP-1, 11.1mm glucose plus 100 nM insulin or 10 µM forskolin plus IBMX (1µM) in the presence or absence of BAPTA-AM (100µM). Whole-cell lysates were then analysed by Western blotting using specific antibodies. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244 and phosphorylated PKB Ser473. The results are mean ± S.E.M for three independent experiments. .**, P< 0.01 and ***, P <0.001 compared with the corresponding treatment in absence of BAPTA.

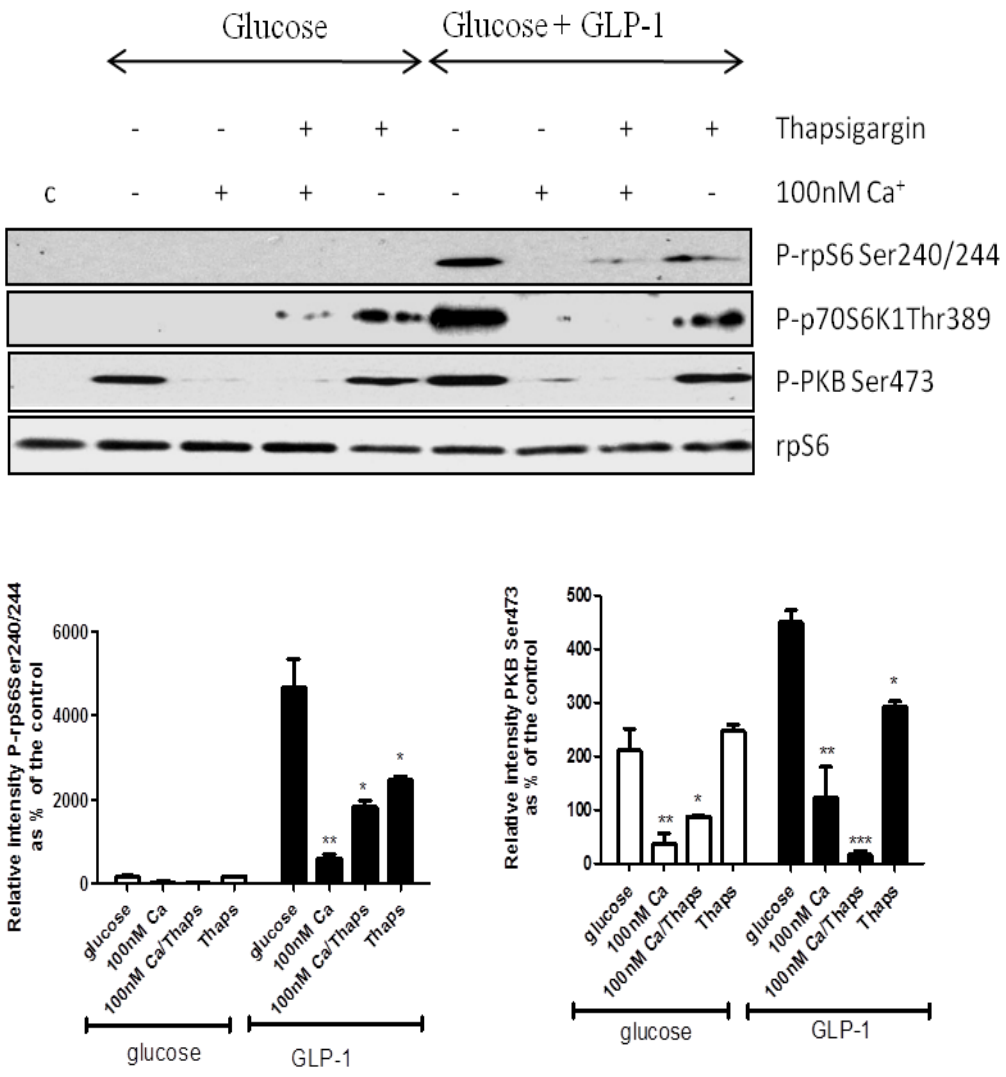


Figure 3.8A. The release of intracellular calcium may be involved in GLP-1 induced phosphorylation of rpS6 in INS1E cells. INS1E cells were pre-incubated for 1 h with KRB containing (0.25x) amino acids**.** Cells were then treated for one hour with, 7.8 mM glucose, 7.8 mM glucose plus GLP-1 in presence or absence of KRB containing nominal calcium (100nM) or nominal calcium in the presence of 1 µM thapsigargin. Cells were then lysed. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phosho-rpS6 Ser240/244, phospho-S6K1 Thr389 and phospho-PKB Ser473. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244 and phosphorylated PKB Ser473. The results are mean $\pm$ S.E.M for three independent experiments. *, P<0.05, **, P< 0.01 and ***, P <0.001 compared with the corresponding treatment.

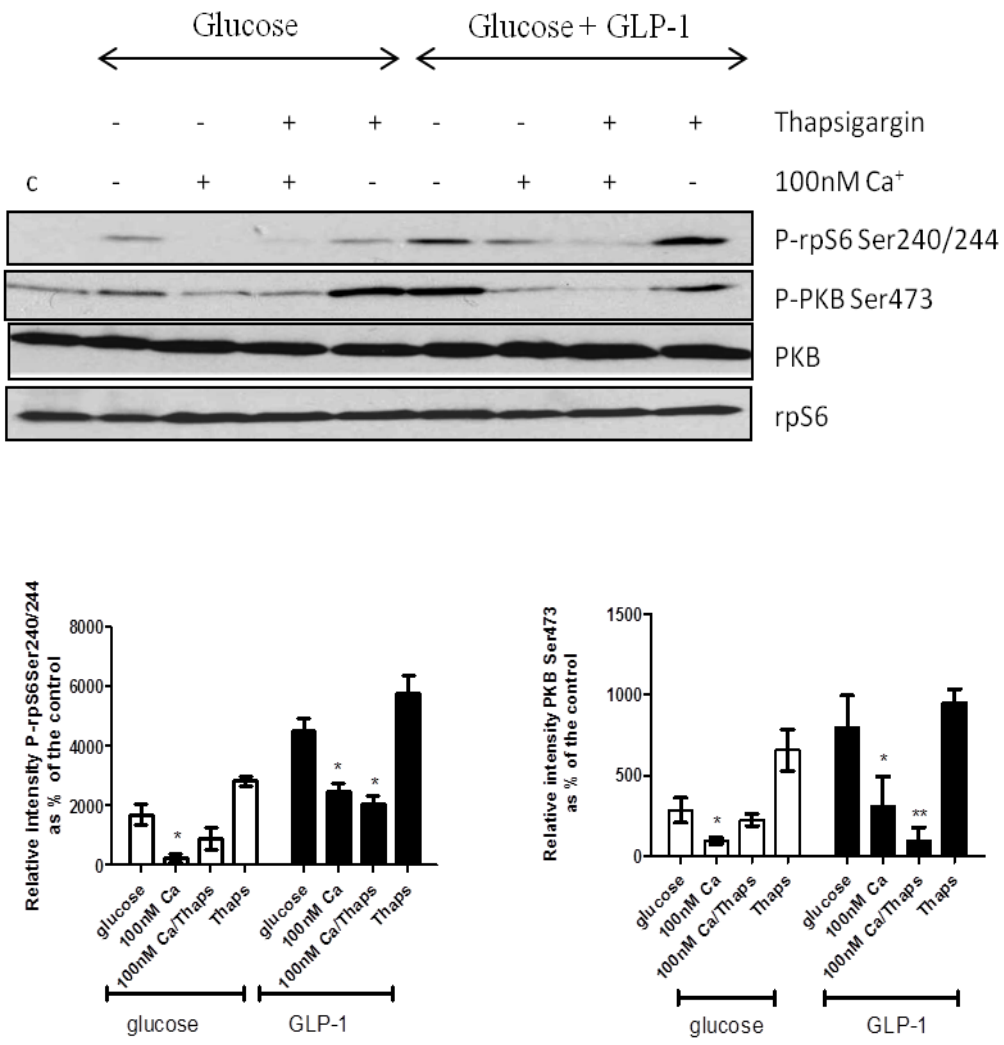


Figure 3.8B. The release of intracellular calcium may be involved in GLP-1 induced phosphorylation of rpS6 in MIN6 cells. MIN6 cells were pre-incubated for 1 h with KRB containing (0.125x) amino acids**.** Cells were then treated for 1h with, 20 mM glucose, 20 mM glucose plus GLP-1 in presence or absence of KRB containing nominal calcium (100nM) or nominal calcium in the presence of 1 µM thapsigargin. Cells were then lysed. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phosho-rpS6 Ser240/244 or phospho-PKB Ser473. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244, phosphorylated PKB Ser473, PKB and rpS6. The results are mean ± S.E.M for three independent experiments. . *, P<0.05 and **, P< 0.01 compared with the corresponding treatment.

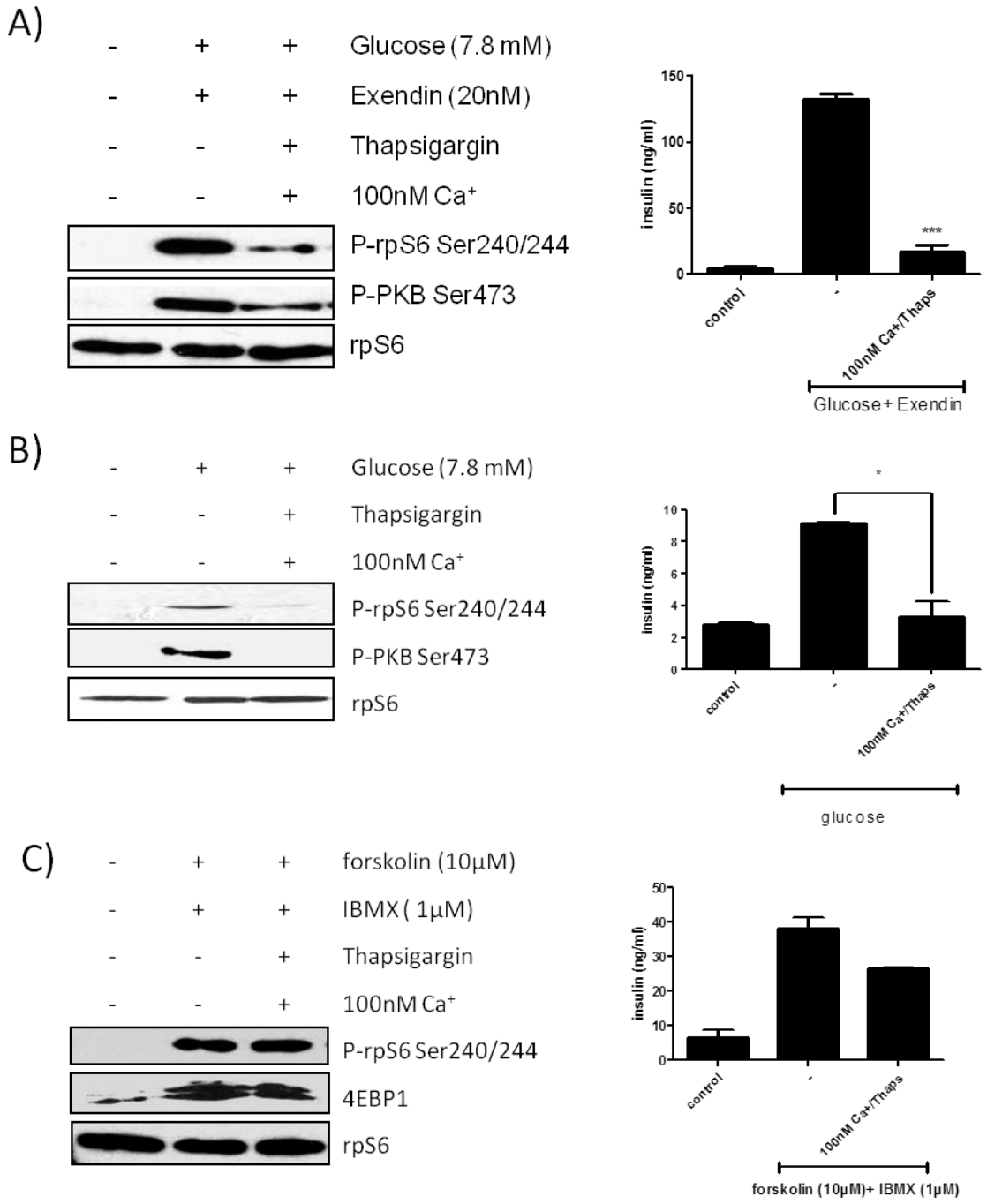


Figure 3.9. Role of intracellular calcium in glucose, exendin and forskolin induced phosphorylation of rpS6 Ser240/244 in rat islets of langerhans. Islets were pre-incubated for 1 hour with KRB containing (0.125x) amino acids**.** Cells were then treated for one hour with (A) glucose (7.8mM) plus exendin (20nM) or (B) glucose or (C) forskolin (10µM) and IBMX (10µM) in presence or absence of KRB containing nominal calcium in the presence of 1 µM thapsigargin. Cells were then lysed. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phosho-rpS6 Ser240/244 or phospho-PKB Ser473. The left panel shows the secretion of insulin by rat islets exposed to different treatments. The results are mean ± S.E.M for three independent experiments. *, P <0.05 and ***, P <0.001, compared with the corresponding treatment in absence of KRB containing nominal calcium in the presence of 1 µM thapsigargin.

3.2.4 Role of AMPK in GLP-1 induced activation of mTOR signalling pathway

The AMP-activated protein kinase (AMPK) is a key physiological energy sensor that coordinates many metabolic pathways to balance energy supply and demand and eventually modulate cellular and organ growth. mTORC1 activity can be modulated by AMPK through the phosphorylation of TSC2 and increasing the GAP activity towards Rheb, leading to inhibition of mTOR activity. To investigate the role of AMPK in rpS6 phosphorylation, MIN6 cells were treated with aminoimidazole carboxamide ribonucleotide (AICAR), an AMPK activator as a control. I tried to use INS1E cells to do this experiment. However, I did not manage to get phosphorylation of AMPK and ACC upon stimulation with glucose, GLP-1 and AICAR. The antibodies of AMPK and ACC that I used in western blotting were rat specific so it should have worked in INS1E cells. Thus, I think the reason for not getting phosphorylation of AMPK in these cells might be attributed to the low expression of AMPK in INS1E cells. Therefore, I used MIN6 cells. Stimulation of MIN6 cells with glucose and GLP-1 led to the phosphorylation of rpS6 with a concomitant decrease in the phosphorylation of AMPK at Thr172 as well as the phosphorylation of its downstream target, acetyl CoA carboxylase (ACC) on Ser79 (Figure 3.10). Infection of MIN6 cells with recombinant adenovirus encoding either dominant-negative or constitutive active AMPK had no effect on GLP-1 stimulated rpS6 phosphorylation (Figure 3.10 and 3.11). Infection of INS1E cells with dominant negative AMPK was not able to stimulate the phosphorylation of rpS6 at Ser240/244. Meanwhile, Activation of AMPK by infecting MIN6 cells with the constitutive active mutant of AMPK did not block GLP-1 induced phosphorylation of rpS6.

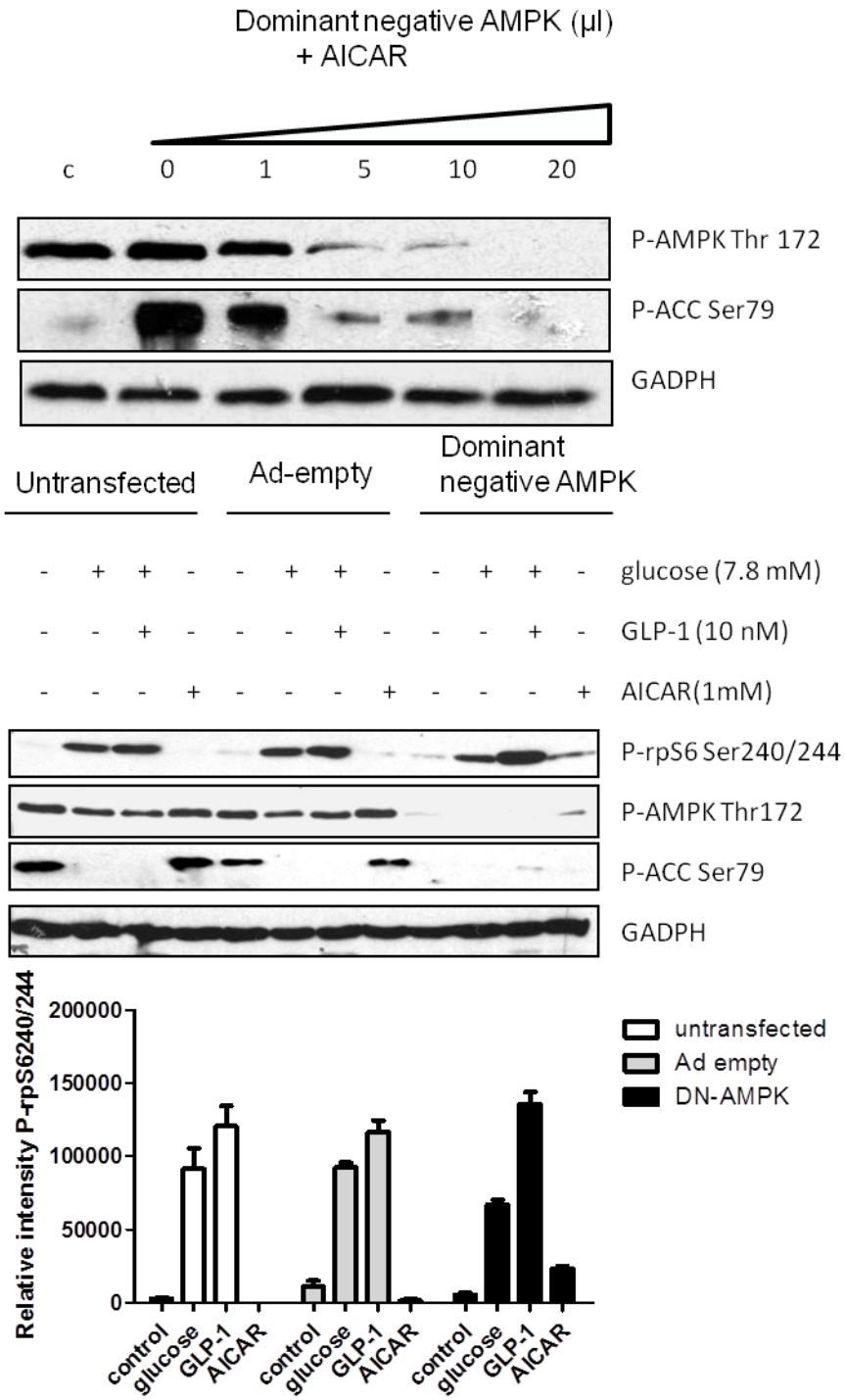


Figure 3.10. Expression of dominant-negative AMPK had no effect on GLP-1 stimulated rpS6 phosphorylation. (A) Titration of dominant-negative AMPK virus in MIN6 cells. (B) MIN6 cells were uninfected or infected with the recombinant adenovirus expressing dominant-negative AMPK for 48 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, cells were incubated with KRB containing 0.25x amino acids and then incubated for 1 hour with glucose (7.8 mM) or glucose (7.8 mM) plus GLP-1 (10nM) or AICAR (1 mM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-AMPK Thr172, or phospho-ACC Ser79 and GADPH as a loading control. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244. The results are mean ± S.E.M for three independent experiments.

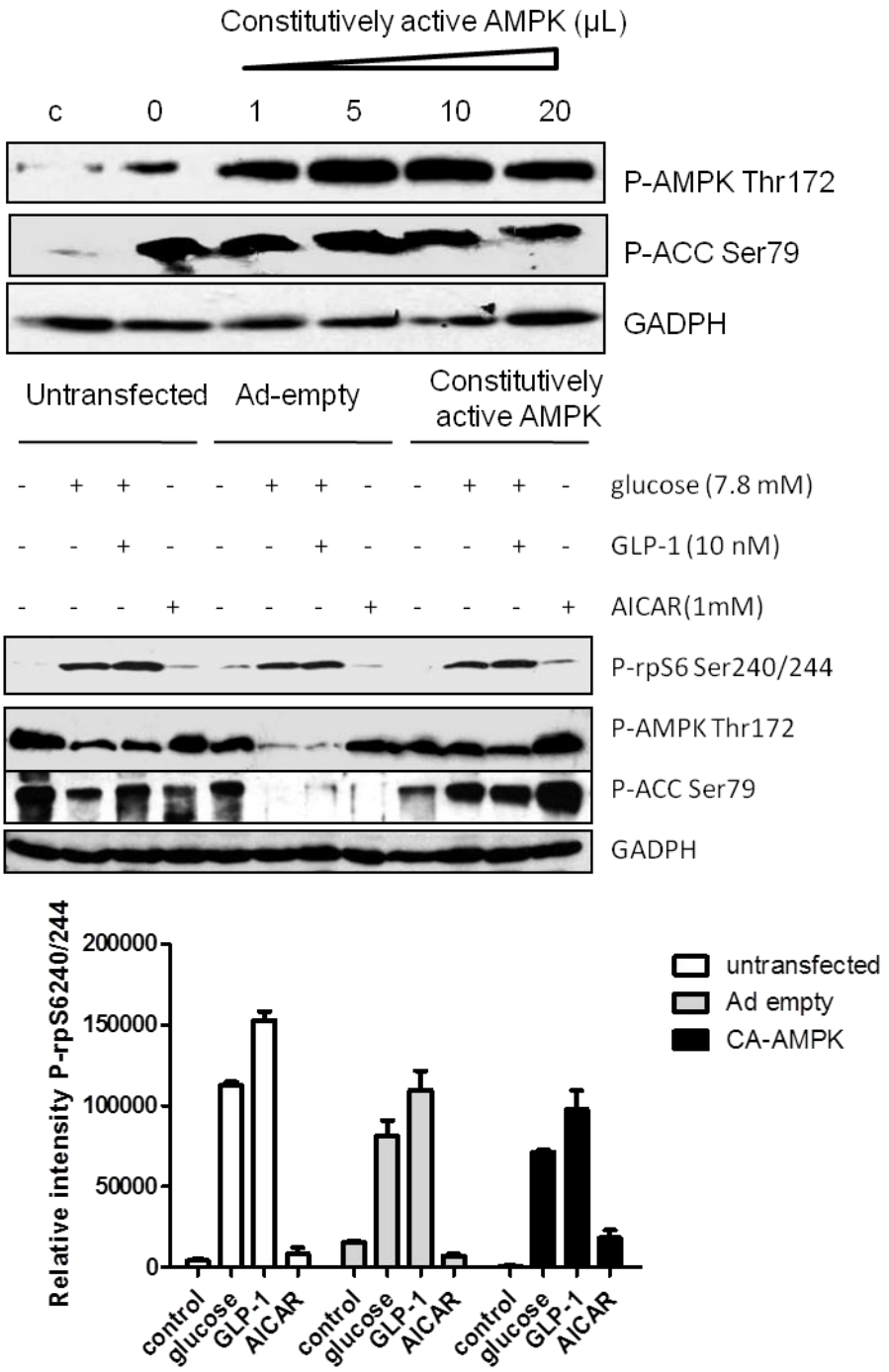


Figure 3.11. Overexpression of constitutively-active AMPK had no effect on GLP-1 stimulated rpS6 phosphorylation. (A) Titration of constitutively active AMPK virus in MIN6 cells. (B) MIN6 cells were uninfected or infected with the recombinant adenovirus expressing constitutively active AMPK for 48 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, cells were incubated with KRB containing 0.25x amino acids and then incubated for 1 hour with glucose (7.8 mM) or glucose (7.8 mM) plus GLP-1 (10nM) or AICAR (1 mM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-AMPK Thr172, or phospho-ACC Ser79 and GADPH as a loading control. The lower panel shows quantified data of phosphorylated rpS6 Ser240/244. The results are mean ± S.E.M for three independent experiments.

3.2.5 Role of PKB in GLP-1 induced activation of mTOR signalling pathway

Activation of PKB leads to the phosphorylation of several downstream targets, including multiple sites on TSC2, which forms a functional complex with TSC1. Phosphorylation of TSC2 impairs the ability of the TSC1–TSC2 complex to act as a GAP towards the small GTPase Rheb, allowing Rheb–GTP to accumulate and consequently activates mTORC1 (Inoki et al., 2003). To determine the role of PKB in the activation of mTORC1, INS1E cells were infected with increasing concentrations of a recombinant adenovirus expressing a constitutive active form of PKB. 48 hr after infection, cells were incubated with KRB containing 0.25x amino acids. An increase of phosphorylation of rpS6 was observed in INS1E cells expressing the constituitive active form of PKB (Figure 3.12), which was sensitive to rapamycin. Inhibitors of PI3K and hVps34 did not affect the phosphorylation of rpS6 stimulated by constitutive activation of PKB. Therefore, PKB activation can activate mTORC1 as evidenced by the increased phosphorylation of rpS6 at Ser 240/244.

To determine if the GLP-1 stimulated rpS6 phophorylation is dependent on the activation of PKB, INS1E cells were incubated with increasing concentration of AKTi 1/2, a potent PKB inhibitor. Cells were then treated by glucose (7.8mM) plus GLP-1 for one hour. The phosphorylation status of PKB, rpS6, PRAS40, p70S6K1 and mTOR were determined using phospho-specific antibodies. As expected, AKTi1/2 suppressed the phosphorylation of PKB at both phosphorylation sites; Ser473 and Thr308 in a dose-dependent manner which paralleled changes in the phosphorylation of S6K, rpS6 and mTOR (Figure 3.13).

To confirm these findings in primary tissue, isolated rat islets of Langerhans were pre-incubated for 1hr in KRB supplemented with amino acids. Cells were incubated for 30 minutes with AKTi1/2 (1μM) before treatment with glucose (7.8 mM) plus Exendin (20nM). Exendin induced phosphorylation of mTOR at Ser2481, S6K1 at Thr389 rpS6 at Ser240/244, TSC2 at Thr1462, PRAS40 at Thr246 and GSK3 at Ser21. The PKB inhibitor, AKTi1/2, blocked the phosphorylation of PKB at Ser473 and thus inhibited its activity as marked by inhibition of GSK3, a readout of PKB activity (Figure 3.14). AKTi also inhibited the phosphorylation of TSC2 induced by exendin so this results in stabilizing the association of TSC2 with TSC1. The

formation of TSC1-TSC2 complex inhibited the phosphorylation of mTOR at 2481 and blocked the ability of mTOR kinase to phosphorylate Thr389 of S6K1. Therefore, GLP-1 stimulates the activation of mTOR and its downstream targets via a PKB-dependent mechanism through the phosphorylaion of TSC2.

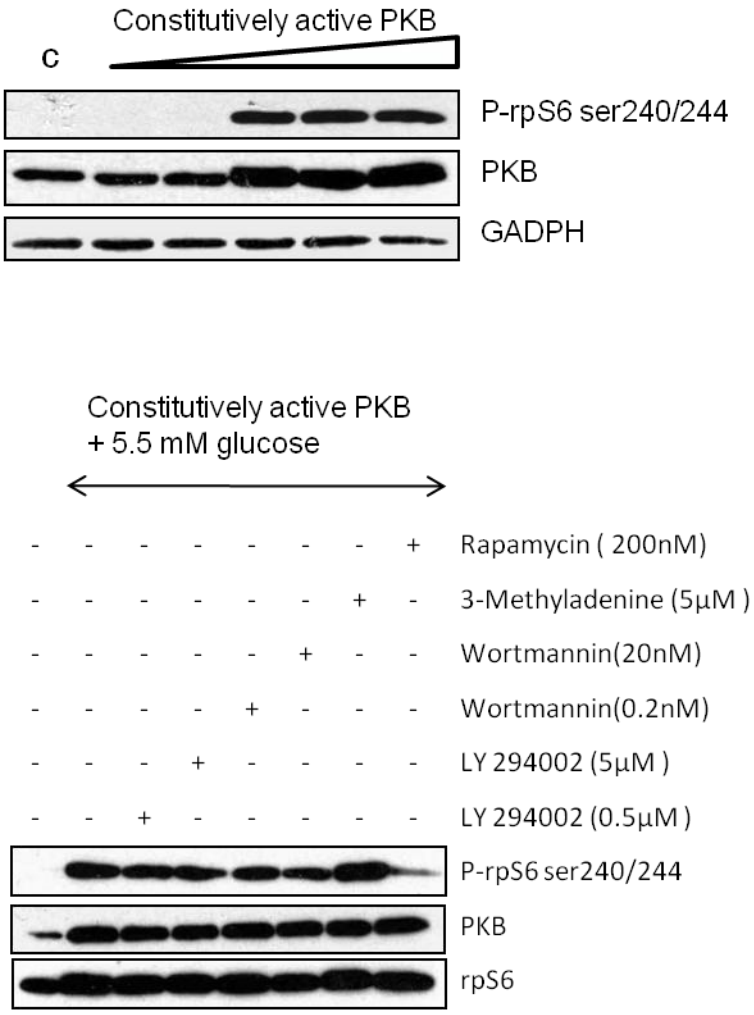


Figure 3.12. hvps34 inhibitor and PI3K inhibitors has no effect on phosphorylation of rpS6 induced by constitutively active PKB in INS1E cells. A) Titration of constitutively active PKB in INS1E cells. (B) INS1E cells were uninfected or infected with the recombinant adenovirus expressing constitutively active PKB for 48 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, cells were incubated with KRB containing 0.25x amino acids and cells were incubated for 30 minutes with different inhibitors as indicated before treatment with glucose (5.5 mM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, PKB and rpS6 as a loading control.

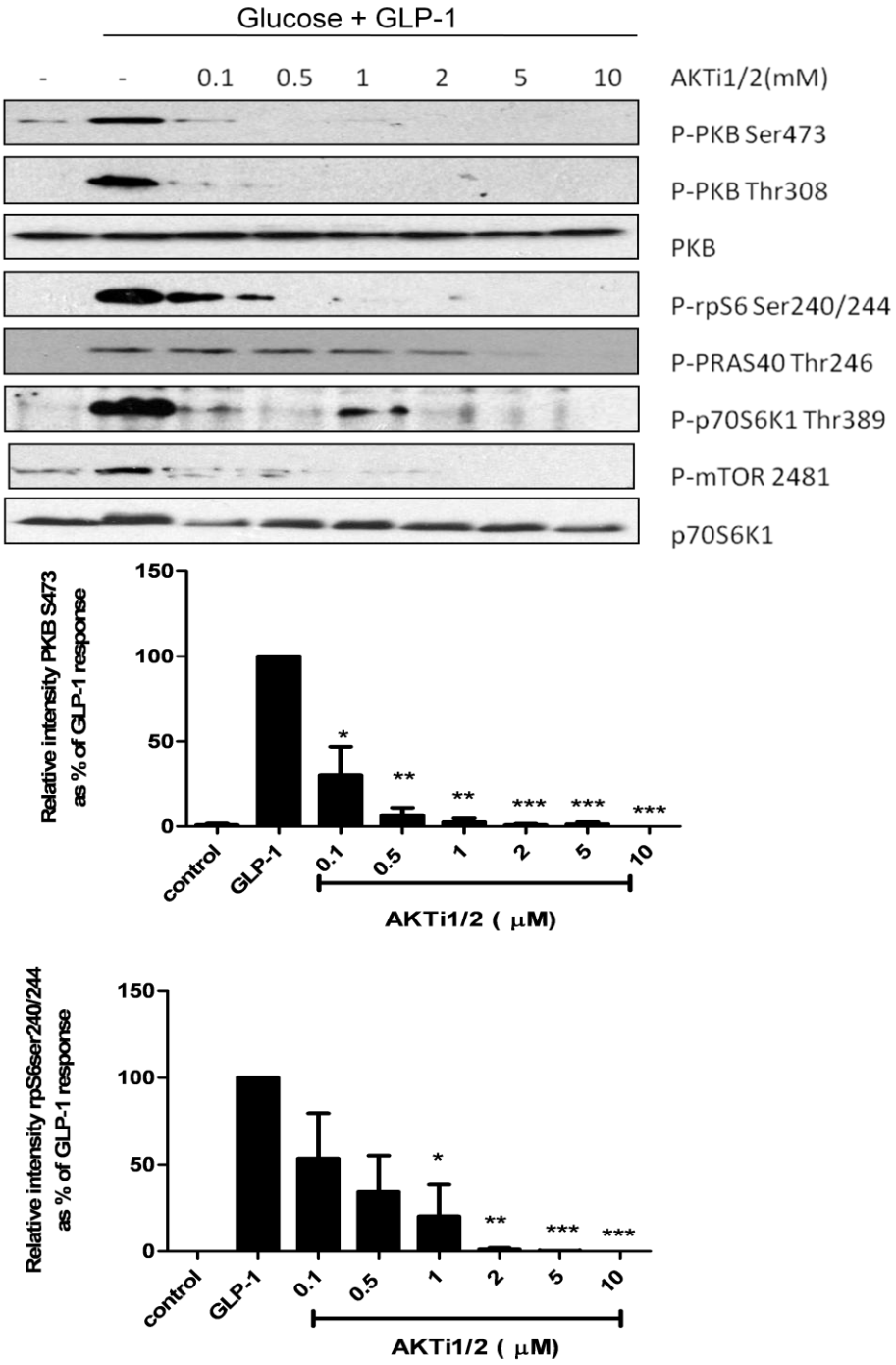


Figure 3.13 Role of PKB in GLP-1 induced mTORC1 activation in INS1E cells. Overnight serum-starved INS1E cells were incubated for 30 min with increasing concentrations of AKTi 1/2 (PKB inhibitor) and then stimulated with glucose (7.8 mM) and GLP-1 (10 nM) for 1 hour. Cells were then lysed and proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB (Ser473 or Thr308), phospho-rpS6 Ser 240/244, phospho-PRAS40, phospho-S6K1 (Thr389) and total PKB as a loading control. The bottom panel shows quantified data of phosphorylated PKB Ser473 and phosphorylated rpS6 Ser 240/244. Results are representative of three experiments. . *, P <0.05 ,**, P< 0.01 and ***, P <0.001 compared with GLP-1 treatment in absence of AKTi1/2.

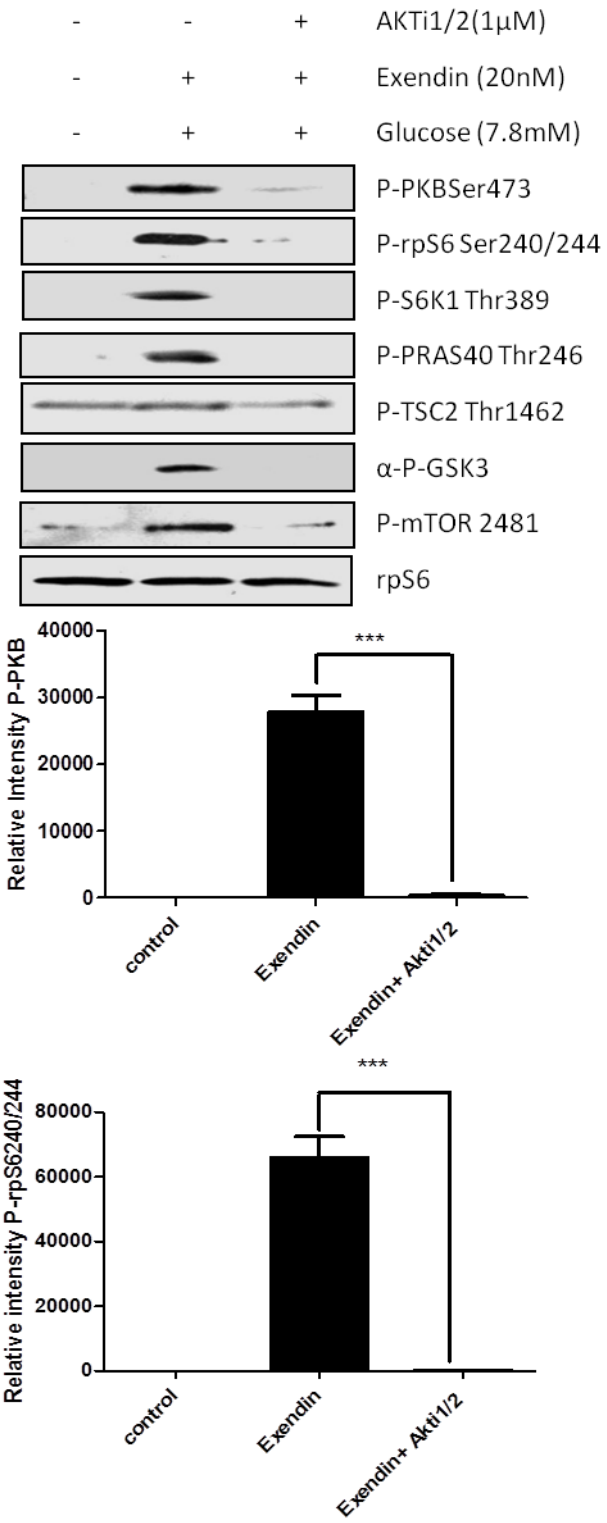


Figure 3.14. Role of PKB in GLP-1 induced mTORC1 activation in Islets of Langerhans Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, the medium was removed and replaced with KRB containing 0.125x amino acids. Cells were incubated for 30 minutes with AKTi1/2 (1µM) before treatment with glucose (7.8mM) plus Exendin (20nM). Lysates were then analysed by Western blotting using specific antibodies. The bottom panel shows quantified data of phosphorylated PKB Ser473 and phosphorylated rpS6 Ser240/244.

3.3 Discussion

GLP-1 is an incretin hormone and its effects are mediated via the interaction with the GLP-1 receptor. Such interaction enables coupling to Gs-containing heterotrimeric G-proteins leading to the activation of adenylate cyclase and the increase in the production of cAMP. cAMP is an important mediator of GLP-1 action on acute molecular events regulating β-cell function since it activates further signalling pathways. In pancreatic β-cells, GLP-1 activates mTORC1, via a poorly understood mechanism. In this report, I provide evidence that, in the pancreatic β-cell line, INS1E cells, and primary rat islets glucose-dependent GLP-1 activation of mTORC1 is mediated via a mechanism independent of both AMPK and Erk but dependent on the activation of PKB. Furthermore, I show that activation of mTORC1 requires the release of intracellular calcium.

3.3.1 Role of cAMP in mTOR signalling pathway

Several studies have demonstrated that cAMP augments glucose–dependent insulin secretion (Kwon et al., 2004a; Pyne and Furman, 2003) and can activate mTOR as evident by the dose-dependent phosphorylation of S6K1 (Kwon et al., 2004a). However, the mechanism of cAMP activation of mTOR is poorly understood. In a similar context, my results show a dose dependent phosphorylation of rpS6 induced by forskolin and this was further augmented in the presence of IBMX (Figure 3.3A), concomitant with an increase in insulin release (Figure3.3B). It has been postulated that the effect of cAMP on insulin secretion is mediated through the activation of voltage-sensitive Ca^{+2} channels, calcium induced Ca^{+2} release, activation of ryanodine receptors and promoting β-cell lipolysis (Pyne and Furman, 2003). Therefore, it is possible that the autocrine effect of insulin mediates cAMP signalling to mTORC1. However, my results suggest that cAMP induced activation of mTORC1 seems to be independent on the autocrine effect of insulin as forskolin can still cause activation of mTORC1 at conditions that reduce insulin secretion and in the presence of BAPTA. Likewise, the McDaniel group also showed that cAMP induced activation of S6K was not affected by diazoxide; an activator of K_{ATP} channel that inhibits insulin secretion (Kwon et al., 2004a). cAMP induces a dose dependent phosphorylation of Erk1/2 which parallels rpS6 phosphorylation and is reduced by

MEK inhibitor PD184352 (Figure 3.4). Therefore, cAMP dependent activation of mTOR may be mediated by Erk. However, PD184352 was reported to activate AMP-activated protein kinase (AMPK) (Moore et al., 2009) and increased AMPK activity was found to block glucose-stimulated mTORC1 signalling (Gleason et al., 2007). Using recombinant adenovirus encoding mitogen-activated protein kinase phosphatase 3 (MKP3) to inhibit Erk signalling did not affect the phosphorylation of rps6 induced by forskolin or GLP-1 (Figure 3.5). This indicates that activation of Erk is not the major determinant in cAMP induced mTORC1 activation. Indeed, a recent paper by Kim et al has proposed a mechanism for cAMP activation of mTORC1 (Kim et al., 2010). They reported that forskolin increased the phosphorylation of S6K1 at Thr389 and 4EBP1 at Thr37/46 and this phosphorylation was reduced by knockdown of Rheb using shRNA or overexpression of dominant negative form and silencing phosphodiesterase 4D (PDE4D). They proposed that there is a physical interaction between Rheb and PDE4D and this interaction is reversed by cAMP to allow Rheb to bind to mTOR which in turn induces the activation of mTORC1 and cap-dependent translation (Kim et al., 2010). Therefore, cAMP can activate mTORC1 via a mechanism involving Rheb and phosphodiesterase 4D.

In the present study, I showed that that the activation of mTORC1 by cAMP is not secondary to activation of PKB. Forskolin induced dose-dependent increase in the phosphorylation of PKB at Ser473. The increased Ser473 phosphorylation suggests that forskolin may also activate mTORC2, which is responsible for this phosphorylation. However, the addition of IBMX inhibited forskolin stimulated PKB phosphorylation. It is possible that excessive activation of mTORC1 by forskolin and IBMX serves as a negative-feedback loop to monitor and limit PKB signalling.

3.3.2 Role of calcium in GLP-1 induced activation of mTOR signalling pathway

Calcium plays an important role in mitogenic signalling in β-cells beside its insulinotropic effect. It has been shown that GLP-1 potentiates Ca^{+2} influx through voltage gated Ca^{+2} channels which in turn triggers Ca^{+2}- induced Ca^{+2} release (Gromada et al., 2004). Here, I demonstrate a central role of calcium influx in the phosphorylation of rpS6, as blocking the influx of extracellular Ca^{+2} across the plasma membrane using nominal calcium buffer reduced the phosphorylation of

rpS6 (Figure 3.8). Similarly, Kwon et al. showed that BAPTA-AM blocked the phosphorylation of S6K1 induced by Exenatide (Kwon et al., 2004a).

Moreover, I show that the phosphorylation of rpS6 induced by forskolin did not require an increase in intracellular calcium concentration, which indicates that cAMP can activate mTORC1 via a calcium-independent mechanism. It was reported that GLP-1 induces cAMP production via activation of transmembrane adenylyl cyclases whose activity can be stimulated by calcium (Ramos et al., 2008). Hence, chelating calcium using BAPTA-AM may inhibit GLP-1 stimulated cAMP production. However, I demonstrated that GLP-1 was still able to produce cAMP in the absence of calcium flux. On the other hand, forskolin is an activator of both Ca^{+2}-dependent and Ca^{+2}-independent adenylyl cyclase. In actual fact, the effect of GLP-1 on rpS6 phosphorylation does not entirely mimic forskolin, as an activator of cAMP. It was clearly evident that GLP-1 alone can produce cAMP to similar extent to those produced in presence of glucose (Figure 3.6A). However, in absence of glucose GLP-1 was unable to stimulate the phosphorylation of rpS6 (Figure 3.6B). Unlike forskolin, GLP-1 requires glucose to induce the activation of mTORC1.

3.3.3 Role of AMPK in GLP-1 induced activation of mTOR signalling pathway

Glucose metabolism leads to a concomitant decrease in the intracellular levels of AMP which in turn inactivates AMPK. It is well established that AMPK can inhibit mTORC1 signalling via the phosphorylation of TSC2 on Ser^{1270} and Ser^{1388}, which augments the ability of the TSC1/TSC2 complex to act as a Rheb-GAP, and thus blocks Rheb-dependent mTOR activation (Hardie, 2005; Inoki et al., 2002). It was reported in pancreatic β-cells that AMPK is activated in response to a decrease in glucose concentration leading to a decrease in mTORC1 signalling (Gleason et al., 2007). Here, I observed that the expression of a constitutively active AMPK had no effect on GLP-1 stimulated phosphorylation of rpS6 (Figure 3.11). In addition, expression of a dominant negative AMPK did not potentiate glucose effect on rpS6 phosphorylation. Thus, it is likely that GLP-1 phosphorylation of rpS6 is not mediated by AMPK inhibition alone.

3.3.4 GLP-1 regulates the activation of mTORC1 through a PKB dependent mechanism

I found that GLP-1 promotes the phosphorylation of rpS6 which correlates with activation of S6K1 via a PKB-dependent mechanism. Inhibition the activity of PKB by AKTi1/2 suppresses GLP-1 stimulated phosphorylation of both rpS6 at Ser240/244 and S6K1 at Thr389 (Figure 3.14). This indicates that the activation of PKB by GLP-1 is a major determinant of mTORC1 activity. PKB activates mTORC1 by its direct phosphorylation of TSC2 and inhibition the GAP activity towards Rheb and in turn increase mTOR activity (Inoki et al., 2002). In addition, PKB can phosphorylate PRAS40 at Thr^{246} to promote mTORC1 activation through the dissociation of PRAS40 from mTOR (Sancak et al., 2007). Here, I have shown that treatment with GLP-1 significantly increases the phosphorylation of PRAS40 at Thr^{246} and TSC2 on 1462 and that AKTi effectively inhibits the phosphorylation of PRAS40 and TSC2 (Figure 3.14). GSK3, a downstream target of PKB, has also been implicated in mTORC1 activation. It was demonstrated by the McDaniel group that treatment of human islets with the GSK-3 inhibitors, LiCl or 1-azakenpaullone (1-Akp) enhances DNA synthesis, promotes cell cycle progression and phosphorylates rpS6 in rapamycin sensitive manner and independent to insulin secretion (Liu et al., 2009). These effects were observed in the presence of physiological concentration of glucose thus the authors postulated that both inhibition of GSK3 and decrease of AMPK activity due to enhanced nutrient metabolism are required for inhibiting TSC1/2 and in turn activate the mTOR pathway (Liu et al., 2009). Collectively, I propose that GLP-1 induces activation of PKB evidenced by the phosphoryation of PKB at Ser^{473} and Thr^{308}. Activation of PKB can in turn phosphorylate PRAS40, GSK3 and TSC2 to enhance the ability of the TSC1/TSC2 complex to act as a Rheb-GAP to promote mTORC1 activation (Figure 3.15).

3.3.5 Importance of mTORC1 activation in pancreatic β-cells

Previous studies have shown that nutrients, growth factors and hormones play an important role in augmentation in β-cell mass (Paris et al., 2003; Topp et al., 2004) and they are also able to activate mTORC1 (Bartolome et al., 2010; Kwon et al., 2004a). GLP-1, in the presence of glucose, stimulates β-cell mass (Buteau et al., 1999; Stoffers et al., 2000) and I and others (Kwon et al., 2004a; Moore et al., 2009)

have demonstrated that GLP-1 potentiates glucose stimulated mTORC1 activation. Our lab has shown that agonists to GLP-1R enhance β-cell replication via a rapamycin dependent mechanism (Xie J and Herbert TP).

Available data from rpS6$^{p-/-}$and S6K1$^{-/-}$ mice revealed the phosphorylation of rpS6 plays an important role in the physiological function and growth of pancreatic β-cells (Pende et al., 2000; Ruvinsky et al., 2005). Islets isolated from rpS6$^{p-/-}$ mice are smaller in size compared to those isolated from wild type. These mice exhibit diminished levels of pancreatic insulin content as well decrease in circulating levels of insulin and impaired glucose tolerance (Ruvinsky et al., 2005). Similar phenotypic changes were observed in the S6K1$^{-/-}$ mice (Pende et al., 2000). The decrease in insulin secretion and reduction of β-cell mass play important role in the development of type 2 diabetes. 4EBP-1 and 4EBP-2 double Knock-out mice exhibit an increase in insulin resistance denoted by the decreased ability of insulin to reduce blood glucose levels and these mice tend to develop obesity (Le Bacquer et al., 2007).

mTORC2 has also been shown to play a positive role in the regulation of β-cell mass through an increase in β-cell proliferation. Our lab has demonstrated that the rapamycin toxicity is mediated by the inhibition of PKB and inactivation of mTORC2 (Barlow et al., 2012). Therefore, therapies resulting in PKB and mTORC2 activation may have the potential to reserve pancreatic β-cell viability.

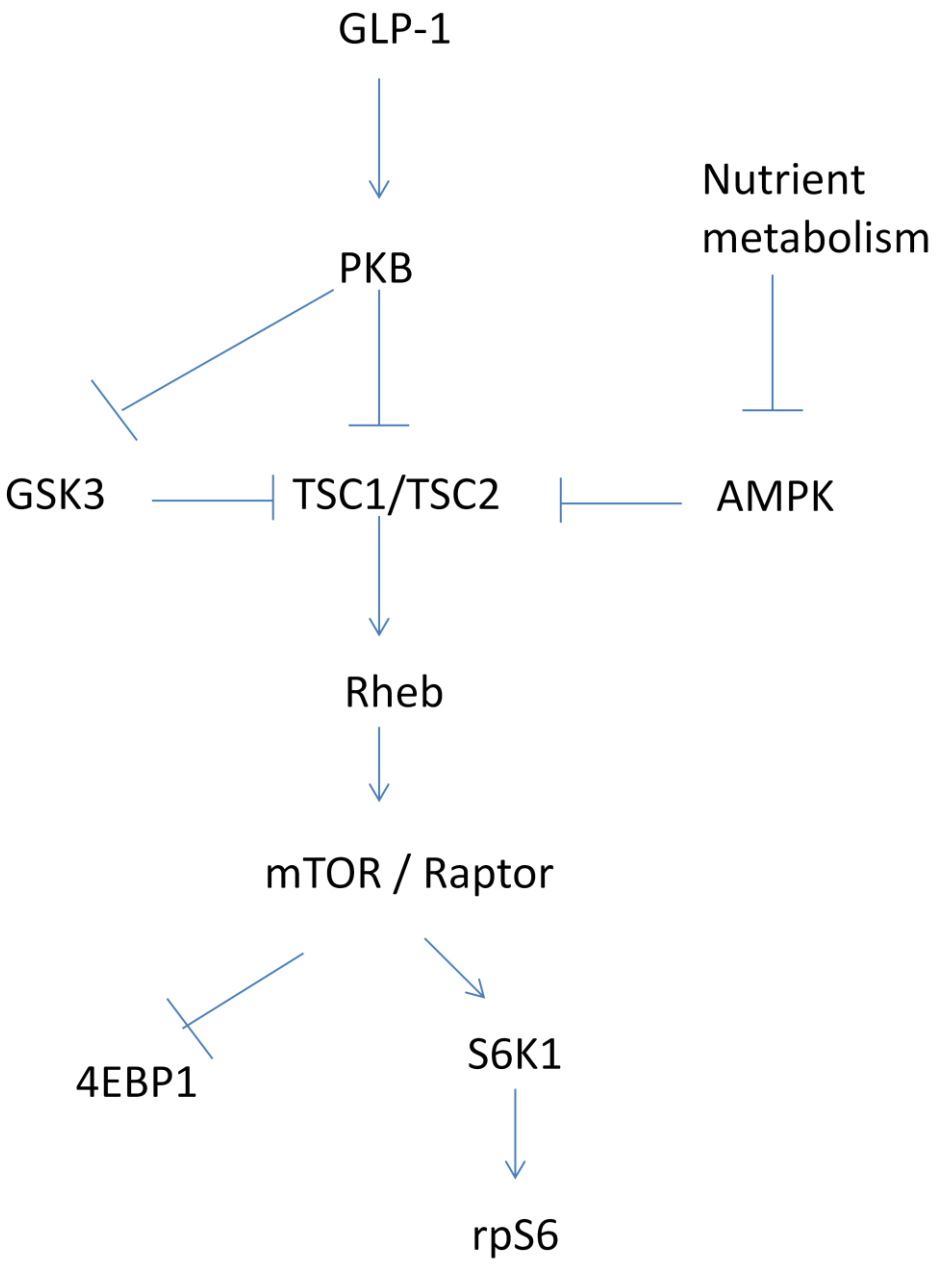


Figure 3.15. proposed mechanism for GLP-1 signalling to mTORC1

CHAPTER 4

Chapter 4: Regulation of PKB by Glucagon Like Peptide-1 in Pancreatic β-cells

4.1 Introduction

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

Human islets were found to express all isoforms of class I and class II PI3K as well as all isoforms of PKB (Muller et al., 2006). Stimulation of pancreatic β-cells with insulin or IGF-1 causes an increase in tyrosine phosphorylation of the β-chain of the insulin receptor and of IRS-2. This results in the recruitment of the p85 a regulatory subunit of PI3K to IRS-2 (Trumper et al., 2000) .

PI3K activity has been shown in several β-cell types (Buteau et al., 2003; Hui et al., 2003; MacDonald et al., 2003). A study on glucose tolerance in PI3Kγ knock-out mice indicated that this mechanism of PI3Kγ is involved in the first phase of insulin secretion (MacDonald et al., 2004). It has been revealed a role for PI3K p110γ in regulation of cortical actin and targeting of insulin granules to the plasma membrane in pancreatic β-cells (Pigeau et al., 2009). This was evidenced by knocking down of p110γ using siRNA or pharmacological inhibition of p110γ with AS605240 which inhibited depolorization-stimulated insulin secretion and Ca^{+2}-evoked insulin exocytosis (Pigeau et al., 2009). These effects were explained by reduced membrane-localized insulin granule pool and increased cortical F-actin that acts as a physical barrier to granule translocation (Pigeau et al., 2009).

In pancreatic β-cells, the PI3K/PKB signalling pathway is stimulated by glucose, hormones and growth factors and this plays an important role in the enhancement of β-cell proliferation and survival (Dickson and Rhodes, 2004). Expression of constitutively active form of PKB in transgenic mice causes a significant increase in both β-cell size and mass, resulting in hyperinsulinaemia and hypoglycaemia (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001). Interestingly, treatment the transgenic mice expressing constitutively active PKB with rapamycin decreases β-cell proliferation and cell mass (Balcazar et al., 2009). The effect of rapamacyin on these mice was shown to be mediated via an mTORC1-dependent mechanism

through the inhibition of cdk4 activity and a decrease in cyclin D2 and D3 levels (Balcazar et al., 2009).

Agonists of the GLP-1 receptor have been reported to potentiate glucose induced PKB phosphorylation in pancreatic β-cells (Trumper et al., 2000) and this activation was PI3K-dependent (Buteau et al., 2003; Trumper et al., 2005). In the pancreatic β-cell line INS(832/13), it was reported that GLP-1 stimulates PI3K through the activation of c-src which activates a metalloprotease which subsequently cleaves membrane bound betacellulin (BTC) (an endogenous EGF-like ligand), which stimulates the epidermal growth factor receptor (EGFR) (Figure 1.4) (Buteau et al., 2003). Transactivation of the EGFR induced by GLP-1 was suggested to mediate GLP-1 stimulated β-cell proliferation (Buteau et al., 2003). It was also reported that GLP-1 stimulation of pancreatic β-cell proliferation is mediated via PI3K dependent activation of the PKCζ isoform (Buteau et al., 2001). However, in human islets that GLP-1 stimulated PI3K activity co-immunoprecipitates with Rap1, suggesting that Rap-1 may activate P13K through a similar mechanism by which Ras activates PI3K (Trumper et al., 2005).

GLP-1 induced activation of PKB has been shown to exert a protective effect against cytokines induced pancreatic β-cell apoptosis (Li et al., 2005). Treatment of INS1E cells with exendin-4 protects against cytokines induced β-cell apoptosis, which is reduced by over-expression of a kinase-dead mutant of PKB (Li et al., 2005). *In vivo,* treatment of the obese db/db mice, an animal model of Type 2 diabetes, with exendin-4 results in up-regulation of PKB expression concomitant with an increase in β-cell proliferation and reduction of β-cell apoptosis (Wang and Brubaker, 2002). In contrast, free fatty acids were shown to decrease the phosphorylation of PKB in INS1 cells which is associated with an increase of apoptosis and these effects can be alleviated by over-expression of constitutively active form of PKB (Wrede et al., 2002). The researchers in this study suggest that the protective effect of PKB in pancreatic β-cells against free fatty acids induced apoptosis is likely mediated by the regulation of the pro-apoptotic downstream targets of PKB; glycogen synthase kinase-3α/β, FoxO1, and p53 (Wrede et al., 2002).

A recent study has demonstrated that chronic treatment of both MIN6 cells and primary rat islets with GLP-1 induced a remarkable increase in IGF-1R expression with a concomitant increase in the release of IGF-2 and an increase of the phosphorylation of PKB at both sites; Ser^{473} and Thr^{308} (Cornu et al., 2009). The released IGF-2 and the up-regulation of IGF-1R expression in response to GLP-1, which is likely mediated by cAMP/ PKA pathway (Cornu et al., 2010), may be responsible for the phosphorylation of PKB and protecting pancreatic β-cells against cytokines-induced apoptosis (Cornu et al., 2009).

Activation of GLP-1R by exendin-4 was shown to promote β-cell proliferation through a mechanism that involves IRS2, a substrate of the insulin and IGF-1 receptor tyrosine kinases (Park et al., 2006). Treatment of human islets with exendin-4 up-regulates the expression of IRS2 which correlates with an increase in the phosphorylation of PKB (Park et al., 2006). Knocking down the expression of IRS2 using siRNA suppresses exendin-4 induced phosphorylation of PKB (Park et al., 2006).

4.1.2 Aims

In pancreatic β-cells, PI3K/PKB was shown to play an essential role in the regulation of vital cell functions including cell growth, survival and proliferation. In addition, it has been reported that GLP-1 induced activation of PKB plays an important role in promoting proliferation and protection against apoptosis in β-cells. Furthermore, I have shown in chapter 3 that GLP-1 induced activation of mTORC1 is mediated via the activation of PKB. Provided that there appears to be discrepancies in the described mechanism by which GLP-1 activates PI3K/PKB signalling cascade, therefore, I aimed in this study to investigate the mechanism by which GLP-1 receptor activation is coupled to the PI3K/PKB signalling pathway in pancreatic β-cells.

4.2 Results

4.2.1 GLP-1 stimulates glucose-dependent phosphorylation of PKB in INS1E cells

To initially characterize glucose dependent GLP-1 signalling, I determined in INS1E cells the activation of PKB in response to GLP1 in the presence of increasing concentrations of glucose. Glucose alone stimulated the phosphorylation of PKB at both sites: Ser473 and Thr308 in a concentration dependent manner which paralleled an increase of GSK3 phosphorylation at Ser21 and Ser9 (Figure 4.1ai). GLP-1 was unable, alone, to stimulate the phosphorylation of PKB but in the presence of increasing concentrations of glucose it significantly augmented the glucose-induced phosphorylation of PKB at both sites (Figure 4.1aii). Furthermore, I measured the insulin released under these conditions; treatment of increasing concentrations of glucose stimulated the production of insulin in a dose dependent manner, an effect that was significantly potentiated in the presence of GLP-1 (Figure 4.1C).

4.2.2 GLP-1-induced phosphorylation of PKB is mediated by class I PI3 kinase in INS1E cells

It is well established that the activation of class I PI3K and the subsequent formation of PtdIns(3,4,5) is crucial for the recruitment of PKB to the cell membrane and its phosphorylation at Thr308 and Ser473 by PDK-1 and mTORC2, respectively (Fayard et al., 2005; Thomas et al., 2002). In addition, I had previously shown that the phosphorylation of rpS6 was dependent on PKB activation (Figures 3.14). Thus, to evaluate whether the phosphorylation of PKB and rpS6 induced by GLP-1 was dependent on PI3K, INS1E cells were pre-incubated with increasing concentrations of PI3K inhibitors; wortmannin and LY294002 and then stimulated with 7.8 mM glucose plus 10nM GLP-1. I chose 7.8 mM glucose since I can see maximum augmentation of GLP-1 to the phosphorylation of PKB (Figure 4.1 B). Pre-treatment with PI3K inhibitors abolished the phosphorylation of PKB and the phosphorylation of rpS6 at Ser240/244 (Figure 4.2).

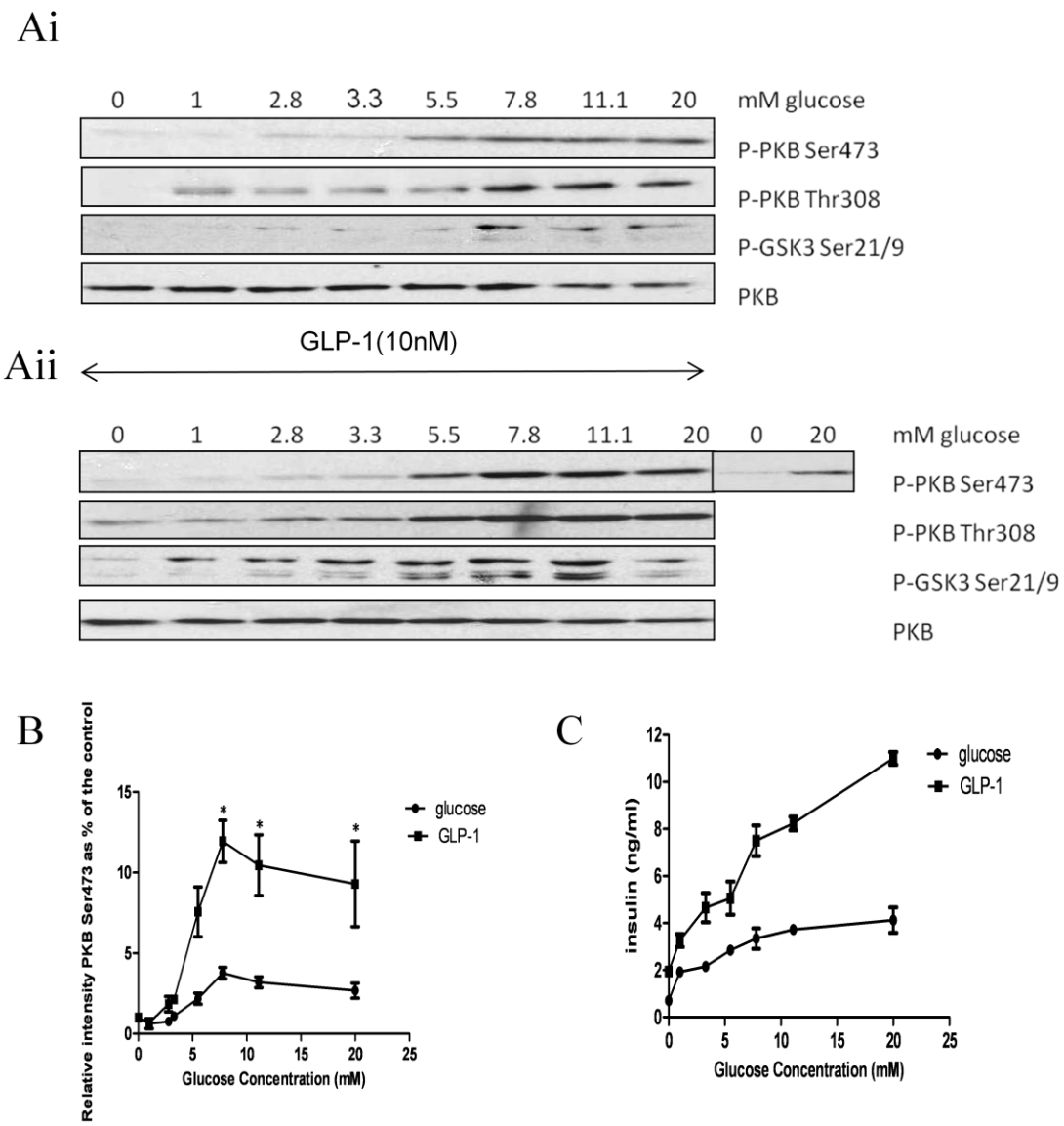


Figure 4.1. Dose response of glucose and glucose plus GLP-1 on the phosphorylation of PKB and GSK-3 in INS1E cells. (A) INS1E cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to treatment with different concentrations of (i) glucose or (ii) glucose plus GLP-1 (10nM), the medium was removed and replaced with KRB containing 0.25x amino acids. Cells were lysed one hour after treatment. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB (Ser473 or Thr308) and phospho-GSK-3 Ser 21/9. (B) Quantified data of phosphorylated PKB Ser473 normalized to total PKB, expressed as % of the control. For densitometric analysis, the quantified 20 mM glucose band was used as a cross reference sample ran on the same gel of GLP-1 samples to compare glucose and GLP-1 phosphorylation of PKB at Ser473 (C) Secretion of insulin by INS1E cells exposed to different concentrations of glucose or glucose plus GLP-1 (10 nM), as indicated. The results representing mean+ S.E.M of n= 3 experiments. * $P<0.05$ compared to the corresponding glucose treatment in absence of GLP-1.

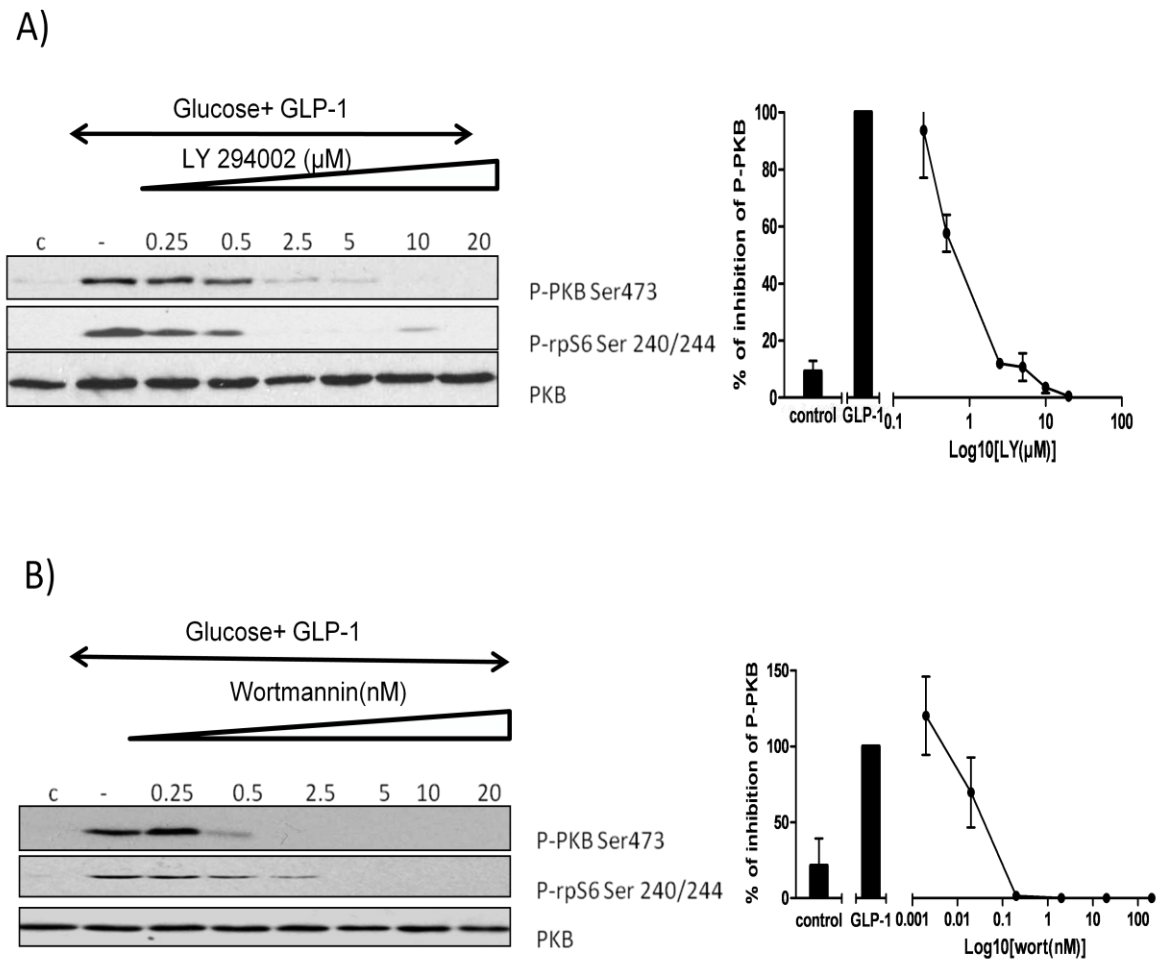


Figure 4.2. GLP-1-induced phosphorylation of PKB is mediated by class I PI3 kinase in INS1E cells. Overnight serum-starved INS1E cells were incubated for 30 min with A) LY 294002 B) wortmannin at the indicated concentrations then they are stimulated with glucose 7.8 mM and GLP-1 (10 nM) for 1 h. Lysates were then analysed by Western blotting using phospho-PKB Ser 473, rpS6 Ser240/244 and PKB as a loading control. The left panel shows quantified data of phosphorylated PKB Ser473. The relative stimulation of GLP-1 over the basal level was taken as 100% and all values are calculated as a percentage of this. The results are mean ± S.E.M for three independent experiments.

Class IA PI3K is composed of the regulatory subunit (p85) and the catalytic subunit (p110). Upon stimulation, p85 subunit of PI3-kinase recruits the p110 catalytic subunit to the membrane. Dominant negative p85, which lacks the binding site of p110, has been shown to inhibit insulin action by blocking the recruitment of p110 (Hara et al., 1994). To provide further evidence that PI3-kinase mediates GLP-1 activation of PKB, I transfected rat islets of langerhans with adenovirus expressing either ad-empty GFP or dominant negative p85 subunit. Transfection of the dominant negative p85 (*lanes 3* and *5*) suppressed GLP-1 and IGF phosphorylation of PKB and rpS6 as shown in Figure 4.3. These data strongly suggest that class IA PI3-kinase activity is required for the GLP-1 stimulation of PKB signalling pathway.

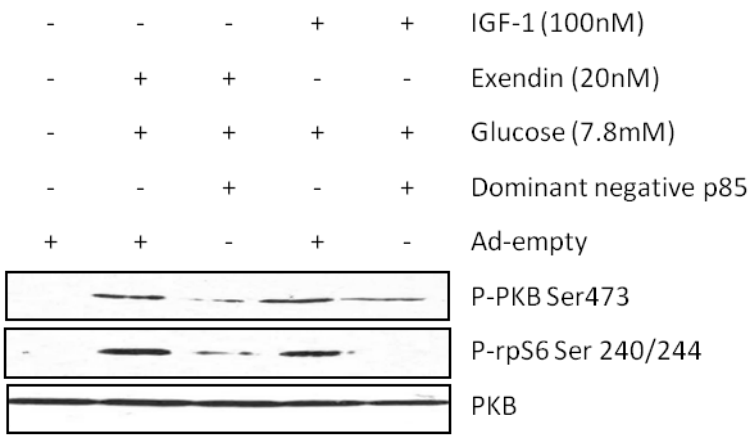


Figure 4.3. Exendin stimulated PKB phosphorylation is mediated via activation of class IA PI3K. Rat Islets of Langerhans cells were infected with the recombinant adenovirus expressing dominant negative p85 or ad-empty for 48 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, cells were incubated with KRB containing 0.125x amino acids and then incubated for 1 h with glucose (7.8mM) plus Exendin (20nM) or glucose (7.8mM) plus IGF-1 (100nM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB Ser473, phospho-rpS6 Ser240/244 and PKB as a loading control.

4.2.3 GLP-1 stimulated PKB activation is independent of EGF-R transactivation

I have demonstrated that GLP-1 potentiates glucose dependent signalling to PKB, however it is unclear how it mediates this effect. A previous study has proposed that c-Src-dependent transactivation of the EGFR in response to GLP-1 releases EGFR ligands leading to activation of PI3K (Figure 1.4) and β-cell proliferation (Buteau et al., 2003).

In order to evaluate the role of EGFR transactivation in GLP-1 induced PKB signalling, I used increasing concentrations EGF-specific inhibitor (AG1478) to evaluate its effect on GLP-1-induced PKB phosphorylation in INS1E cells. Figure 4.4 Ci reveals that pre-treatment with AG1478 had no significant effect on glucose plus GLP-1 induced PKB or rpS6 phosphorylation. As a control, INS1E cells were treated with EGF (20ng/ml) for 5 minutes when I got the maximal phosphorylation of Erk1/2 (as seen in figure 4.4A). AG1478 effectively blocked EGF-induced Erk1/2 activation at 2.5 nM as shown in figure 4.4Bi.

To confirm this result, I used another pancreatic β-cell line; MIN6 cells. Similarly, AG 1478 (5 nM) did not affect the phosphorylation of PKB induced by glucose, glucose plus GLP-1 or insulin. Furthermore, AG1478 did not inhibit glucose or glucose plus GLP-1 induced Erk1/2 phosphorylation in MIN6 cells. In contrast, this concentration of AG 1478 efficiently inhibited EGF-induced Erk1/2 activation as demonstrated in figure 4.5.

To confirm these results in primary cells, I tested the effect of AG1478 (5 nM) on the phosphorylation of PKB and rpS6 stimulated by glucose and GLP-1 in rat islets of Langerhans. AG1478 had no effect on glucose or glucose plus GLP-1 stimulated phosphorylation of PKB and rpS6. In contrast, the phosphorylation of PKB and rpS6 induced by EGF was significantly reduced by AG1478 as demonstrated in figure 4.6.

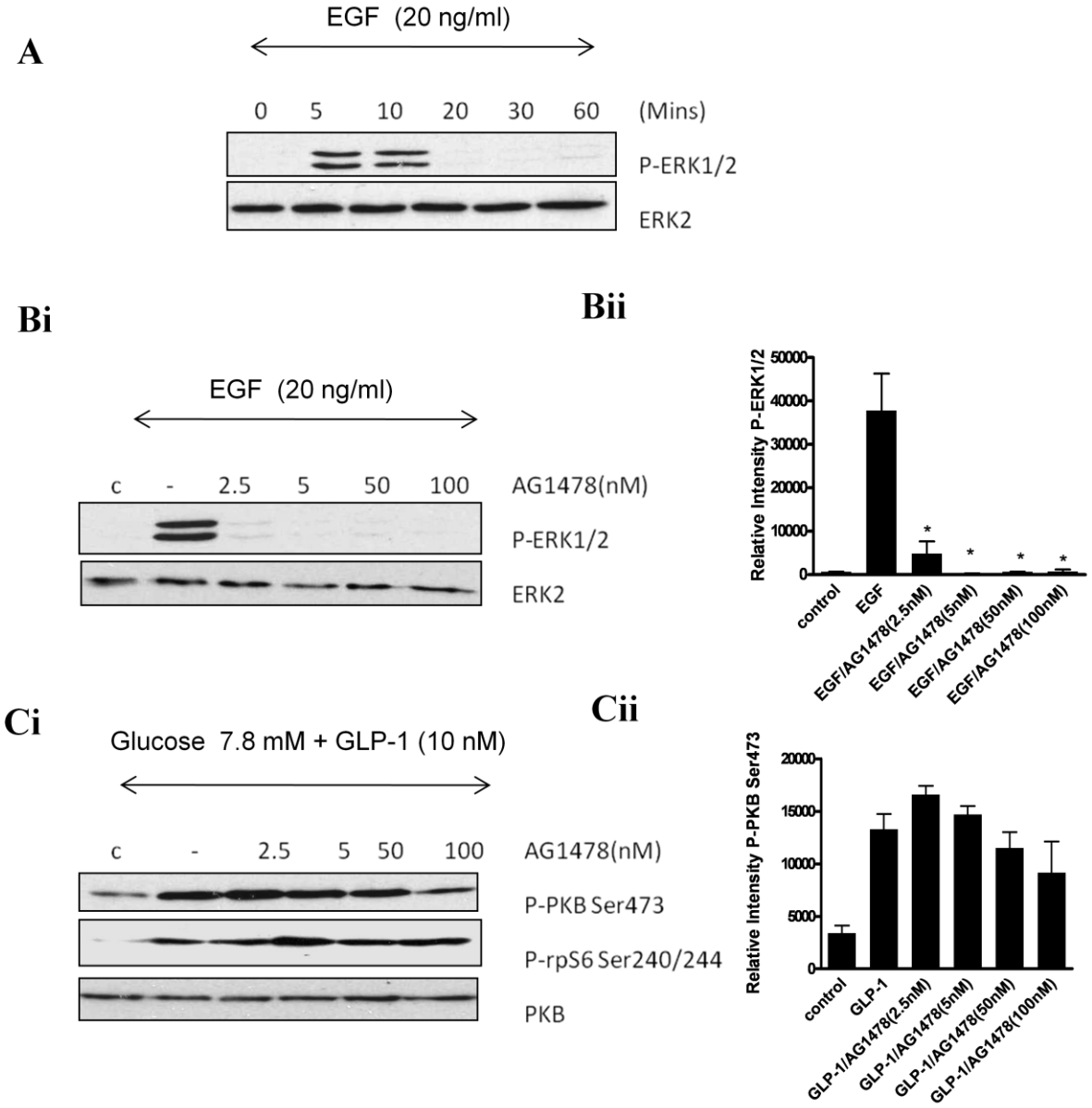


Figure 4.4. GLP-1 stimulated PKB activation in INS1E cells is independent of EGF-R transactivation. INS1E cells were incubated overnight in CMRL-1066 medium containing glutamine. One hour prior to treatments, the medium was removed and replaced with KRB containing 0.25x amino acids. Cells were lysed and proteins were separated by SDS-PAGE, blotted on a PVDF membrane and probed with specific antibodies. (A) Time course of the phosphorylation of Erk1/2 and PKB Ser473 in INS1E cells treated with EGF (20 ng/ml). (B) Dose–response effect of EGF-specific inhibitor (AG 1478) on the EGF-induced stimulation of Erk1/2 phosphorylation. (C) Dose–response effect of EGF-specific inhibitor (AG 1478) on the GLP-1 induced stimulation of PKB phosphorylation. A representative Western blot is shown in the right panel and quantification is presented in the left one. The results are mean ± S.E.M for three independent experiments. * P<0.05 compared to GLP-1 treatment.

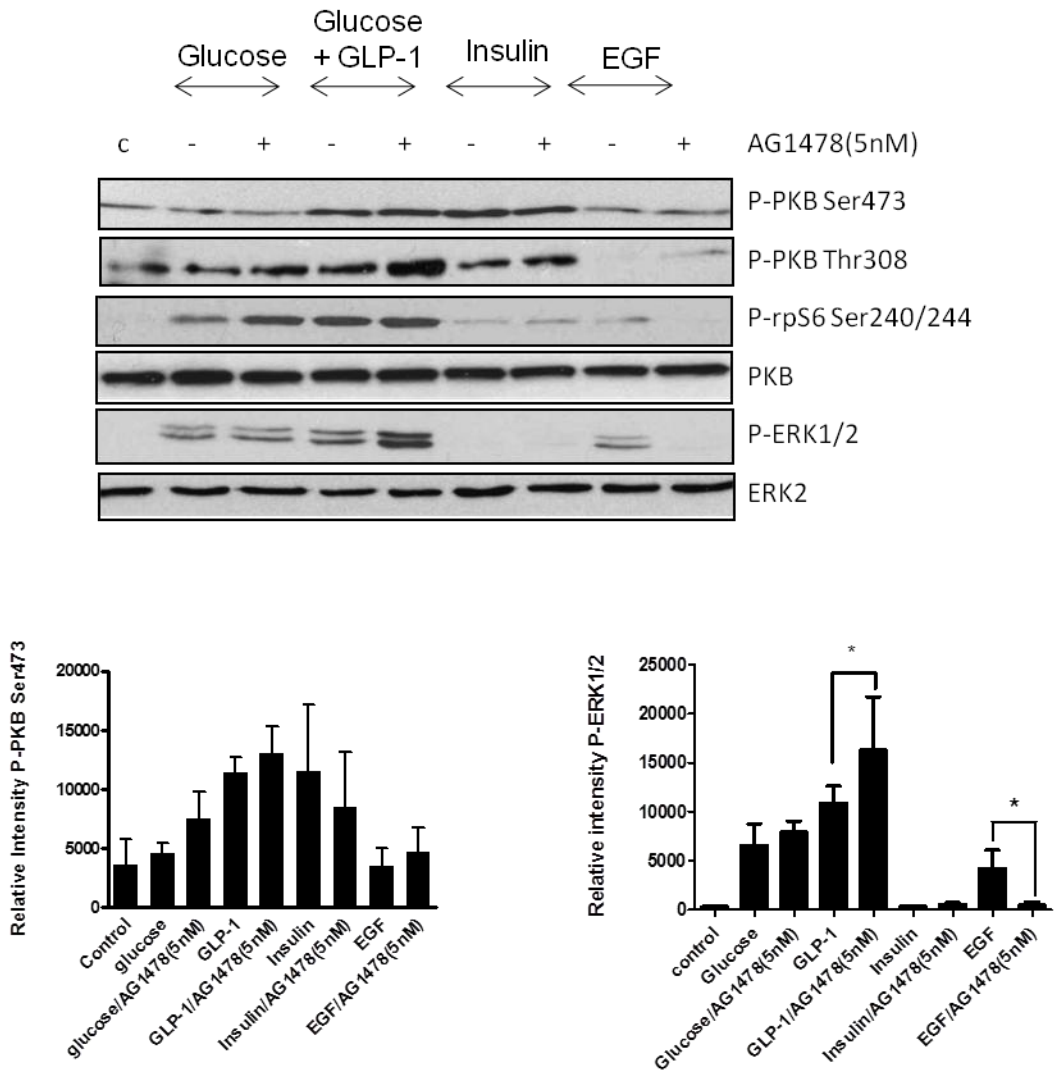


Figure 4.5. Glucose, GLP-1 and Insulin stimulated ERK and PKB activation in MIN6 cells is independent of EGF-R transactivation. MIN6 cells were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, the medium was removed and replaced with KRB containing 0.125x amino acids. Cells were incubated for 30 minutes with AG 1478 (5nm) before treatment with glucose (20mM) or glucose (20mM) plus GLP-1 (10nm) or insulin (100 nM). Whole-cell lysates were then analysed by Western blotting using specific antibodies. A representative Western blot is shown in the upper panel and quantification is presented below. The results are mean + S.E.M for three independent experiments. * $P<0.05$ compared to the corresponding treatment in the absence of AG1478.

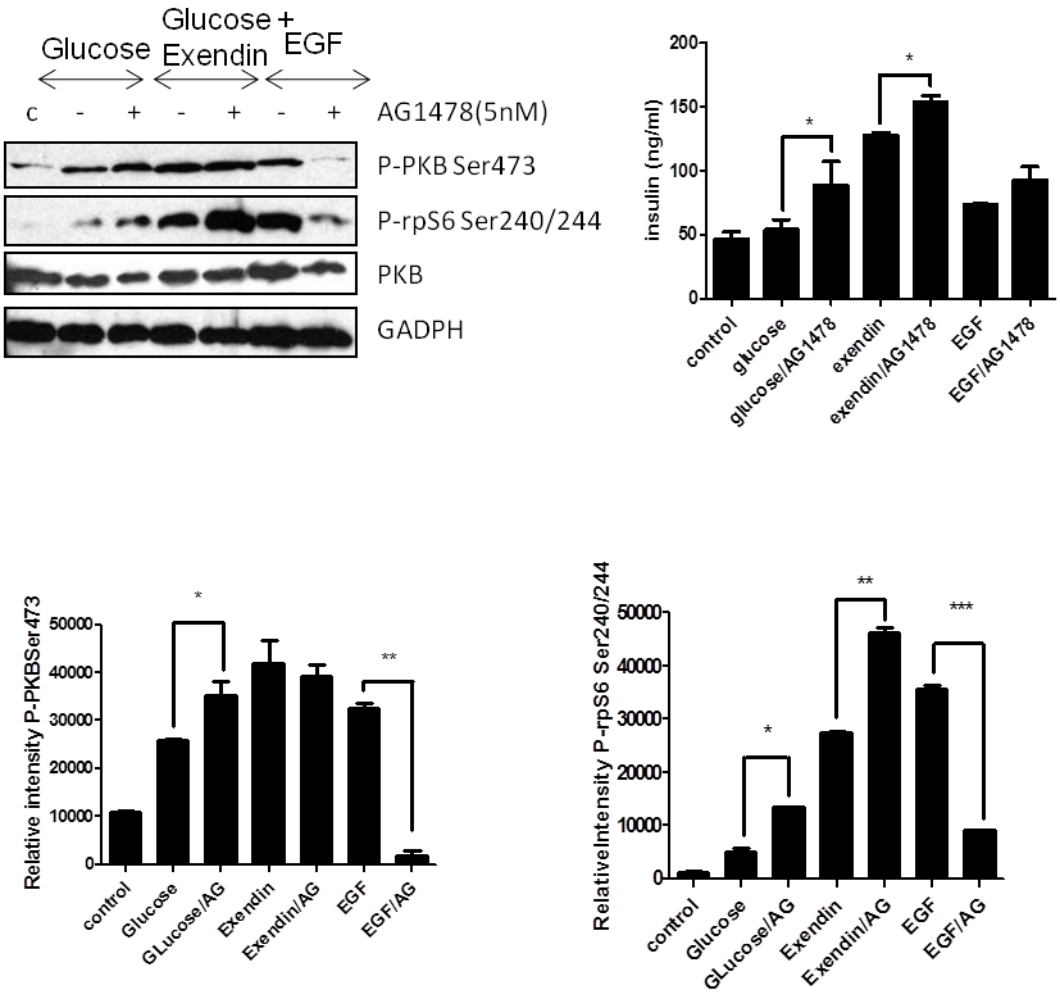


Figure 4.6 . Glucose and GLP-1 PKB activation in Rat Islets of Langerhans is independent of EGF-R transactivation. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, the medium was removed and replaced with KRB containing 0.125x amino acids. Cells were incubated for 30 minutes with AG 1478 (5nM) before treatment with glucose (7.8mM) or glucose (7.8mM) plus Exendin (20nM) or EGF (20ng/ml). Lysates were then analysed by Western blotting using specific antibodies. A representative Western blot is shown in the right upper panel and quantification is presented below. The left upper panel shows the insulin secreted by rat islets exposed to different conditions. The results are mean ± S.E.M for three independent experiments. * P<0.05, **, P<0.01, ***, P<0.001 compared to the corresponding treatment in the absence of AG1478.

I also detected the insulin released under these different stimulation conditions. As expected, exendin potentiated glucose induced insulin secretion. Interestingly, there was a slight increase in the insulin released in the presence of AG1478 upon stimulation with different treatments. The increase of insulin release was concomitant with a potentiation of PKB and rpS6 phosphorylation induced by glucose and GLP-1 (Figure 4.6).

To investigate the effect of AG1478 (5 nM) on GLP-1 induced activation of PI3K, I measured phospho-tyrosine (pY) associated PI3K activity in INS1E cell lysates treated with 7.8 mM glucose plus GLP-1 in the presence and absence of AG1478 (5nM). GLP-1 stimulated PI3K activity was unaffected by AG1478 (5nM), indicating that this dose of AG1478 did not affect PI3K activity induced by GLP-1 and that GLP-1 can stimulate PI3K activity independently of EGF transactivation (Figure 4.7).

To conclude, my results suggest that GLP-1 stimulated PKB phosphorylation is independent of EGFR transactivation.

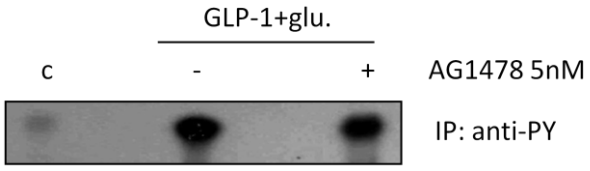


Figure 4.7. Effects of EGFR inhibitor on GLP-1–induced PI 3-kinase activation. INS1E cells were serum starved overnight and then incubated for one hour in KRB containing 0.25xaa. Cells were incubated for 30 minutes with or without AG 1478 (5nM) before treatment with glucose (7.8 mM) plus GLP-1 (10nM). PI 3-kinase activity associated with phosphotyrosine was assayed as described in Material and Methods.

4.2.4 GLP-1 stimulated PKB phosphorylation in INS1E cells and rat islets of Langerhans may be mediated by the autocrine effect of insulin/IGF

In pancreatic β-cells, glucose metabolism leads to an increase in intracellular calcium primarily mediated by an influx of calcium through L-voltage calcium channels, an effect that is augmented by GLP-1 (Gromada et al., 2004; Gromada et al., 1998). Such rise in intracellular calcium stimulates the increase of secreted insulin and IGF (Gromada et al., 1998). To assess whether insulin secretion is a major determinant in GLP-1 stimulated PKB phosphorylation, INS1E cells were incubated for 1 h in modified KRB containing 0.25x amino acids. Next, they were treated with 7.8 mM glucose or 7.8 mM glucose plus GLP-1 in presence or absence of diazoxide; an activator of K_{ATP} channel that inhibits insulin secretion, nifedipine (L-voltage gated calcium channel blocker that can inhibit insulin secretion) or PI3K inhibitors; LY294002 and wortmannin. As shown in figure 4.8, diazoxide inhibited both glucose and GLP-1 induced PKB phosphorylation at both sites; Ser473 and Thr308. It was reported that glucose mediates activation of mTOR via the autocrine effect of insulin (Kwon et al., 2004a) so as expected, diazoxide also abolished glucose stimulated rpS6 phosphorylation at Ser240/244. Similarily, GLP-1 induced phosphorylation of rpS6 was significantly reduced by diazoxide. The inhibitory effect of diazoxide on the phosphorylation of PKB and rpS6 paralleled its inhibition of insulin secreted upon stimulation with either glucose or GLP-1. Nifedipine partially reduced the phosphorylation of PKB stimulated by both glucose and GLP-1. Conversely, nifedipine had little effect on GLP-1 induced phosphorylation of rpS6 or GSK3. However, nifedepine did not inhibit the insulin secreted by INS1E cells upon stimulation with glucose or GLP-1. It was previously shown that cAMP can mobilize Ca^{+2} from intracellular stores (Kwon et al., 2004a), thus there is an insulin release despite blocking of L-type calcium channels due to cAMP effect released from glucose metabolism and GLP-1 binding to its receptor.

To investigate the role of insulin secretion in the phosphorylation of PKB in primary cells, rat islets of langerhans were incubated for 60 minutes in 7.8 mM glucose alone or 7.8 mM glucose plus exendin (20nM) in the presence or absence of diazoxide. Both glucose and exendin stimulated phosphorylation of PKB at Ser473 was blocked by diazoxide. Furthermore, the exendin stimulated rpS6 and GSK3 phosphorylation

was also reduced by diazoxide in parallel with the inhibition of glucose and exendin stimulated insulin secretion (Figure 4.9).

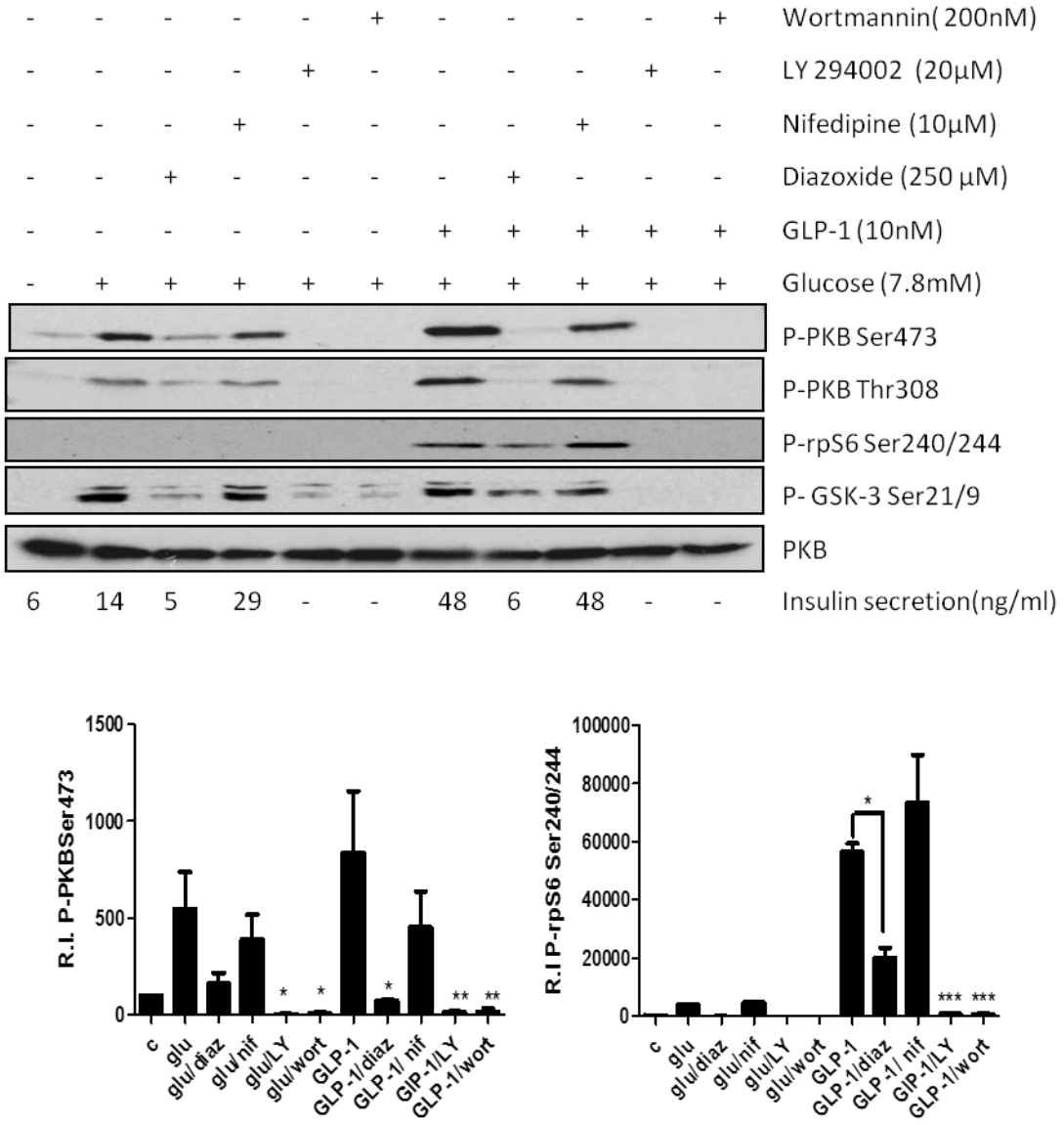


Figure 4.8. GLP-1 stimulated PKB phosphorylation in INS1E cells may be mediated by the autocrine effect of insulin/IGF. INS1E cells were incubated for one hour in modified KRB containing 0.25x amino acids. Next, they were treated with 7.8 mM glucose, 7.8 mM glucose plus GLP-1 in the presence or absence of 250 µM diazoxide, 20µM LY294002, or 200nM wortmannin. Proteins were resolved on SDS-PAGE and western blotted using phospho-PKB (Ser473 or Thr308), phospo-GSK-3 Ser9/21 and phospho-rpS6 Ser240/244. The lower panel shows quantified data of phosphorylated PKB Ser473 and phosphorylated rpS6 Ser240/244. The results are mean $\pm$ S.E.M for three independent experiments. * P<0.05, **, P<0.01, ***, P<0.001.

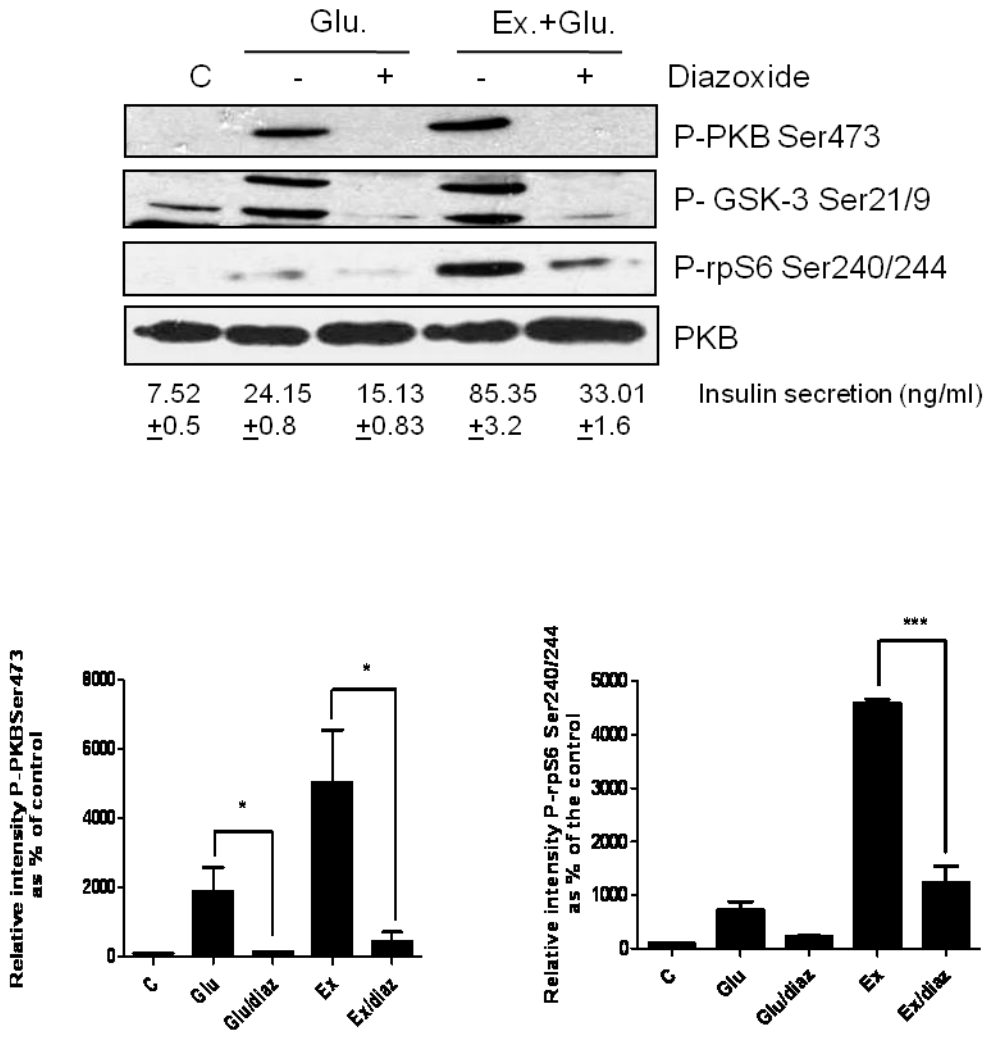


Figure 4.9. GLP-1 stimulated PKB phosphorylation in Islets of Langerhans may be mediated by the autocrine effect of insulin/IGF. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. One hour prior to stimulation, the medium was removed and replaced with KRB containing 0.125x amino acids. Cells were treated with glucose (7.8mM) or glucose (7.8mM) plus Exendin (20nM) in the presence or absence of 250 µM diazoxide. The lower panel shows quantified data of phosphorylated PKB Ser473 and phosphorylated rpS6 Ser240/244. The results are mean ± S.E.M for three independent experiments. *, P<0.05, ***, P<0.001.

Collectively, my data shows that GLP-1 potentiate glucose induced insulin secretion and hence potentiate glucose stimulated PKB and rpS6 phosphorylation. Therefore, I suggest that GLP-1 induced activation of PKB/mTORC1 may be mediated via the autocrine effect of insulin/IGF.

4.2.5 The Phosphorylation of PKB by GLP-1 is independent of the insulin receptor

I have provided evidence that the autocrine effect of insulin/IGF may be responsible for GLP-1 stimulated PKB activation in pancreatic beta cells. Therefore, I proposed that the insulin released after glucose and GLP-1 stimulation can cause the activation of insulin receptor. To determine the involvement of the insulin receptor in GLP1-stimulated PKB activation, INS1E cells were transfected for 72 hours with siRNA against IR beta subunit (Rat insulin receptor On-TARGETplus SMARTpool siRNA, J-080102-10-0010, NM_017071, Dharmacon). The transfection of INS1E cells with IR siRNA effectively knocked down IR expression by about 80% as detected by western blotting without affecting IGF-1R levels as shown in figure 4.10 while the transfection agent or scrambled control siRNA had no effect. While the silencing IR siRNA significantly inhibited insulin stimulated PKB activation, knock down of IR expression had no significant effect on GLP1 stimulated PKB phosphorylation and activation of mTORC1 (Figure 4.10).

4.2.6 The Phosphorylation of PKB by GLP-1 is dependent upon activation of the IGF-1 receptor

The insulin receptor is similar in structure to the insulin-like growth factor 1 receptor (IGF-1R), which have in common an extracellular ligand - binding domain that activates an intracellular tyrosine kinase domain. Three well-defined ligands can potentially activate insulin-like signalling system includes: insulin, insulin-like growth factor 1 (IGF-1), and insulin-like growth factor 2 (IGF-2) (Siddle, 2011). My data revealed that insulin receptor was not involved in GLP-1-stimulated PKB phosphorylation while reduction of insulin receptor expression blocked insulin-induced PKB phosphorylation. Therefore, insulin is unlikely to be responsible for the autocrine activation of PKB induced by GLP-1. Beta cells also express IGF-1R and secrete IGF (Van Schravendijk et al., 1987) , thus I next tried to investigate the role of IGF-1R in PKB activation induced by GLP-1, INS1E cells were transfected with

IGF-1R siRNA (On-Targetplus SMARTpool siRNA, J-091936-10-0010, NM_052807 Dharmacon) or scrambled siRNA using lipofectamine at a concentration of 100 nM for 72 hours. IGF-1R silencing was demonstrated by western blotting (Figure 4.11, lanes 5 and 6). Preventing IGF-1R expression suppressed both IGF-1 and GLP-1-induced PKB phosphorylation and the phosphorylation of rpS6.

To further confirm these data in primary cells, dispersed rat islets of langerhans were transfected with siRNA against IGF-1R using lipofectamine at a concentration of 100 nM for 72 hours. Likewise, knocking down of IGF-1R blocked both IGF-1 and exendin induced phosphorylation of PKB and rpS6 (Figure 4.12). Collectively, these data suggest that GLP-1 activation of PKB is dependent on secretion and IGF-1R signalling so I can propose that the autocrine effect of IGF is responsible for GLP-1 activation of PKB and mTORC1.

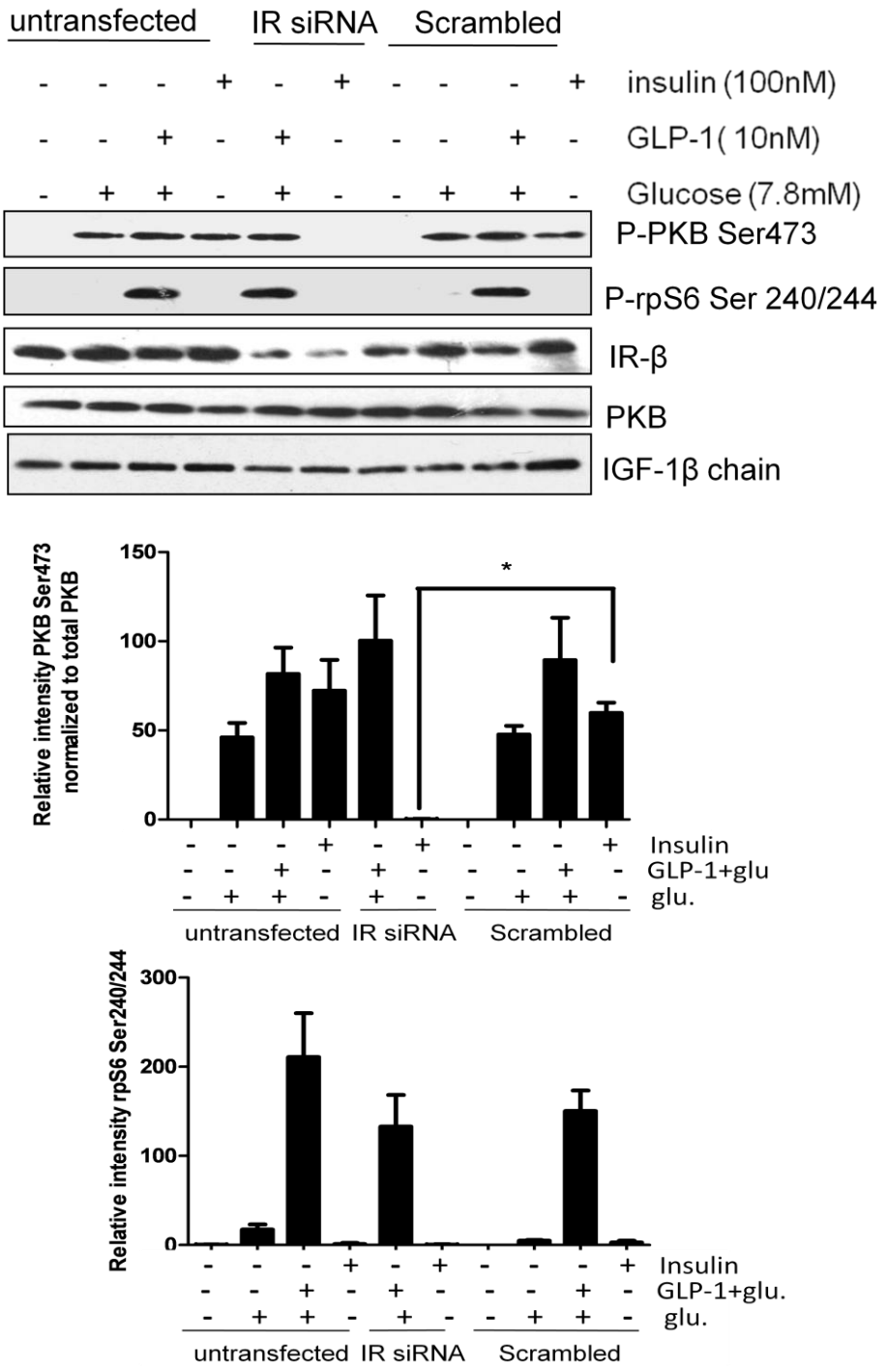


Figure 4.10. GLP-1 stimulated PKB phosphorylation in INS1E cells is independent of the insulin receptor in INS1E cells. INS1E cells were transfected with siRNA against IR-β receptor or scramble RNAi for 72 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. Cells were then washed with KRB containing 0.25x amino acids and incubated for 30 minutes with glucose (7.8mM) or glucose (7.8mM) plus GLP-1 (10nM) or insulin(100nM) . Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB Ser473, phospho-rpS6 Ser240/244. The lower panel shows quantified data of phosphorylated PKB Ser473 normalized to total PKB and phosphorylated rpS6 Ser240/244. The results are mean ± S.E.M for three independent experiments.*,P<0.05.

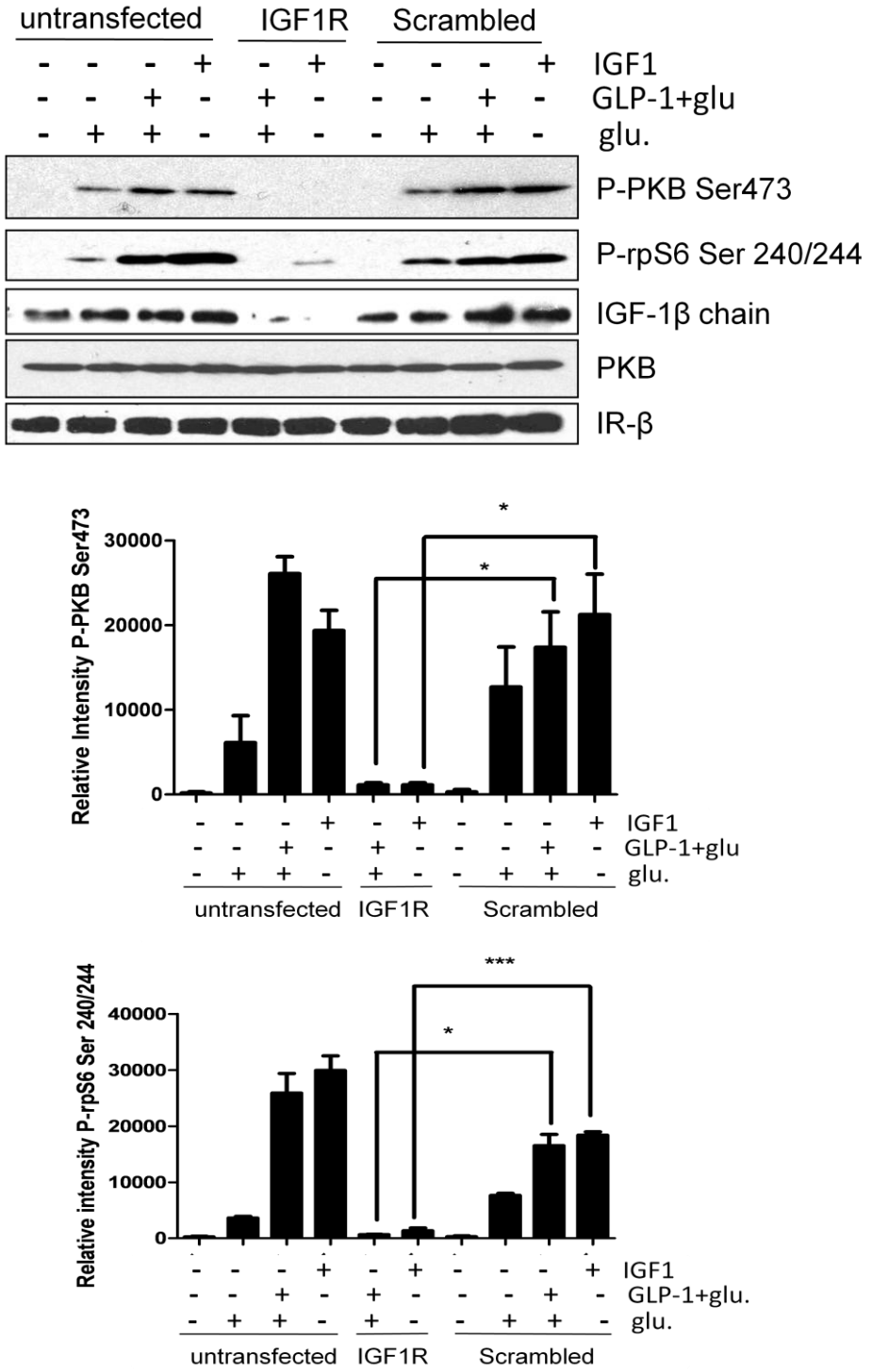


Figure 4.11. GLP-1 stimulated PKB phosphorylation in INS1E cells is dependent upon the activation of the IGF-1 receptor. INS1E cells were transfected with siRNA against IGF-1 receptor or scramble RNAi for 72 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. Cells were then washed with KRB containing 0.25x amino acids and incubated for 30 minutes with glucose (7.8mM) or glucose (7.8mM) plus GLP-1 (10nM) or IGF-1 (100nM) . Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB Ser473, phospho-rpS6 Ser240/244. The lower panel shows quantified data of phosphorylated PKB Ser473and phosphorylated rpS6 Ser 240/244. The results are mean $\pm$ S.E.M for three independent experiments.*P<0.05, ***P<0.001

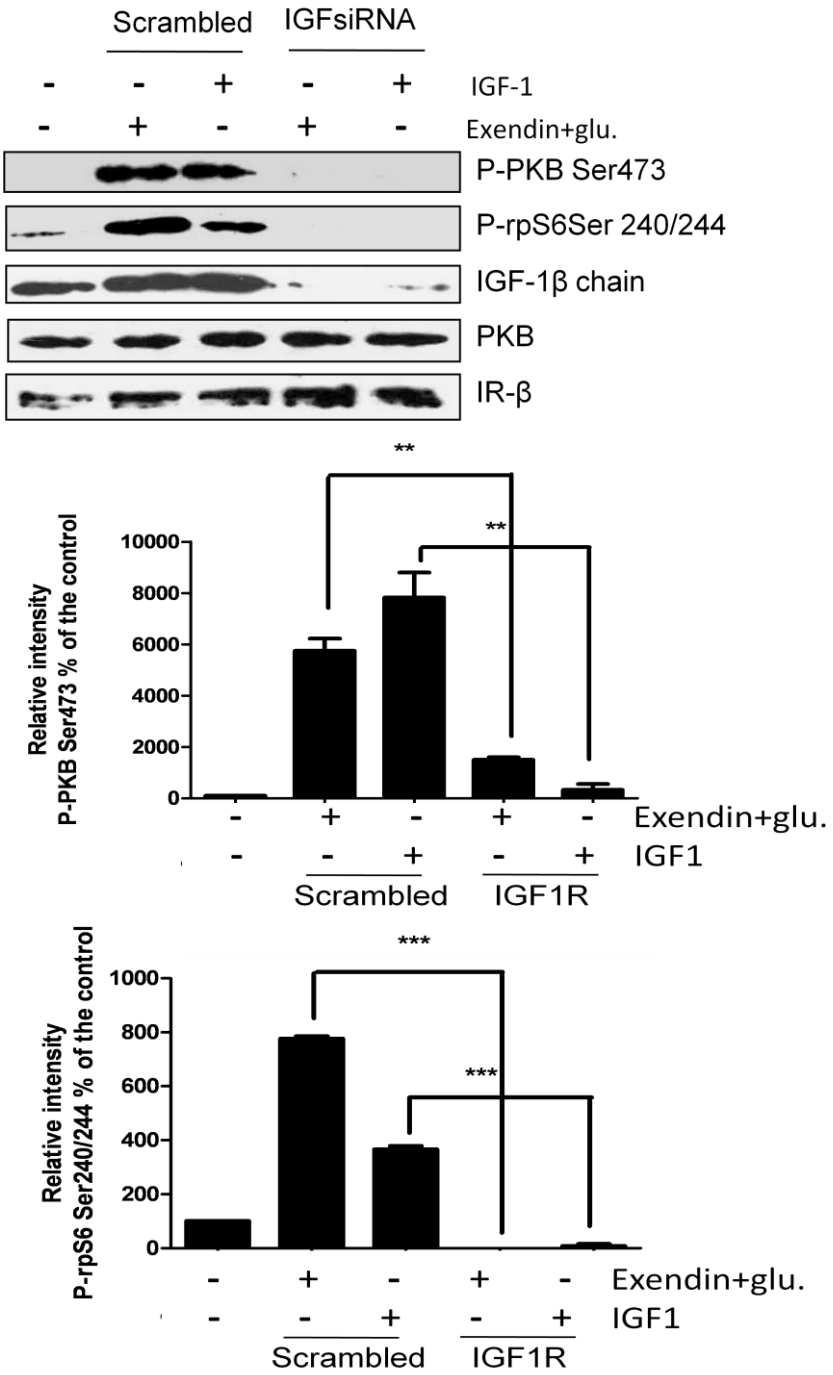


Figure 4.12. GLP-1 stimulated PKB phosphorylation in dispersed rat islets mediated via by the autocrine effect of IGF-1. Dispersed Rat Islets of Langerhans cells were transfected with siRNA against IGF-1 receptor or unrelated RNAi for 72 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. Cells were then washed with KRB containing 0.125x amino acids and incubated for 30 minutes with glucose (7.8mM) or glucose (7.8mM) plus GLP-1 (10nM) or IGF-1 (100 nM). Whole-cell lysates were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-PKB Ser473, phospho-rpS6 ser240/244. The lower panel shows quantified data of phosphorylated PKB Ser473and phosphorylated rpS6 Ser 240/244. The results are mean ± S.E.M for three independent experiments.**P<0.01, ***P<0.001

4.3 Discussion

4.3.1 PKB activation by GLP-1

In this study, I have shown that GLP-1 and glucose act synergistically to phosphorylate PKB in a concentration-dependent manner in INS1E cells. These findings are consistent with the results of a study reported by Trumper et al (Trumper et al., 2000). They showed that stimulation of INS-1 cells with GLP-1 at different glucose concentrations led to a three-fold increase in PKB phosphorylation at 2.5 mM and 5 mM glucose whereas at 10 mM and 20 mM glucose they detected a two-fold increase in PKB phosphorylation compared to respective controls. Although it is predictable that GLP-1 potentiates glucose-stimulated PKB phosphorylation, there is little evidence in the literature demonstrating that, especially in primary cells. I show that GLP-1 potentially augments glucose-induced phosphorylation of PKB in isolated rat islets (Figure 3.2). In human islets, it was reported that glucose at a concentration of 15mM induced the phosphoryation of PKB and this was further potentiated with 10 nM of GLP-1, though such potentiation was insignificant (Trumper et al., 2005).

4.3.2 GLP-1 stimulated PKB activation is independent of EGF-R transactivation

It has been suggested that GPCR can activate the EGFR either through the release of EGFR ligands released by the activation of metalloproteinases (Gavi et al., 2006) or through the phosphorylation of the EGFR by the non-receptor tyrosine kinase c-src on Tyr845, thus forming docking sites for proteins containing SH2 domains (Biscardi et al., 1999). For that reason, EGFR activation/phosphorylation can lead to the recruitment and subsequent activation of Class 1 PI3Ks. In INS(832/13) cells, the activation of PI3K by GLP-1 was shown to be dependent on transactivation of EGFR, which was demonstrated to be mediated by the activation of c-src and consequent activation of metalloproteases which cleave membrane bound BTC (endogenous EGF-like ligands), allowing transactivation of the EGFR (Buteau et al., 2003). Using inhibitors of PI3K to β-cells, I have shown that glucose and GLP-1 stimulated PKB phosphorylation is dependent on PI3K (Figure 4.2), implying a common mode of PKB activation. Furthermore, my data demonstrates that inhibition of EGFR by the selective EGFR inhibitor, AG1478, had no effect on GLP-1 stimulated PKB in INS1E cells, MIN6 cells and rat islets of langerhans. (Figures 4.4, 4.5 and 4.6). There are a number of possible reasons for the difference between my results compared to the

published data of Buteau et al. Firstly, the researchers (Buteau et al., 2003) stimulated pancreatic β-cells with GLP-1 for a longer period (18 hours) while I was able to detect GLP-1 induced phosphorylation of PKB after only 20 minutes from addition of GLP-1 (Figure 3.1). Therefore, this long period of stimulation with GLP-1 can activate other signalling pathways for example increased expression of the IGF-1 receptor following GLP-1 treatment reaches its peak after 18 hours (Cornu et al., 2009) . Secondly, researchers in the same study (Buteau et al., 2003) used AG1478 at concentration of 250 nm to examine the role of EGFR transactivation in mediating GLP-1 activation of PI3K. However, I showed that 5 nm of AG1478 is adequate to inhibit EGFR signalling to Erk1/2. Moreover, when I used 100 nm of AG1478, it inhibited both glucose and insulin induced phosphorylation of PKB indicating that AG1478 has only limited specificity at high concentrations. A further possible reason for this difference that they used a different cell line; INS(832/13). Moreover, they provided a further evidence to prove that the activation of PI3K by GLP-1 is mediated through c-src when they showed that src inhibitor blocked GLP-1 induced PI3K activation. However, work in our lab demonstrated that using src inhibitor did not affect the acute (1 h) activation of PKB by GLP-1 in MIN6 cells (Moore C and Herbert TP, unpublished data). It was previously shown in HEK293 over-expressing GLP-1R that GLP-1 induces Erk1/2 activation through a mechanism that involves EGF transactivation (Syme et al., 2006). However, I have demonstrated that the phosphorylation of Erk1/2 induced by GLP-1 was not affected by EGF-specific inhibitor. Yet, in pancreatic β cells GLP-1 stimulates Erk 1/2 activation independent of the transactivation of EGF. Hence, based on the evidence provided in this chapter, I postulate that acute GLP-1 treatment induced phosphorylation of PKB is independent of EGFR transactivation.

4.3.3 GLP-1 stimulated PKB phosphorylation in pancreatic β-cells may be mediated by the autocrine effect of IGF

It has been postulated that the mechanism by which glucose-induced an increase in PKB/mTORC1 activation in β-cells might be via secreted insulin positively feeding back on the β-cell to activate insulin signal transduction pathways (Xu et al., 1998b). In an insulin-secreting cell line, the beta TC3 cells, glucose induces the tyrosine phosphorylation of the insulin receptor beta-subunit and blocking insulin secretion by

either epinephrine or by removing extracellular Ca^{+2} inhibits this effect (Rothenberg et al., 1995). In addition, both glucose and insulin stimulate the tyrosine phosphorylation of insulin receptor substrate 1(IRS-1) and promote the association of IRS-1 with the p85 regulatory subunit of PI3K (Rothenberg et al., 1995). In this context, similar results were shown in rat pancreatic islets (Velloso et al., 1995). However, a recent study has reported that glucose can signal to mTORC1 independently of IR/PKB signalling pathway in clonal cell lines generated from insulin receptor-deficient β-cells (Bartolome et al., 2010). They showed that in $IR^{-/-}$cells glucose failed to phosphorylate PKB at Ser^{473} and TSC2 at Thr^{1462} but stimulated S6K1 at Thr^{389} and this phosphorylation was completely blunted by the addition of U0126, an MEK inhibitor (Bartolome et al., 2010). Therefore, they suggested that glucose can signal to mTORC1 via MEK/Erk activation dependent mechanism. In contrast, I have shown that glucose and GLP-1 induced activation of PKB/mTORC1 are independent to Erk activation (Figure 3.5). Similar results were shown by our group in MIN6 cells that glucose and GLP-1 can activate mTORC1 independent to MAPK/Erk activation (Moore et al., 2009). I think the difference in results presented by our group and results presented in $IR^{-/-}$ cells is attributed to the activation of Erk under conditions of knocking down of insulin receptor which might be a compensatory or adaptive mechanism to get mTORC1 activation.

In INS1 cells, GLP-1 increases the tyrosine phosphorylation of IRS-2, which co-immunoprecipitates with p85, the regulatory subunit of PI3K (Trumper et al., 2000). In addition, GLP-1 increases PI3K activity in Gab-1 (growth factor receptor bound 2 (Grb2)-associated binder-1) immunoprecipitates (Trumper et al., 2000). Gab-1 acts as an adaptor protein for Src homology-2 (SH2) domain-containing proteins that include the regulatory subunit p85 of PI3K (Rocchi et al., 1998). Gab-1 undergoes tyrosine phosphorylation at Tyr472 by insulin receptor and that this phosphorylation regulates its binding to p85 regulatory subunit of PI3K (Rocchi et al., 1998). Therefore, GLP-1 recruits several downstream components of insulin/IGF signalling. My data demonstrates that glucose and GLP-1 induced phosphorylation of PKB/mTORC1 is likely dependent on secretion since blocking secretion by diazoxide inhibits PKB phosphorylation. Moreover, phosphorylation of PKB induced by glucose and GLP-1 in INS1E cells was partially inhibited by nifedipine (Figure 4.8). It was reported that the calcium signalling through L-type channels represent only 25% of

the calcium current in INS1E cells as INS1E cells were found to express multiple non–L-type voltage dependent calcium channels including P/Q type, R type and T-type (Mears, 2004). Thus, insulin secretion is not blocked by nifedipine and explains why I can detect phosphorylation of PKB in the presence of nifedipine.

A rise in intracellular calcium is believed to trigger insulin exocytosis. In my study, I demonstrate that glucose and GLP-1 induced activation of PKB are inhibited by both BAPTA-AM (calcium chelator) and incubation with nominal calcium buffer which blocks the influx of extracellular Ca^{+2} across the plasma membrane (Figure 3.7, 3.8 and 3.9). In this context, it has been previously shown that blockade of Ca^{+2} influx by EGTA interfered with glucose-induced insulin release (Frodin et al., 1995). Thus, these results provide further evidence that glucose and GLP-1-induced activation of PKB is dependent on secretion. Because insulin is the major hormone released from β-cells in response to nutrients, hormones and neuronal stimuli, I thought that the autocrine effect of insulin may be responsible for the activation of PKB by GLP-1. However, my data shows that GLP-1-induced PKB phosphorylation was suppressed by knocking-down IGF-1R but not IR expression.

Several reports have revealed that insulin is not the only metabolically principal hormone stored in and secreted from the pancreatic β-cells, IGF-2 can be also co-localized with insulin inside β-cell granule (Buchanan et al., 2001; Hoog et al., 1997). There is some evidence in the literature demonstrating the physiological role of IGF-2 in the control of β-cell mass especially during the embryonic development. It was reported that the expression of IGF-2 is more pronounced in foetal pancreatic cells compared to adult pancreas suggesting that IGF-2 may be implicated in cell growth and cell differentiation during embryonic stage (Portela-Gomes and Hoog, 2000). In an animal model of Type 2 Diabetes, Goto–Kakizaki (GK) rats, the production of IGF-2 and IGF-1R is impaired and that in vitro IGF-2 supplementation increases cell proliferation (Calderari et al., 2007). Furthermore, during the neonatal period, pancreatic β-cells follow a programmed turnover including a stage of β-cell loss by apoptosis in which it was reported that IGF-2 expression was reduced (Petrik et al., 1999). It was also reported that IGF-2 stimulates islet cell growth and promotes the survival of transplanted islet cells in rodents (Robitaille et al., 2003). β-cell-specific IGF-1R knockout mice display normal growth and development of β-cells, but show

impairment of glucose tolerance and defective glucose-stimulated insulin secretion (Kulkarni et al., 2002). On the other hand, transgenic expression of IGF-2 in mice results in an increase of β-cell mass, however, these mice display some features of type 2 diabetes including hyperinsulinaemia, mild hyperglycaemia, and impaired glucose and insulin tolerance tests (Devedjian et al., 2000). A study in intercrossed heterozygous mice of IGF-IR/IRS-1/IRS-2 knockouts has demonstrated that IGF-1 promotes β-cell development and survival via an IRS-2 signalling pathway dependent mechanism (Withers et al., 1999). In addition, IGF-1 was shown to induce the proliferation of INS1 cells, in a glucose dependent manner, via IRS-induced activation of PI3K and mTORC1 (Hugl et al., 1998). IGF-1 was reported to prevent pancreatic β-cell death and this was concomitant with the activation of PKB and mTORC1 signalling pathway (Liu et al., 2002). Consistent with the literature, I have demonstrated that IGF can also activate PKB and mTORC1 in both INS1E cells and isolated rat islets of Langerhans (Figure 4.11 and Figure 4.12). Based on the evidence provided here that knocking down of IGF-1R inhibits PKB and mTORC1 activation, I think that IGF might be useful as a pharmaceutical agent for preventing pancreatic β-cell apoptosis, stimulation of β-cell growth and delay the onset of diabetes.

A recent study revealed that chronic stimulation (18 hours) with GLP-1 induced PKB phosphorylation in MIN6 cells is mediated by the activation of IGF-1 receptor signalling and the subsequent secretion of IGF-2 (Cornu et al., 2009). The authors showed that the up-regulation of IGF-1R by GLP-1 follows a slow kinetics reaching the maximum at 18 hours which parallels GLP-1 stimulation of PKB (Cornu et al., 2009). These observations are consistent with my results that show the role of IGF-1R in GLP-1 signalling to PKB, however, I have shown that GLP-1 can rapidly activate PKB (after only 20 minutes) and there was no change in the protein expression of IGF-1R upon GLP-1 treatment. Therefore, I hypothesize that GLP-1 activation of PKB is mediated by the activation of IGF-1R and the autocrine effect of IGF. Collectively, these results provide a link between GLP-1 and IGF signalling pathway and could have some physiological implications on β-cell mass. It could be a therapeutic approach to modulate IGF-1R and IGF-2 expression to prevent beta cell loss.

4.3.4 Importance of PI3K/PKB in GLP-1 signalling in pancreatic β-cell function

GLP-1 activates PI3K and PKB signalling pathway and enhances β-cell replication. GLP-1 was shown to promote cell proliferation in INS-1 cells which is abrogated by inhibiting PI3K (Buteau et al., 1999). Inhibition of PKB also prevents GLP-1 stimulated INS-1 cells proliferation (Wang et al., 2004). *In vivo* studies have also revealed the correlation between PKB activation and GLP-1 stimulated β-cell proliferation and delayed the onset of diabetes in Leptin$^{db/db}$ mice (Wang and Brubaker, 2002). Together, these data highlight the importance of PI3K/PKB in mediating GLP-1 stimulated β-cell proliferation.

GLP-1 can protect against cytokines induced apoptosis via a PKA dependent activation of PKB. The mechanism by which PKB prevents cytokines mediated β-cell apoptosis was reported by Cornu and his colleagues (Cornu et al., 2009). They have shown that GLP-1 induces the expression of the IGF-1R and the expression and secretion of IGF-2 and that the reduction of IGF-1R signalling through inhibition of IGF-2 or IGF-1R expression prevents GLP-1 dependent cytoprotection. Furthermore, it was demonstrated that the up-regulation of IGF-1R expression by GLP-1 was PKA dependent (Cornu et al., 2010). In this context, over-expression of dominant negative CREB induces diabetes as a result of reduction of β-cell mass which is correlated with reduction of IRS-2 expression (Jhala et al., 2003). Therefore, GLP-1 signalling via a PKA dependent mechanism promotes cell survival by increasing IGF-2 expression and secretion, IGF-1R expression, IRS-2 expression and activation of PKB.

CHAPTER 5

Chapter 5: The effect of the SNAT2 transporter and glutamine withdrawal on mammalian target of Rapamycin (mTOR) signalling

5.1 Introduction

5.1.1 Mechanisms of amino acids regulation of mTORC1

Classically, growth factors can regulate mTORC1 activity via either PI3K/PKB pathway or the Ras/MAPK pathway by phosphorylation of TSC2 leads to the functional inactivation of the TSC1-TSC2 complex. This complex has a GAP (GTPase-activating protein) domain that induces the intrinsic GTPase activity of the small G-protein Rheb, thus stimulate the conversion of Rheb into its GDP- bound inactive state. Rheb is a potential activator of mTORC1 which may be mediated via direct binding to mTOR (Bhaskar and Hay, 2007).

Rheb has been implicated in amino acids-dependent activation of mTORC1. This was evident by the finding that over expression of Rheb can overcome the decreased S6K1 phosphorylation observed upon amino acid starvation (Long et al., 2005b). This effect of Rheb is not via regulating amino acid transport but through a direct interaction with mTOR (Long et al., 2005a). Indirectly, Rheb can regulate mTORC1 activation through stimulation of phospholipase D which catalyzes the conversion of phosphatidylcholine (PC) into phosphatidic acid (PA). PA is a positive effector of mTORC1 activation. This is based on the observation of using l-butanol to inhibit PA accumulation or silencing phospholipase D inhibits mTORC1 activation (Fang et al., 2003; Fang et al., 2001) .

Another component of the pathway by which amino acids regulate mTORC1 activity is the Rag GTPases (Sancak et al., 2008). The rags are small GTP-binding proteins and consists of four proteins; RagA, RagB, RagC and RagD. In HEK293 cells, it was observed that rags are co-immunoprecipitated with endogenous and coexpressed mTORC1 suggesting a physical association between ragGTPase and raptor. They investigated the functionality of this interaction by the expression of mutant Rag-GTPase. Mutants that are permanently bound to GDP (Rag^{GDP}) inhibit the phosphorylation of S6K1 whereas the mutants that permanently bound to GTP (Rag^{GTP}) increase the activation of mTORC1and maintain mTORC1 activity even in the absence of amino acids. Furthermore, Knockdown of RagGTPase using shRNA

decreases amino acid stimulation of S6K1. The authors postulated that amino acids regulate the activity of the mTORC1 pathway through the Rag proteins, and mediate the translocation of mTORC1 to the same intracellular compartment that contains its activator Rheb (Sancak et al., 2008).

In addition to Rheb and RagGTPAse, there are some reports highlighted the role of vps34 as signalling intermediate in amino acid regulation of mTORC1 (Gulati et al., 2008; Nobukuni et al., 2005). In HEK293, knockdown of hvps34 using siRNA blocks both insulin and amino acids-induced activation of mTORC1 without affecting the phosphorylation of PKB at Ser473. Moreover, overexpression of a dimeric FYVE domain, which competes for intracellular PI3P-docking sites, sequesters PI3P and inhibits mTORC1 activation (Nobukuni et al., 2005).

5.1.2 Aims

In pancreatic β-cells, the role of amino acid transporters in the regulation of amino acid-dependent regulation of mTORC1 is unknown. Thus, the aim of this study is to investigate the role of SNAT2, the most abundant amino acid transporter in pancreatic β-cells, in amino acids signalling to mTORC1.

5.2 Results

5.2.1 Glutamine is Essential for Rapamycin-Sensitive mTORC1 Signalling

Glutamine is the most abundant amino acid in the body and is the major substrate for the SNAT2 transporter. In order to assess the role of glutamine in the regulation of mTORC1 activation, I have characterized the effect of glutamine withdrawal on insulin-stimulated phosphorylation of rpS6 at Ser240/244. Intact islets of Langerhans were isolated as described in material and methods then they were serum starved overnight in CMRL-1066 containing glutamine. Next morning, the medium was removed and replaced with EBSS containing 2 or 20 mM glucose and 1x essential amino acids. Islets were then treated with or without insulin (100nM) plus or minus glutamine (200nM) for 30 minutes. Insulin, in the presence of 20 mM of glucose, stimulated the phosphorylation of rpS6 at Ser240/244 which was significantly inhibited by glutamine deprivation (Figure 5.1). In addition, glutamine starvation in the presence of 20mM glucose reduced insulin-stimulated phosphorylation of PKB at Ser473 by about 30% (Figure 5.1).

To confirm that the effect of glutamine withdrawal on the phosphorylation of rpS6 is mediated through inhibiting the activity of mTORC1, islets of Langerhans were treated with insulin (100nM) in presence or absence of rapamycin (200nM) for 30 minutes. In the presence of rapamycin, the phosphorylation of rpS6 at Ser240/244 as well as the phoshorylation of S6K1 at Thr389 was inhibited (Figure 5.2). 4EBP1 generally appears as three migrating bands (α, β and γ) when separated by SDS-PAGE and analysed by immunoblotting. The nonphosphorylated α-form migrates most rapidly. Increases in the phosphorylation of the intermediate β form to the most highly phosphorylated γ form decreases migration of 4EBP1 when separated by SDS-PAGE. Treatment of rat islets with 20mM glucose plus insulin resulted in enhanced phosphorylation of 4EBP1 γ (lane 2, Figure 5.2) and the concomitant decrease of the intermediate migrating 4EBP1 β compared with glucose treatment. Rapamycin inhibited the phosphorylation of 4EBP1γ as demonstrated by the decrease in SDS-PAGE mobility shift (Figure 5.2, compare lanes 2 and 3). In contrast, the phosphorylation of PKB at Ser473 was not affected by rapamycin.

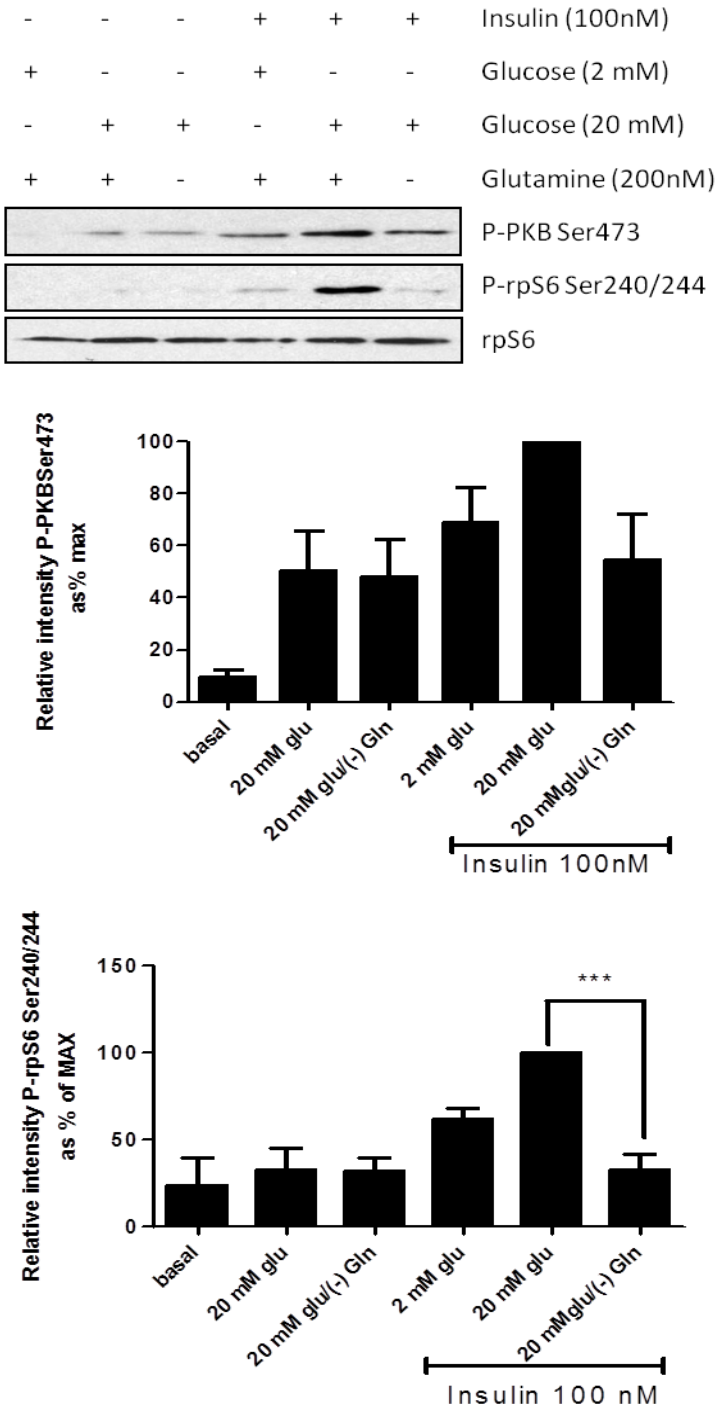


Figure 5.1. Effect of extracellular L-glutamine (L-Gln) starvation on mammalian target of rapamycin (mTOR)-dependent signalling in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 2 or 20 mM glucose and 1x essential amino acids. Islets were incubated for 30 minutes with or without 100 nM insulin plus or minus glutamine (200 nM). Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser 473 and rpS6 as loading control. The results are mean $\pm$ S.E.M for three independent experiments. ***, $P<0.001$.

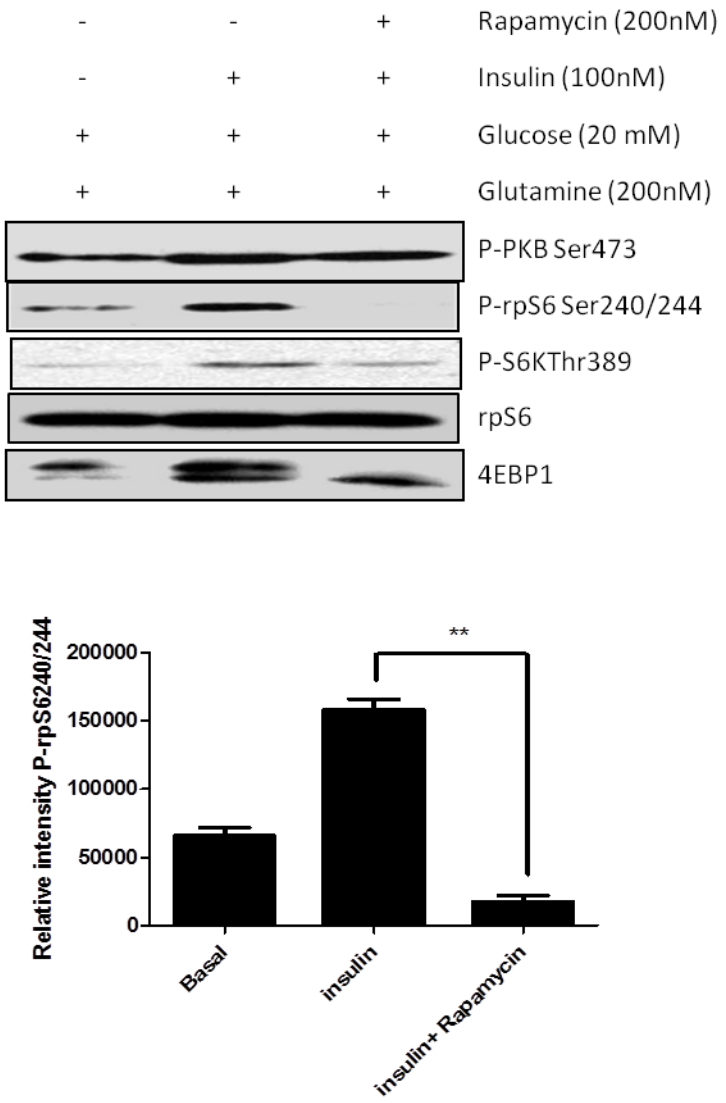


Figure 5.2. Effect of rapamycin on insulin signalling to mammalian target of rapamycin (mTOR)-dependent signalling in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose and 1x essential amino acids. Islets were incubated for 30 minutes with or without 100 nM insulin plus or minus rapamycin (200 nM). Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser 473, phospho-S6K1Thr389, 4EBP1 and rpS6 as loading control. The results are mean $\pm$ S.E.M for three independent experiments. **, $P < 0.01$.

It is well established that mTORC1 can regulate cap-dependent translation mediated by its downstream target; 4EBP1. This protein binds to and inhibits the function of the cap-binding translation factor eIF4E. 4EBP1 prevents the association between eIF4E and eIF4G, thus blocks the formation of initiation factor complex (eIF4F) required for cap-dependent translation. Insulin by phosphorylation of 4EBP1 decreased its affinity for eIF4E leading to its dissociation from eIF4E and allowing eIF4E to bind to eIE4G. This was evident by the increase of association of eIF4G with ^{m7}GTP beads as shown in Figure 5.3 (lane2). The recruitment of eIF4G to the eIF4E cap complex was inhibited by glutamine deprivation or rapamycin (lanes 3 and 4, Figure 5.3). Taken together, it can be concluded that glutamine is required for insulin-induced activation of mTORC1.

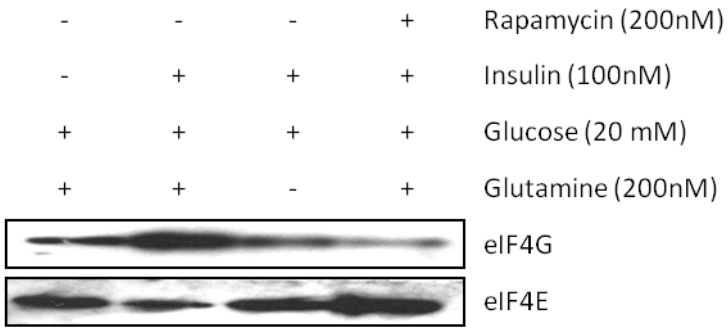


Figure 5.3. The recruitment of the eukaryotic translation initiation factor 4G (eIF4G) to the eIF4E cap complex is sensitive to L-glutamine in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose and 1x essential amino acids. Islets were incubated for 30 minutes with or without 100 nM insulin plus or minus glutamine (200 nM) or plus or minus rapamycin (200 nM). Proteins were resolved by SDS-PAGE and levels of eIF4G associated with ^{m7}GTP beads analyzed using western blotting (N=1).

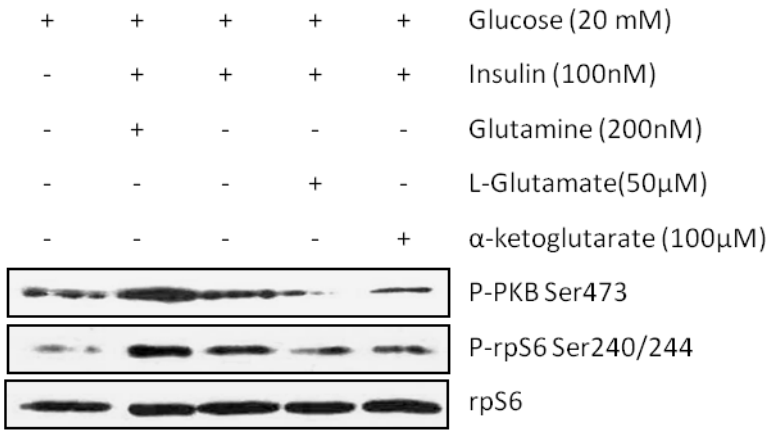
5.2.2 Glutamine metabolites unable to activate mTORC1

In mammalian cells, L-glutamine is converted to L-glutamate through the activity of the glutaminase enzyme. In turn, it will enter tricarboxylic acid (TCA) cycle via a process known as glutaminolysis (Newsholme et al., 2003). Thus, glutamine can enhance metabolic flux. In order to investigate whether glutamine effect on mTOR is via an increase in cellular energy, I sought to use L-glutamate and α-ketoglutarate (products of glutaminolysis) and examine their effect on mTORC1 activation in glutamine starved cells. Addition of L-glutamate and α-ketoglutarate were unable to restore insulin stimulated phosphorylation of rpS6 (Figure 5.4A). Because I had concerns about cell permeability of L-glutamate and α-ketoglutarate, I confirmed the results using the ester form of L-glutamate and α-ketoglutarate. Similarly, L-Glutamic acid dimethyl ester hydrochloride or Dimethyl α-ketoglutarate cannot restore insulin induced mTORC1 activation (Figure 5.4B). Therefore, glutamine-regulated activation of mTORC1 is not mediated by increased glutaminolysis.

5.2.3 Inhibition of SNAT2 antagonizes mTORC1 activity

The transport of glutamine across the cell membrane is mediated by at least four different transporters; Na^+-dependent systems A, ASC, N and the Na^+-independent system L. SNAT2 is the most abundant isoform of SLC38A family transporters expressed in islets and has high affinity for glutamine. Thus, to investigate the role of SNAT2 in regulation of mTORC1 activity, I first used MeAIB; an inhibitor to SNAT2 transporter. Islets of Langerhans were treated with insulin in presence or absence of 10 mM MeAIB and plus or minus glutamine. The phosphorylation of rpS6 at Ser240/244 was significantly reduced in presence of MeAIB and upon glutamine starvation (Figure 5.5). On the other hand, MeAIB did not affect the phosphorylation of PKB (Figure 5.5). Therefore, this suggests that amino acids dependent insulin phosphorylation of rpS6 is dependent on SNAT2.

A)



B)

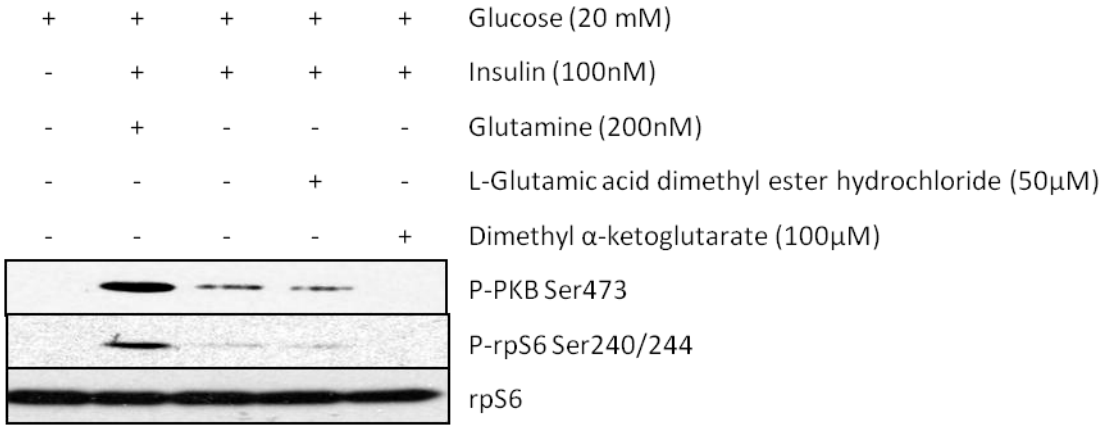


Figure 5.4 Glutamine metabolites fail to activate mTOR signalling in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose and 1x essential amino acids. **(A):** Islets were incubated for 30 minutes with 100 nM insulin with or without glutamine (200 nM) or L-Glutamate (50µM) or α-ketoglutarate (100µM). The results are representative of two independent experiments. **(B)** Islets were incubated for 30 minutes with 100 nM insulin with or without glutamine (200 nM) or L-Glutamic acid dimethyl ester hydrochloride (50µM) or Dimethyl α-ketoglutarate (100µM). Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244 and phospho-PKB Ser473 and rpS6 as loading control. N=1.

5.2.3.1 Silencing SNAT2

To provide further evidence that SNAT2 is required for insulin-induced activation of mTORC1, I applied RNA interference technology for silencing of SNAT2 gene. I got two sequences of double stranded RNAs; the first was provided by Dr Alan Bevington and this siRNA that has the forward sequence 5'-CUGACAUUCUCCUCCUCGUdTdT and the second was purchased from Dharmacon and has the sequence GGGAUCAUGUAGACGCAAA. Dispersed Islets of Langerhans were transfected using lipofectamine at a concentration of 100nM from each siRNA for 72 hours and SNAT2 silencing was demonstrated by RT-PCR (Figure 5.6 A and B). SNAT2 expression was knocked-down by the silencing SNAT2 siRNAs while the transfection agent or scrambled control siRNA had no effect (Figure 5.6 A and B). Each of the SNAT2-targeted siRNAs blocked the phosphorylation of rpS6 at Ser240/244 and that of S6K1 at Thr389 (Figure 5.7 A and B). Interestingly, the phosphorylation of PKB was inhibited by knocking down of SNAT2. These results indicate that mTORC1 and PKB activation requires SNAT2 expression.

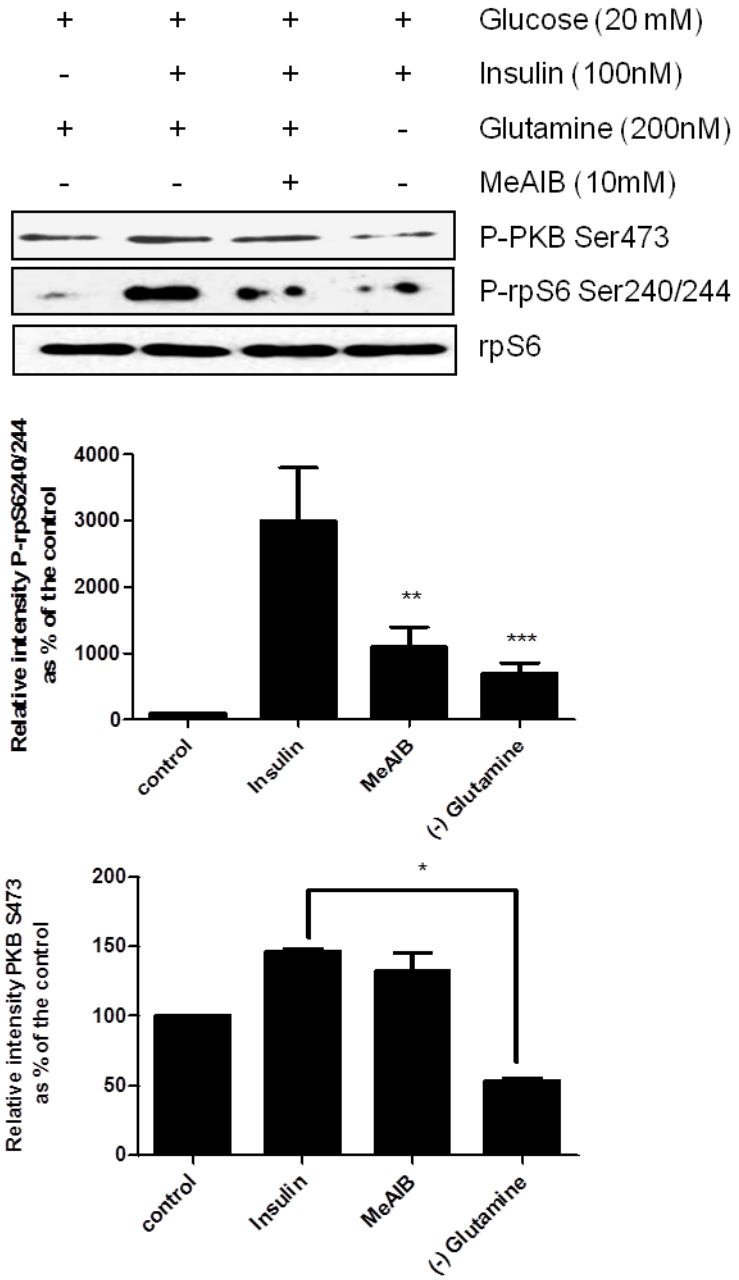
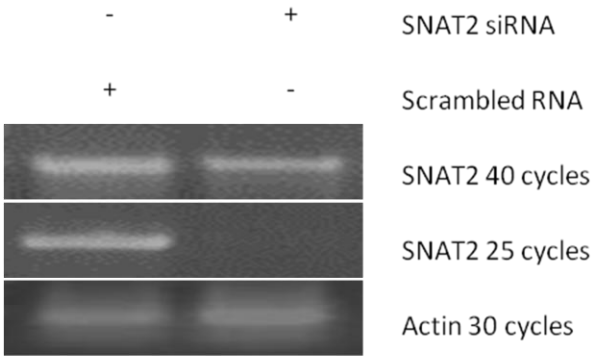


Figure 5.5: Effect of 30 minutes of inhibition of SNAT2 transporters with 10 mM methylaminoisobutyrate (MeAIB) or extracellular L-glutamine (L-Gln) starvation on mammalian target of rapamycin (mTOR)-dependent signalling in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose and 1x essential amino acids. Islets were incubated for 30 minutes with 100 nM insulin with or without MeAIB (10mM) or glutamine (200 nM). Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser 473 and rpS6 as loading control. The results are mean $\pm$ S.E.M for three independent experiments. *, P <0.05; **, P < 0.01; and ***, P < 0.001 compared with insulin.

A)



B)

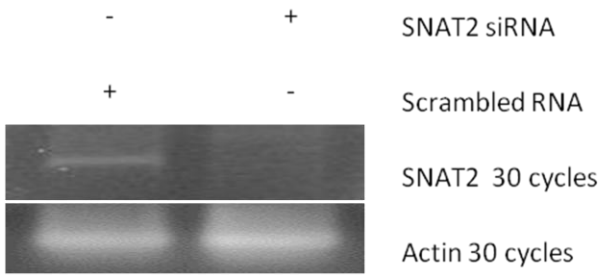


Figure 5.6. The expression of SNAT2 in dispersed islets transfected with either control or SNAT2 siRNA. Dispersed Rat Islets of Langerhans cells were transfected with siRNA against SNAT2 using two different target sequences (A) 5'-CUGACAUUCUCCUCCUCGUdTdT and (B) GGGAUCAUGUAGACGCAAA or unrelated RNAi for 72 hours. RNA was isolated as described in the material and methods and the expression of SNAT2 was done by reverse transcriptase-PCR using specific oligonucleotide primers. The PCR products were resolved by 1.5 % agarose gel containing ethidium bromide and visualized under ultraviolet fluorescence. The expression of actin was used as loading control.

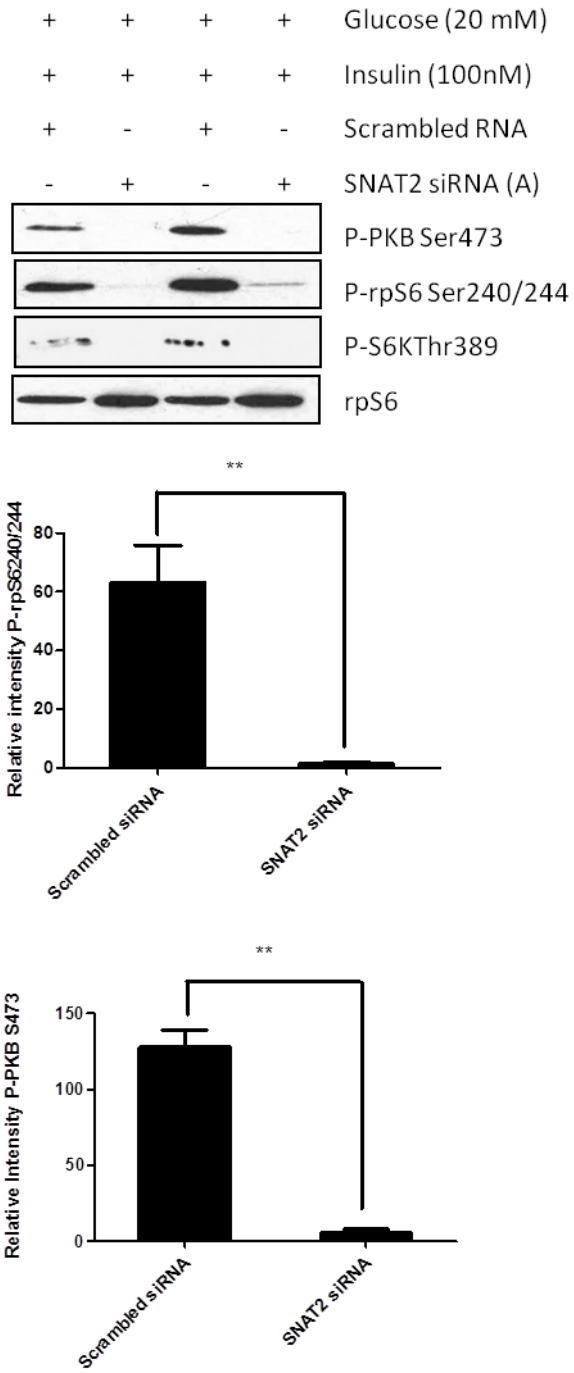

+ + + + Glucose (20 mM)
+ + + + Insulin (100nM)
+ - + - Scrambled RNA
- + - + SNAT2 siRNA (A)
P-PKB Ser473
P-rpS6 Ser240/244
P-S6KThr389
rpS6
**
Relative intensity P-rpS6240/244
80
60
40
20
0
Scrambled siRNA
SNAT2 siRNA
**
Relative Intensity P-PKB S473
150
100
50
0
Scrambled siRNA
SNAT2 siRNA

Figure 5.7 (A)

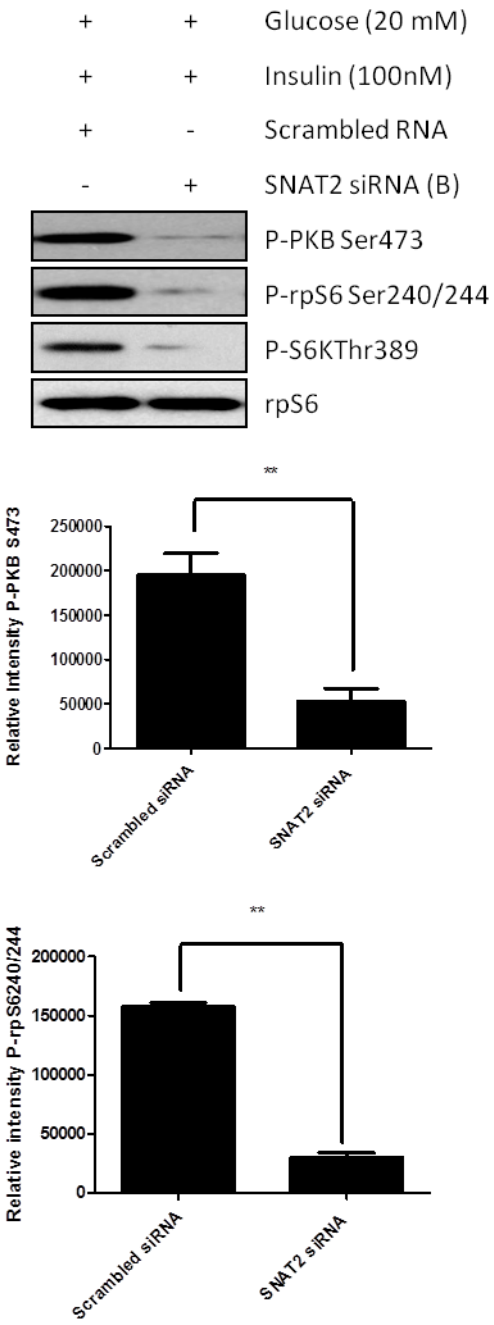


Figure 5.7 (B): Effect of SNAT2 siRNA on mTOR signalling in rat islets of Langerhans. Dispersed Rat Islets of Langerhans cells were transfected with two different sequences of siRNA against SNAT2 (A) 5'-CUGACAUUCUCCUCCUCGUdTdT and (B) GGGAUCAUGUAGACGCAAA or unrelated RNAi for 72 hours. Cells were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose and 1x essential amino acids . Islets were incubated for 30 minutes with 100 nM insulin with glutamine (200 nM). Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phosphor-S6K1 Thr389 and phospho-PKB Ser473 and rpS6 as loading control. The results are mean $\pm$ S.E.M for three independent experiments. **, P <0.01.

5.2.4 LAT1 is required for amino acid sensing to mTORC1

LAT1 transporter is responsible for the influx of branched side-chain amino acids such as Leucine. It has been proposed that LAT1 can couple with SNAT2 to transport essential amino acids into the cell in exchange for the efflux of intracellular glutamine (Nicklin et al., 2009). Furthermore, it has been reported that Leucine promotes β-cell proliferation through mTORC1 activity (McDaniel et al., 2002; Xu et al., 2001). Thus, I sought first to interrogate the requirement of LAT1 in mediating mTORC1 activity. I used 2-aminobicyclo(2,2,1)hepatane carboxylic acid (BCH); an inhibitor of LAT1. BCH suppressed the phosphorylation of rpS6 Ser240/244 suggesting inhibition of mTORC1 activity (Figure 5.8). Moreover, the phosphorylation of PKB was also inhibited by BCH. Similarly, withdrawal of essential amino acids inhibited the phosphorylation of rpS6 Ser240/244 and PKB at Ser473 (Figure 5.8). Therefore, these results demonstrated that branched-side amino acids are essential for mTORC1 and PKB activation and secondly LAT1 activity is required for the activity of mTORC1 and PKB.

The above results may support the idea that there may be coupling between SNAT2 and LAT1 which precedes mTORC1 activation. To test this hypothesis, islets of Langerhans were treated with MeAIB, BCH and under conditions of glutamine or branched-side amino acids starvation for 30 minutes. The lysates were then analysed for the intracellular free amino acid concentrations using HPLC. The components and the concentration of amino acids present in the minimum essential medium (MEM) are listed in Table 5.1. Under the control conditions, the four major free amino acid pools in the cells were glutamine, glutamate, glycine and alanine. It was observed that the intracellular levels of glutamine went down significantly upon glutamine withdrawal and treatment with BCH (Figure 5.9). The observation that inhibition of LAT system reduced intracellular glutamine suggests the coupling of system L with another transport system to maintain the In/out glutamine gradient. Inhibition of SNAT2 with MeAIB partly depletes the intracellular glutamine concentration (Figure 5.9). It was also noticed the intracellular concentrations of glutamate, alanine and glycine were reduced upon withdrawal of glutamine and L-system amino acids and inhibition of SNAT2 and LAT1 carriers. As expected, BCH and starvation of system L substrates depleted the intracellular levels of leucine and isoleucine.

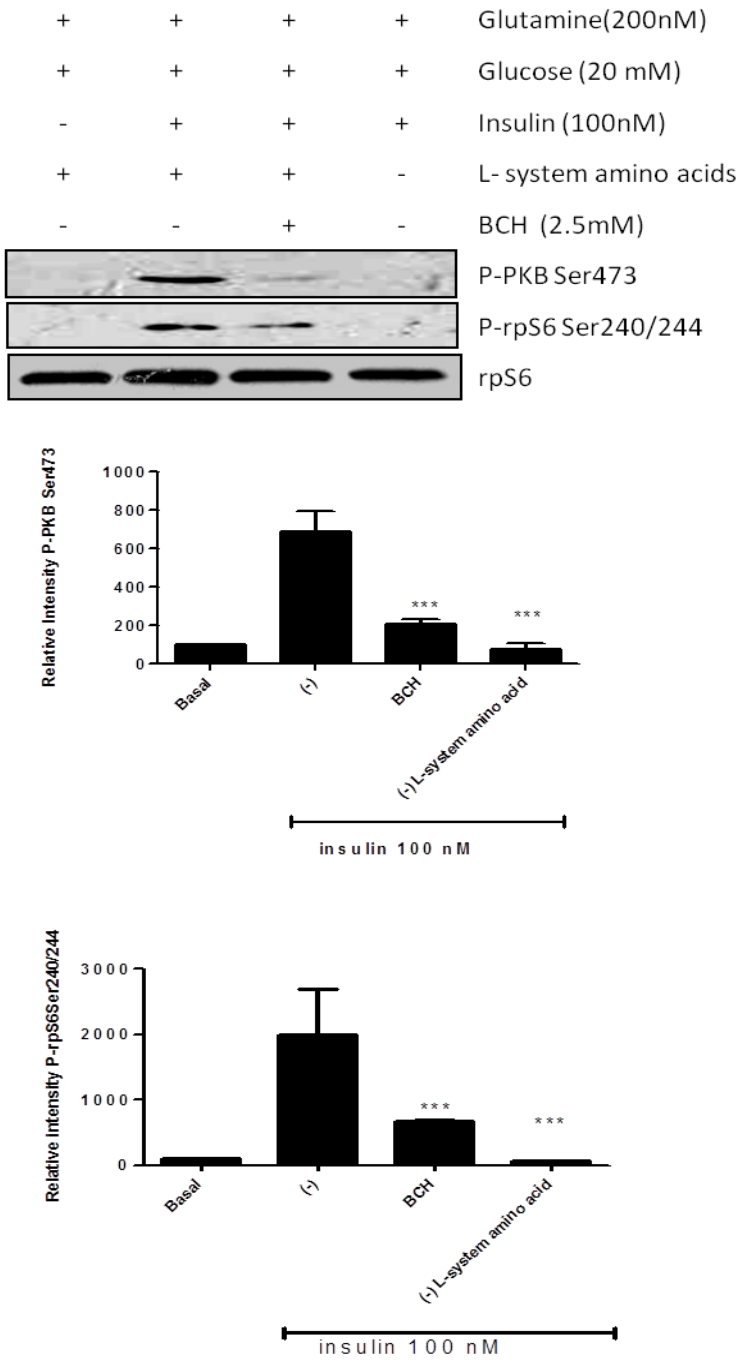
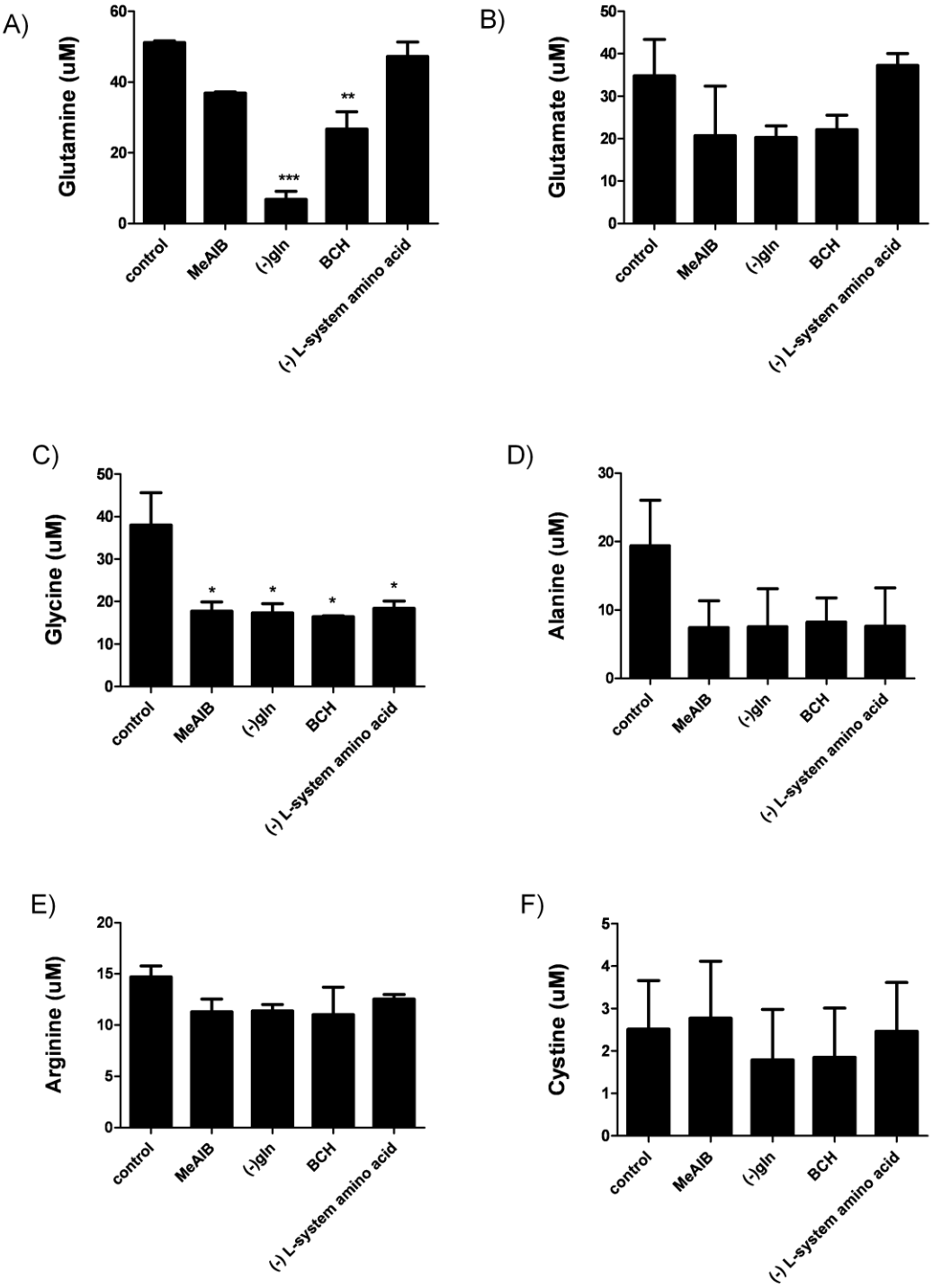


Figure 5.8 : Effect of 30 minutes of inhibition of L-type amino acid transporter 1 (LAT1) with 2.5 mM 2-aminobicyclo-(2,2,1)heptanecarboxylic acid (BCH) or extracellular system L amino acids starvation on mammalian target of rapamycin (mTOR)-dependent signalling in rat islets of Langerhans. Islets of Langerhans were serum starved overnight in CMRL-1066 medium containing glutamine. The medium was then removed and replaced with EBSS containing 20 mM glucose, glutamine and 1x essential amino acids. Islets were incubated for 30 minutes with 100 nM insulin with or without BCH (2.5mM) or L-system amino acids. Proteins were resolved by SDS-PAGE, blotted on a PVDF membrane and probed with antisera against phospho-rpS6 Ser240/244, phospho-PKB Ser473 and rpS6 as loading control. The results are mean $\pm$ S.E.M for three independent experiments. ***, $P < 0.001$;compared with insulin.

Table 5.1 Components of minimum essential medium (MEM)

Amino Acids	Concentration in fresh MEM (μ mol/L)
L-Ala	Nil
L-Arg	600
L-Asn	Nil
L-Asp	Nil
L-cys	200
L-Glu	Nil
L-Gly	Nil
L-His	200
L-Ile	400
L-Lys	400
L-Met	100
L-Phe	200
L-Pro	Nil
L-Ser	Nil
L-Thr	400
L-Trp	50
L-Tyr	200
L-Val	400


A)
Glutamine (uM)
60
40
20
0
***
**
control
MeAIB
(-)gln
BCH
(-) L-system amino acid
B)
Glutamate (uM)
50
40
30
20
10
0
control
MeAIB
(-)gln
BCH
(-) L-system amino acid
C)
Glycine (uM)
50
40
30
20
10
0
*
*
*
*
control
MeAIB
(-)gln
BCH
(-) L-system amino acid
D)
Alanine (uM)
30
20
10
0
control
MeAIB
(-)gln
BCH
(-) L-system amino acid
E)
Arginine (uM)
20
15
10
5
0
control
MeAIB
(-)gln
BCH
(-) L-system amino acid
F)
Cystine (uM)
5
4
3
2
1
0
control
MeAIB
(-)gln
BCH
(-) L-system amino acid

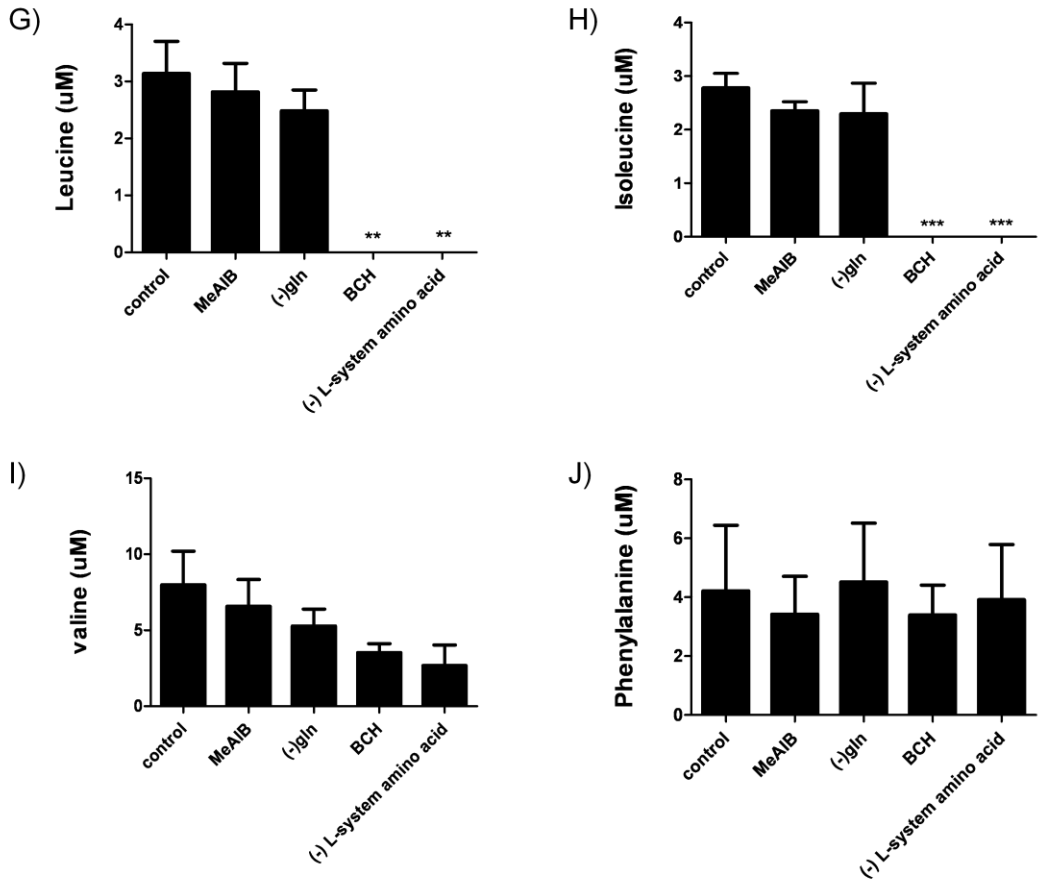


Figure 5.9. Effect of extracellular L-glutamine (L-Gln) or System L substrates starvation or inhibition of SNAT2 with 10 mM methylaminoisobutyrate (MeAIB) or inhibition of LAT1 transporter with 2.5 mM 2-aminobicyclo-(2,2,1)heptanecarboxylic acid (BCH) on intracellular free amino acid profile in rat islets of Langerhans. After 30 minutes of incubation with MeAIB or BCH or in absence of extaracellular glutatamine or System L substrates, islets were rapidly chilled on ice, rinsed three times with ice-cold 0.9% (wt/vol) NaCl, and deproteinized by scraping in 50 µl of 0.3 M perchloric acid. Precipitated protein was sedimented (10 min, 4°C, 140000x *g*) and supernatant was used for determination of amino acids on an Agilent 1100 high-performance liquid chromatograph with Zorbax Eclipse AAA column (4.6 x 75 mm, 3.5 µm) at 40°C with *o*-phthalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate precolumn derivatization and ultraviolet and fluorimetric postcolumn detection. The results are mean ± S.E.M for three independent experiments. *, P <0.05; **, P <0.01; and ***, P < 0.001 compared with control.

Glutamine starvation or incubation with MeAIB for 30 minutes did not affect the intracellular levels of system L substrates including leucine and isoleucine. It is difficult to see the effect of MeAIB or glutamine withdrawal on the concentrations of other L system substrates because of their low concentrations therefore they are hardly detected in primary pancreatic β-cells.

5.3 Discussion

5.3.1 Amino Acid Status in Diabetes

5.3.1.1 General Functions of Amino Acids

Amino acids, as constituents of protein, are required for the synthesis of bioactive molecules such as hormones and neurotransmitters. Amino acids act as substrates for gluconeogenesis and ureagenesis (Kimball and Jefferson, 2002). Beside this important role in protein synthesis, amino acids act as signalling molecules, mainly can activate mTOR signalling pathway (Kimball and Jefferson, 2006). In β-cells, amino acid metabolism is known to enhance insulin secretion (Newsholme et al., 2007).

5.3.1.2 Effects of Diabetes on Amino acid Levels

Insulin deficiency or impairment of insulin action affects the levels of amino acids as insulin is responsible not only for glucose metabolism but lipids and amino acid metabolism as well (Tessari et al., 2011). Insufficient amino acids has been considered as an important element in the pathogenesis of some complications of diabetes (Anuradha, 2009).

The effect of insulin deficiency on the amino acid profile in plasma and heart muscle of diabetic rats was first demonstrated by Scharff and Wool in 1966. Following induction of diabetes and the subsequent insufficiency of insulin, the concentration of alanine, valine, isoleucine, leucine and taurine were increased, tyrosine and tryptophan were decreased and some amino acids were unchanged (Scharff and Wool, 1966). The amino acid profile in plasma, erythrocytes, leucocytes and platelets of diabetic patients was demonstrated by some reports (Carl et al., 2002; Franconi et al., 1995; Szabo et al., 1991). In patients with type 1 diabetes, the levels of taurine in the plasma and platelets were decreased (Franconi et al., 1995). Similarly, it was reported that taurine content in the plasma of patients with type 2 diabetes was reduced (De Luca et al., 2001). On the other hand, the levels of threonine, alanine and isoleucine were increased in the plasma of type 2 diabetic subjects (De Luca et al., 2001). In platelets of type 1 diabetic subjects, the levels of aspargine, glycine,

taurine, alanine, valine, cysteine, leucine, phenyl alanine and lysine were significantly reduced (De Luca et al., 2001).

5.3.2 Glutamine, SNAT2 and LAT1 and its substrates regulate mTORC1 signalling pathway

Glutamine is the most abundant non-essential amino acids in the body. It is estimated that glutamine constitutes more than 60% of the free amino acids. Depending on the cell type, the intracellular glutamine concentration ranges between 2 and 20 mM and is present in concentration of 0.7 mM in the plasma (Curi et al., 2005b). Consistence with this, my results has shown that pancreatic β-cells contain large pool of glutamine (Figure 5.9 A). Glutamine plays diverse regulatory roles in several cell processes including cell metabolism, protein synthesis and cell integrity. In pancreatic β-cells, glutamine has a prominent role in regulation of insulin and glucagon release which is dependent on the transport of glutamine mainly by SNAT2 transporters (Gammelsaeter et al., 2009). It was also demonstrated in β-cells that amino acids are critical elements in regulation of the activity of mTORC1 (Kwon et al., 2004a). Here, I have shown that glutamine is an essential factor that allows insulin to activate mTORC1 in rapamycin dependent manner (Figure 5.1).This is evident by the inhibition of the phosphorylation of rpS6 upon glutamine withdrawal. In addition, glutamine deprivation blocked the recruitment of eIF4G to eIF4E (Figure 5.3). Interestingly, the phosphorylation of PKB at Ser473 was also reduced upon glutamine starvation suggesting that glutamine can regulate not only mTORC1 but mTORC2 as well. This finding is similar to the results published recently that showed that amino acids can signal to mTORC2 via activation of class I PI3K (Tato et al., 2010). However, the exact mechanism by which this occurs is still unclear. One possible mechanism is that glutamine can increase the intracellular sodium through its transport by SNAT2 causing membrane depolorization and activation of calcium channels as explained before in section 5.1.3.2.4. In turn, calcium can phosphorylate PKB at Thr308 via activation of Ca^{+2}/calmodulin-dependent protein kinase kinase (CaM-KK) (Yano et al., 1998). Phosphorylation of PKB at Thr308 should result in activation of PKB (Alessi et al., 1997) and hence can activate its downstream targets. In addition, it is well documented the role of hvps34 in amino acids sensing to mTORC1 in calcium dependent manner (Nobukuni et al., 2005).

Amino acids are secretagogues for insulin secretion in pancreatic β-cells so one can think that the endogenous insulin released in response to amino acids is responsible for activation of mTORC1. Nevertheless, addition of exogenous insulin (100nM) and in presence of 20 mM of glucose to rat islets in my experiments was not able to activate mTORC1 in the absence of amino acids (glutamine or L-system amino acids). Consistent with this finding, it has been shown that amino acids can signal to mTORC1 independent to the upstream insulin signalling pathway (Xu et al., 1998a). In this study, amino acids stimulate the phosphorylation of 4EBP1 under conditions that block insulin secretion; incubation at 24° C and in the presence of two tyrosine kinases inhibitors: genistein and herbimycin A (Xu et al., 1998a).

A summary of my hypothesis of the mechanism that explains how SNAT2 couples with PKB/mTORC1 signalling pathway is illustrated in figure 5.10. This proposed mechanism is supported by my data shown in Figure 3.9 that calcium chelators blocked mTORC1 activation. A further relevant explanation is that SNAT2 can couple to integrin $\alpha_3\beta_1$ (McCormick and Johnstone, 1995) that in turn can be translocated with adhesion kinases that are powerful activators of PI3K (Thamilselvan et al., 2007). The fact that glutamine withdrawal ultimately produces a decrease in its concentration on both sides of the plasma membrane (Figure 5.9A) indicates the importance of its transporters in regulation of mTORC1 signalling.

Selective silencing of SNAT2 with siRNA resulted in significant impairment of PKB and mTORC1 in the presence of insulin (Figure 5.7). This is strongly suggests that this transporter is a key player in regulation of PKB and mTORC1 signalling. In L6 myoblasts, knocking-down SNAT2 expression decreased PI3K activity and hence PKB phosphorylation (Evans et al., 2008). Moreover, they found that silencing of SNAT2 resulted in the depletion of glutamine. It has been proposed that SNAT2 can act as nutrient receptor besides its function as a transporter (Hyde et al., 2007). SNAT2 can regulate its own expression by being a sensor to amino acids availability that may be transduced to signalling pathway (Hyde et al., 2007). Therefore, I hypothesize that amino acid sensing by SNAT2 can signal to PI3K and thus regulate the activity of PKB and in turn mTORC1 (Figure 5.10).

In this study (Figure 5.5), I have demonstrated that inhibition of the flux through SNAT2 by treatment with MeAIB did not exactly mimic the effects of glutamine withdrawal or SNAT2 silencing on PKB/mTORC1 signalling pathway. MeAIB was shown to inhibit insulin-stimulated phosphorylation of rpS6 but did not affect PKB phosphorylation. This could be explained as follows: MeAIB is an amino acid analogue so it acts as competitive inhibitor and is transported by SNAT2 causing its activation by a transceptor–like mechanism (Hyde et al., 2007). Coupling of SNAT2 and MeAIB can generate a permissive signal to PI3K that allows activation of PKB. Furthermore, such coupling may also induce a rise in the intracellular calcium leading to activation of PKB. Another possibility is that MeAIB was shown to induce an increase in cell volume of INS1E cells (Archana Nair and Terry Herbert, unpublished data) and cell swelling can induce the activation of PKB (Webster et al., 2002). In this case, MeAIB may act as direct inhibitor to mTORC1. In addition, MeAIB partly depletes glutamine (Figure 5.9 A) which can also explain why MeAIB inhibits mTORC1 activation**.**

In the present study, I have observed that glutamine metabolites are not able to restore mTORC1 activation (Figure 5.4). These data are similar to that of Nicklin et al who reported that L-glutamine-regulated activation of mTORC1 is a proximal event and does not result from increased glutaminolysis (Nicklin et al., 2009). The researchers in this study have proposed that there is a coupling between SNAT2 and LAT1 transporters that mediate glutamine activation of mTORC1 (Nicklin et al., 2009). However, in my current study, inhibition of SNAT2 or glutamine deprivation has a minimal effect on the intracellular concentration of Leucine, isoleucine and valine; substrates of L-system amino acids. I think that the coupling between SNAT2 and LAT1 is not the model of mTORC1 regulation by amino acids in pancreatic β-cells despite the observation of impairment of mTORC1 activity by inhibition of LAT1 or deprivation of extracellular L-system substrates (Figure 5.8). It is possible that glutamine and system L substrates signal independently by different mechanisms to mTORC1.

Supporting evidence that LAT1 transporter couples to mTOR independent to SNAT2 is the observation in figure 5.9A which shows that intracellular glutamine concentration is reduced upon inhibition of L- system transporter by BCH. It is

actually a surprising finding as one may expect an increase in glutamine level if there is a coupling between SNAT2 and LAT system. Therefore, I propose that LAT1 may be coupled with another transport system that can also responsible for glutamine influx. Moreover, glutamine can be a substrate for LAT1 carrier so the levels of glutamine are reduced by BCH. Collectively, LAT1 could also determine the intracellular levels of glutamine and maintain In/Out glutamine gradient which might explain its role in regulation of glutamine.

Glutamine is considered as an important precursor for peptide and protein synthesis as well as nucleotide and nucleic acid synthesis by providing a source of carbon and nitrogen (Newsholme et al., 2003). Thus, it is important for the cell to have enough glutamine in order to maintain normal cell function. Inhibition of SNAT2 or glutamine starvation as well as inhibition of LAT1 or deprivation of its substrates partially depletes some amino acids such as alanine, glycine and glutamate (Figure 5.9). The decrease of alanine, glycine and glutamate under conditions of inadequate intracellular concentrations of glutamine could be explained as follows. Glutamate is the immediate product of glutamine metabolism that is formed by the action of glutaminase enzyme (Newsholme et al., 2003). Therefore, a decrease in glutamine will consequently affect the level of glutamate. Alanine and glycine are non essential amino acids that can be synthesized using metabolic intermediates. Alanine is formed by transamination of pyruvate with glutamate (Elliott, 2009). Thus, the reduction of glutamate will decrease the intracellular alanine concentrations. Glycine comes from serine which is in turn derived from the glycolytic intermediate, 3-phosphoglycerate (Elliott, 2009). Alanine and glycine are substrates of SNAT2 transporter so it is expected that their levels decrease by inhibition of SNAT2 by MeAIB. Interestingly, the levels of alanine and glycine are reduced by inhibiting L system despite being not transported by this system. It might be explained by that the cell utilises alanine and glycine as sources of carbon and nitrogen under conditions of inadequate amount of glutamine.

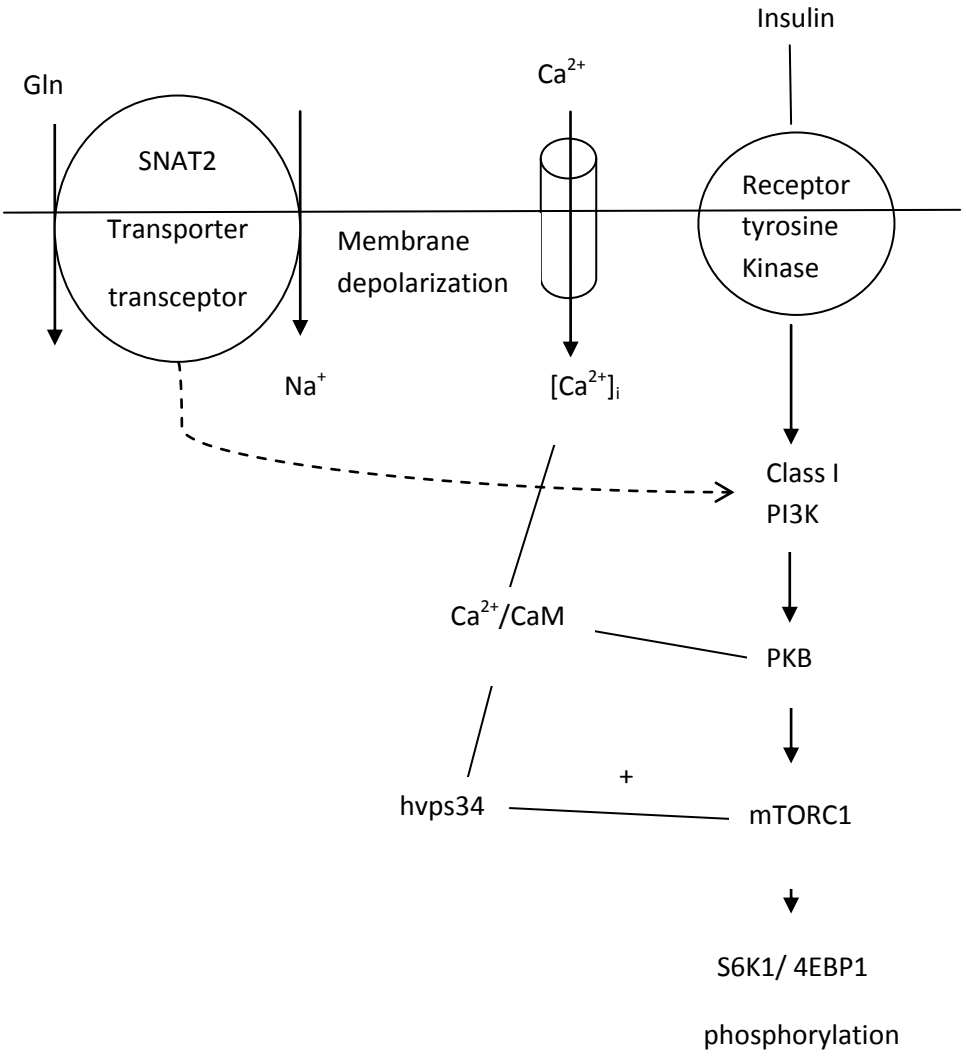


Figure 5.12 Proposed model depicting the role of glutamine/SNAT2 in regulating mTOR complex 1 glutamine stimulation leads to an increase in [Ca^{+2}]i, which increases the interaction of Ca^{+2}/CaM with hVps34 through its conserved CaM-binding motif, resulting in hVps34 activation, the production of PI(3)P, and a conformational change in the hVps34-associated mTOR Complex 1 leading to its activation. SNAT2 may function as a hybrid transporter-receptor (transceptor) that may sense to PI3K signalling pathway.

5.3.3 Conclusion so far and Future directions

This report has highlighted the role of amino acids on regulation of mTORC1 signalling in pancreatic β-cells. Furthermore, it revealed the importance of SNAT2 transporter to control mTORC1 activity and in turn management of vital cell functions including protein synthesis, cell proliferation and cell growth. Therefore, it would be interesting to investigate the effect of SNAT2 inhibition either by MeAIB or knocking down its expression using siRNA on protein synthesis, cell size and proliferation. It would be also interesting to determine the effect of over-expression of SNAT2 on mTORC1 signalling pathway in pancreatic β-cells. It is worth addressing the role of SNAT2 transporter as well as amino acids in the pathogenesis of Diabetes Mellitus. This study has highlighted the role of LAT1 in the regulation of mTORC1 which seems to act independently of SNAT2. Therefore, further experiments are required to address the mechanism by which LAT1 regulate mTORC1 activity in pancreatic β-cells. Another interesting experiment is to investigate the expression levels of SNAT2 and LAT1 and their activities in animal models of diabetes or diabetic subjects. In addition, based on the data presented here, SNAT2, LAT1 and amino acids are potential targets for treatment of Diabetes Mellitus. Further studies are to be performed to investigate the effect of amino acids supplement in diabetic subjects.

CHAPTER 6

Chapter 6: Final Discussion

6.1 Overview

Mammalian target of rapamycin (mTOR) is a highly conserved protein kinase which integrates signals from nutrients (glucose and amino acids), hormones and growth factors to regulate cellular growth and proliferation (Howell and Manning, 2011). Previous findings demonstrated that GLP-1 stimulates β-cell proliferation and protects cells against cytokine induced apoptosis via activation of mTORC1 and PKB (Kwon et al., 2004a; Li et al., 2005; McDaniel et al., 2002). Furthermore, GLP-1R agonists in the presence of glucose are able to enhance β-cell replication via an mTORC1-dependent pathway (Kwon et al., 2004a). However, it is not fully understood how GLP1 receptor activation leads to the activation of PKB/mTORC1 although it has been reported that this is mediated by EGFR transactivation (Buteau et al., 2003). In this thesis, I provide evidence that GLP-1 in the presence of glucose activates mTORC1 in rat islets of Langerhans and β-cell lines and this is likely mediated via the activation of PKB. GLP-1 also potentiates glucose-stimulated mTORC1 activation via a calcium and PI3K dependent mechanism. The ability of GLP-1 to stimulate PKB and mTORC1 is mediated via the activation of the IGF-1 receptor, possibly through the autocrine effect of IGF.

I have presented some data in Chapter 5 of this thesis suggesting that mTORC1 is coupled to at least two plasma membrane amino acid exchangers (SNAT2 and System L transporters) in isolated rat islets of Langerhans. The main functional effect of amino acid transporters is that they act primarily by maintaining an intracellular/extracellular concentration gradient of their amino acid substrates, notably L-Glutamine as a main substrate of SNAT2 and L-leucine as a main substrate of System L transporter. Incubation of rat islets in medium without Glutamine or System L amino acids is sufficient to prevent mTORC1 activation in response to insulin.

GLP-1 and amino acids are potent secretagogues for insulin secretion in pancreatic β-cells. Coupling of GLP-1 to its own receptor enhances insulin secretion through the

stimulatory G- protein (G_s) coupled activation of adenylate cyclase and the consequent increase in intracellular cyclic AMP. cAMP has a potential influence on insulin secretion through the activation of PKA and Epac. GLP-1 may augment the glucose effect to close ATP-sensitive K^+ channels by activation of PKA resulting in consequent depolarization of the plasma membrane and opening of voltage-sensitive calcium channels (Gromada et al., 1998). In addition, GLP-1 can increase the ATP sensitivity of the K_{ATP}-channel and subsequently increasing the responsiveness of channel closure (Suga et al., 2000). GLP-1 can also antagonise Kv currents in rat pancreatic β-cells (MacDonald et al., 2002) in a cAMP dependent manner (MacDonald et al., 2003). Therefore, GLP-1 promotes the depolarization of β-cell membrane through inhibition of the K_{ATP}- and Kv-channels and consequently activates the voltage-gated Ca^{+2}- channels with resulting Ca^{+2} influx and initiation of Ca^{+2}- dependent insulin exocytosis (Gromada et al., 2004). Furthermore, GLP-1 induces the mobilization of Ca^{+2} from intracellular stores; from Ins (1,4,5)P_3 – sensitive Ca^{+2} store via a PKA-mediated event and from Ryanodine receptors via Epac (Gromada et al., 2004). Elevations in intracellular calcium are alone sufficient to initiate insulin secretion (Jones et al., 1989). Preventing calcium influx by removal of extracellular calcium abolishes nutrient-induced insulin secretion (Figure 3.9) and this was concomitant with inhibition of PKB/mTORC1 signalling pathway, indicating a potential role of secretion to drive GLP-1 signalling to PKB/mTORC1.

Many amino acids can stimulate insulin secretion in the presence of glucose. However, there are a few amino acids such as leucine, lysine and arginine that can trigger insulin secretion in the absence of glucose and therefore act as initiators to insulin secretion. Leucine enters pancreatic β-cell through System L amino acid transporter and stimulates a biphasic increase in insulin secretion. Leucine metabolism in the pancreatic β-cell decreases the potassium permeability which results in membrane depolorization and activation of L-type calcium channels through which calcium enters the β-cell to induce insulin release (Yang et al., 2010). Accumulation of charged amino acids such as Lysine and arginine inside the pancreatic β-cell can directly depolarizes the cell membrane leading to calcium influx and insulin exocytosis (Newsholme et al., 2007). Glutamine may potentiate glucose-induced insulin secretion through multiple mechanisms including its metabolism and the subsequent closure of K_{ATP} channel, depolorization of cell membrane and

opening of calcium voltage channels that result in insulin release. Moreover, glutamine is co-transported with Na^+ via SNAT2 carrier so it may cause membrane depolorization occurring due to Na^+ transport and in turn enhance insulin secretion by activating voltage gated Ca^{+2} channels (Newsholme et al., 2007). My results have demonstrated that withdrawal of amino acids including glutamine and leucine or inhibition of their transport inhibits PKB/mTORC1 signalling pathway.

A summarized proposed mechanism that may explain how GLP-1 and amino acids signal to PKB and mTORC1 signalling pathway is shown in Figure 6.1. Both GLP-1 and amino acids can induce the depolarization of pancreatic β-cell membrane and stimulate the activation of the voltage-gated calcium channels, Ca^{+2} influx and insulin exocytosis. IGF-2 can be localised with insulin inside β-cell granules (Hoog et al., 1997). Therefore, it could be the autocrine effect of insulin and /or IGF-2 which is responsible for GLP-1 and amino acids induced activation of PKB/mTORC1. Those ligands (insulin/ IGF) bind to their own receptors leading to their autophosphorylation and activation of mTORC1 signalling via PI3K/PKB pathway. I have shown that the GLP-1 induced activation of PKB and mTORC1 was dependent on signalling through IGF-1R (Figure 4.12) and therefore the phosphorylation of PKB and rpS6 are mediated via the autocrine effect of IGF. Alternatively or in addition to the autocrine effect of IGF in the activation of PKB/mTORC1 signalling pathway, calcium is also important to mediate nutrient activation of PKB. Calcium can activate PKB through its phosphorylation of Ca^{+2}/CaM protein kinase (Yano et al., 1998). Activation of PKB results in the phosphorylation of TSC2 and I have demonstrated that GLP-1 induces the phosphorylation of TSC2 at Thr^{1462} in PKB dependent manner in pancreatic β-cells (Figure 3.14). Phosphorylation of TSC2 impairs the ability of the TSC1–TSC2 complex to act as a GAP towards the small GTPase Rheb, allowing Rheb–GTP to accumulate. Rheb–GTP potentially activates mTORC1. In addition, the rise of intracellular calcium by amino acids or GLP-1 promotes the binding of Ca^{+2} /CaM to hVps34 through its conserved CaM-binding motif (that is essential for lipid kinase activity). Activation of hVps34 induces a conformational change in mTORC1 that is required for the displacement of FKBP38 by Rheb-GTP and hence derives the activation of mTORC1 (Gulati et al., 2008).

In chapter 5 of my thesis, I have shown the potential role of SNAT2 in the activation of PKB and mTORC1. Beside its function as a transporter, SNAT2 can act as a nutrient receptor as it may regulate its own expression based on nutrient availability (Hyde et al., 2007). SNAT2 can activate PI3K through its coupling to integrin $\alpha_3\beta_1$ (McCormick and Johnstone, 1995) that in turn can be translocated with adhesion kinases that are powerful activators of PI3K (Thamilselvan et al., 2007). Although there is no available data on changes of expression or activity of SNAT2 in diabetic subjects, it was demonstrated that the activity of SNAT2 and LAT1 transporters were increased in gestational diabetes (Jansson et al., 2002). Very recently, it was shown that the expression and the activity of SNAT2 were up-regulated under conditions of induction of ER stress by arsenite (Oh et al., 2012). It is well documented that ER stress is closely associated with β-cell dysfunction and peripheral insulin resistance and consequently lead to the development of type 2 diabetes mellitus. It is possible that the pancreatic β-cells respond to the changes of amino acid concentrations occurring as a result of diabetes by alteration of the expression or the activity of amino acid transporters including System A and System L. Deficiency of amino acids can up-regulate the expression of SNAT2 and hence the activity of SNAT2 is increased under starvation conditions and this is known as adaptive regulation. Therefore, I postulate that at early incidence of diabetes the expression and the activity of SNAT2 and LAT1 are up-regulated as a kind of compensation to the inadequate amino acid levels. This report has provided some evidence for the contribution of SNAT2 to the activation of PKB/mTORC1 signalling pathway. Thus, it is possible that SNAT2 plays a key role in the regulation of pancreatic β-cell function in terms of regulating β-cell growth and proliferation via an mTORC1 dependent pathway. Future work is required to explore the role of amino acid transporters including SNAT2 and LAT1 in the regulation of pancreatic β-cell function.

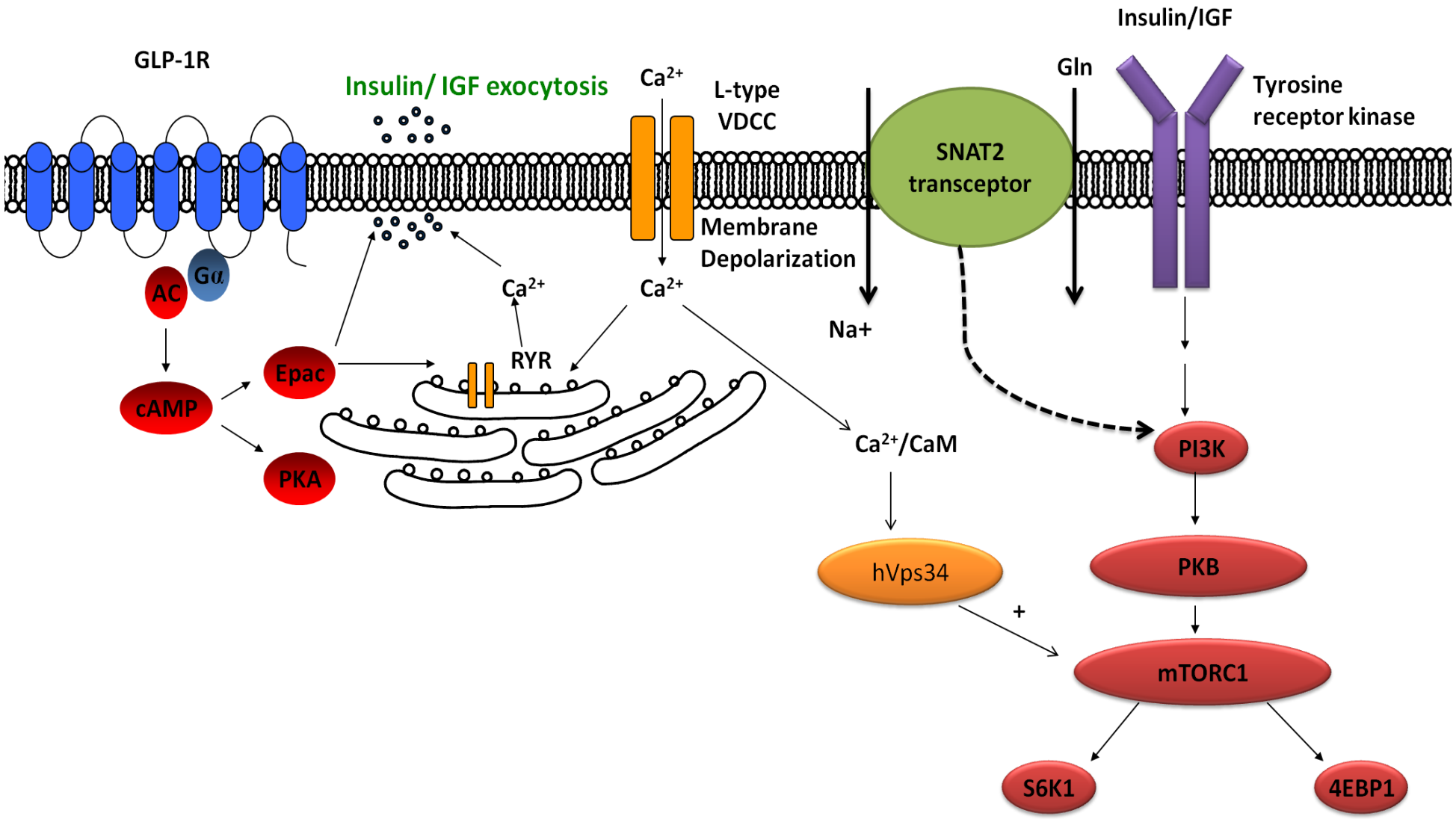


Figure 6.1 Proposed mechanisms for GLP-1 and amino acids induced activation of PKB/mTORC1 signalling pathway

6.2 Clinical relevance

6.2.1 GLP-1 based therapy for treating patients with type 2 diabetes

GLP-1 exerts multiple actions on the pancreatic β-cell thus it is considered as an excellent candidate for treating type 2 diabetic patients. The glucose dependent insulinotropic effect (the incretin effect) of GLP-1 suggests that it is a potential tool for treatment of diabetes with a minimal risk of hypoglycaemia during its administration. Indeed, continuous *iv* infusion of exogenous GLP-1 is capable of normalizing blood glucose concentrations in fasting patients with type 2 diabetes (Nauck et al., 1993). It was also demonstrated that 6 weeks course of subcutaneous infusion of GLP-1 significantly decreased hemoglobin A1c (HbA1c) and body weight, and greatly improved the first-phase insulin response and maximal beta-cell secretory capacity in type 2 diabetics (Zander et al., 2002). Furthermore, GLP-1 has been shown to enhance all steps of insulin biosynthesis as well as insulin gene transcription (Fehmann et al., 1995). Additionally, GLP-1 up-regulates the genes for the cellular machinery involved in insulin secretion, such as the glucokinase and GLUT-2 genes (Wang et al., 1997). Importantly, GLP-1 also has trophic effects on β-cells. It stimulates β-cell proliferation (Stoffers et al., 2000); and enhances the differentiation of new β-cells from progenitor cells in the pancreatic duct epithelium (Zhou et al., 1999). Additionally, GLP-1 has been shown to be capable of inhibiting apoptosis of β-cells, including human β-cells (Buteau et al., 2004). However, GLP-1 itself is not the ideal tool for the treatment of diabetic subjects since it is rapidly degraded within few minutes by the enzyme dipeptidyl peptidase-IV (Holst, 2007). Therefore, GLP-1R analogues such as exenatide, a naturally occurring GLP-1R agonist which resists the cleavage by dipeptidyl peptidase-IV is currently used as a therapeutic agent for the treatment of type 2 diabetes (Wang and Brubaker, 2002). Exenatide is administered subcutaneously and is used as adjunctive therapy with metformin or a sulfonylurea, or both in patients with type 2 diabetes (Iltz et al., 2006). Exenatide enhances glucose homeostasis by reproducing the effects of endogenous GLP-1 through a combination of known mechanisms, including glucose-dependent insulin secretion and suppression of postprandial glucagon secretion. Exenatide has been shown to slow gastric emptying, and decreasing food intake and therefore, reduce body weight (Iltz et al., 2006). In experimental model of diabetes *db/db* mice, exendin-4 reduces the plasma glucose level and body weight and increases β-cell

mass via enhanced β-cell proliferation and reduce apoptosis (Wang and Brubaker, 2002).

Liraglutide is another GLP-1 analogue that has been approved for clinical treatment of type 2 diabetes. It has longer half-life compared to GLP-1 as it is derived from GLP-1 with a substitution of Lys^{34} with Arg^{34} and an attachment of a free-fatty acid derivative which is responsible for the long-acting property of Liraglutide. This free-fatty acid derivative promotes the binding of Liraglutide to plasma protein and delays its clearance by kidney (Chia and Egan, 2008). Similar to GLP-1 and exenatide, Liraglutide is required to be injected subcutaneously. Clinically, monotherapy of Liraglutide decreased plasma glucose levels and body weight and increased insulin secretion (Vilsboll et al., 2007). Furthermore, Liraglutide has been shown to have anti-apoptotic effects against both fatty acids and cytokines in pancreatic rat islets which are mediated via a PI3K dependent mechanism (Bregenholt et al., 2005).

Another strategy for incretin-based therapy is to enhance the effect of endogenous GLP-1 through inhibition of dipeptidyl peptidase-IV, the enzyme responsible for GLP-1 degradation. A study that involved DPP-knockout mice has revealed that those mice have increased fasting incretin levels, decreased plasma glucose levels and a higher insulin levels compared to wild-type mice (Marguet et al., 2000). Examples for DPP-IV inhibitors are Sitagliptin and vildagliptin. Sitagliptin, which is orally administrated, was approved for use as monotherapy or in combination with metformin for treatment of type 2 diabetic subjects (Zerilli and Pyon, 2007). Experimental study in high fat diet/streptozotocin -treated mice has demonstrated that long term treatment with Sitagliptin deceased plasma glucose and HbA1C with concomitant increase in incretin levels and an increase in β-cell mass (Mu et al., 2009). Vildagliptin is a reversible and competitive inhibitor of DPP-IV which can be administered orally alone or with metformin to control symptoms of type 2 diabetes. In the $Irs2^{-/-}$ mice, Vildagliptin has been demonstrated to improve glycaemic control and inhibit apoptosis by increasing β-cell mass (Sato et al., 2012). Saxagliptin is a selective, reversible inhibitor of DPP-IV that can be used alone or in combination with other oral antidiabetics (Tahrani et al., 2009).

Therefore, GLP-1 mimetics and DPP-IV inhibitors can improve glycaemic control and their action is glucose dependent so they do not produce hypoglycaemia besides they aid to preserve the β-cell mass and function and help in weight reduction. These multiple actions give them potential advantages over other treatments for type 2 diabetes therapy.

The protective and proliferative effects of GLP-1 are likely mediated by multiple signalling pathways, including the activation of mTOR and the stimulation of PKB which increases CREB phosphorylation and IRS2 levels (Brubaker and Drucker, 2004; Egan et al., 2003). In this thesis, I have elucidated the mechanism by which mTOR and PKB are activated in response of GLP-1. My study has highlighted the role of IGF-1R in mediating GLP-1 multiple actions in pancreatic β-cells. Therefore, IGF-1R could be a potential target for treatment of diabetes either by increasing ligand concentration or by enhancing IGF-1 binding to the IGF-1 receptor. IGF-1 potentiates insulin action in experimental animals (Jacob et al., 1989; Jacob et al., 1991) and patients with diabetes mellitus. (Cheetham et al., 1993; Moses et al., 1996). A study performed on patients with either severe insulin resistance or type 2 diabetes has demonstrated that post-prandial glucose disposal is partly dependent upon IGF-1 concentrations and administration of IGF-1 results in improved post-prandial glucose usage (Kuzuya et al., 1993). Other clinical trials of IGF-1 administration to type 1 diabetics have revealed a reduction in insulin requirements over 4-8 week periods and a significant reduction in haemoglobin A1C (Quattrin et al., 2001; Thrailkill et al., 1999). Administration of IGF-1 as monotherapy— without insulin or oral hypoglycaemic agents — to patients with type 2 diabetes for 6 weeks reduced haemoglobin A1C by 1.2% (Group, 1996). However, a major problem with the administration of IGF-1 has been the induction of adverse events. These adverse events are dose-dependent, and occur most commonly in patients who have received doses greater than 40 mcg/kg twice a day subcutaneously (Jabri et al., 1994). These include effects related to changes in sodium and water balance, such as oedema, arthralgias, headaches and myalgias. Other complications including Bell's palsy (facial nerve paralysis), pseudotumour cerebri (increased intracranial tension), tachycardia and hypoglycaemia have occurred (Moses et al., 1996).

IGF-1 is bound to a family of proteins known as insulin-like binding proteins (IGFBPs). The predominant form of IGFBP in plasma is IGFBP-3 which accounts for over 75% of the IGF binding capacity. Combined administration of IGF-1 and IGFBP-3 has been shown to decrease the fasting blood glucose level by 35% with a significant reduction in insulin requirements by 47% - 67% (Clemmons et al., 2005). Therefore, IGF-1 could be a potent insulin sensitizer, even when it's administered with its binding protein. It has not been elucidated whether the concomitant administration of IGFBP3 with IGF-1 will result in a reduction in adverse effects. To date, no long-term Phase III trial with IGF-1 in type 2 diabetes has been completed. A potential approach for stimulating the IGF-1R and, therefore, enhances insulin sensitivity by technologies other than by administering the ligand includes the design of IGF-1 like peptide agonists.

6.2.2 Potential Role of Amino Acids in Diabetes Management

In pancreatic β-cells, it has been shown that amino acids augment glucose-stimulated insulin secretion and increase protein synthesis and amino acid transport in target tissues like skeletal muscle (Newsholme et al., 2007). The mechanisms by which amino acids promote insulin secretion include metabolism (e.g L-glutamine and L-Leucine), depolarization of the plasma membrane (e.g L-arginine), or enhancement of mitochondrial function (Newsholme et al., 2007). Furthermore, certain amino acids have been demonstrated to activate mTOR signalling pathway in a dose-dependent manner and in the presence of glucose in pancreatic β-cells (Kwon et al., 2004a). However, it has not been fully elucidated the mechanism by which amino acids activate mTOR signalling pathway, though there are some suggestions that pointed to a possible involvement of Ca^{+2} and vps34 (Nobukuni et al., 2005) . In this thesis, I have demonstrated the effect of withdrawal of glutamine and substrates of system L transporters on reduction of mTOR activity. In fact, clinical studies in patients with type 2 diabetes have shown that the plasma levels of some amino acids including glutamine and arginine were reduced (Menge et al., 2009). It might be that the reduction of amino acid concentration as a consequence of diabetes affects β-cell survival via the reduction of mTOR activity. Therefore, it might be useful to consider amino acid supplementation as a potential strategy to treat or reduce risk of diabetes. Indeed, dietary supplementation of glutamine has

improved skeletal muscle function which is commonly impaired in diabetic subjects and this beneficial effect was through the increase of protein synthesis. Experimental studies in animal models of diabetes have revealed that glutamine supplementation can protect against the development of diabetic complications (Pereira et al., 2011; Tsai et al., 2011). In chapter 5 of my thesis, I have demonstrated the role of two plasma membrane amino acids exchangers; SNAT2 and LAT1 in mediating amino acids signalling to mTOR. Again these transporters could be potential targets for developing of new categories of drugs to protect pancreatic β-cells.

6.3 Concluding Remarks

It has been clearly demonstrated that mTORC1 plays a critical role in the regulation of β-cell mass through stimulating of an increase in β-cell proliferation and size. Nutrients and growth hormones stimulate mTORC1 and consequently stimulate β-cell replication. mTORC2 plays an important role in regulating cell survival via PKB. Recently, our lab has provided an evidence that mTORC2 can regulate β-cell viability and highlighted the key role of PKB in β-cell survival (Barlow et al., 2012). Therefore, it is likely for both complexes of mTOR to be involved in β-cell adaptation for obese subjects or patients suffering from insulin resistance in order to prevent the development of type 2 diabetes. Under conditions of obesity and insulin resistance, mTORC1 may play a key role in β-cell compensation in response to increasing demand for insulin. Development of pharmacological tools to activate mTORC1 or nutrient supplementation including amino acids and IGF may be a potential target to stimulate β-cell mass and function.

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