The Role of Phosphorylation in the Regulation of the Mammalian Target of Rapamycin

A dissertation submitted to the University of London in candidature for the degree of Doctor of Philosophy

By

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Abstract

A key regulator of translation is the mammalian target of rapamycin (mTOR), a protein kinase member of the family of phosphatidylinositol kinase (PIK)-related kinases. mTOR is dually regulated by growth factors and nutrient availability, though the precise mechanisms by which mTOR is regulated are not well understood. The C-terminal of the mTOR catalytic domain has been of regulatory interest by the identification of the insulin stimulated and nutrient sensitive S2448 phosphorylation site. The functional significance of S2448 phosphorylation on the mTOR downstream targets p70 S6 kinase (S6K1) and eIF4E-binding protein 1 (4E-BP1) are unclear.

A novel nutrient responsive mTOR phosphorylation site has been identified at T2446. In contrast to S2448 phosphorylation, T2446 is dephosphorylated when CHO-IR cells are insulin stimulated and phosphorylated when cells are nutrient deprived. Studies show that activation of AMP-activated kinase (AMPK) is concomitant with an increase in mTOR T2446 phosphorylation, paralleled by a decrease in S6K1 phosphorylation. Regulation of T2446 phosphorylation may involve AMPK. Phosphorylation at T2446 and S2448 is mutually exclusive. The functional significance of phosphorylation at T2446 and S2448 on the downstream target S6K1 was investigated by a mutational strategy where each site was substituted with non-phosphorylatable alanine or phospho-mimic glutamic acid. Evidence indicates that although phosphorylation of T2446 and S2448 is mutually exclusive in response to growth factors and nutrients, their individual phosphorylation may not be enough to have a direct effect on downstream S6K1 activity.

Additionally, the tuberous sclerosis complex (TSC) may have positive regulatory effects on insulin signalling. Loss of TSC2 impairs insulin signalling by down-regulating the turnover of insulin receptor substrate-1 (IRS-1) protein, affecting associated class 1a phosphoinositide 3-kinase (PI3K) activity and downstream signalling; including suppression of PKB activation and mTOR S2448 phosphorylation.

Statement

This thesis is an account of research conducted at the Department of Biochemistry and Molecular Biology at University College London, between September 1999 and September 2003. Except where references are given, this thesis contains my own original work, does not exceed the word limit stipulated by the University and is not substantially the same as any I have submitted for any other degree, diploma or examination.

Some of the work presented in this thesis has been published elsewhere:

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Contents

List of Tables

List of Figures

1. Introduction

2. Materials and Methods

3. Identification and characterisation of a novel mTOR phosphorylation site

4. Investigating the role of phosphorylation in mTOR by mutational analysis

5. The involvement of TSC in upstream signalling of mTOR

Abbreviations

4E-BP1	eIF4E Binding Protein 1
5'TOP	5' Tract of Oligopyrimidine
Ab	Antibody
AICAR	5-aminoimidazole-4-carboxamideribonucleoside
AMP	Adenosine Monophosphate
AMPK	AMP-activated kinase
AMPKK	AMPK-kinase
ANT	Adenine Nucleotide Transporters
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
Autorad	Autoradiograph
BCAT	Branched Chain Aminotransferase
BCKD	α-Keto Acid Dehydrogenase
BCR	B-Cell Receptor
BSA	Bovine Serum Albumin
CAM	Calmodulin
cdks	Cyclin-Dependent Kinases
CEF	Chicken Embryo Fibroblasts
CHO-IR	Chinese Hamster Ovary-(stably expressing) Insulin Receptor)
CK2	Casein Kinase II
CoAsy	CoA synthase
DMSO	Dimethylsulfoxide
DNA-PK_{cs}	DNA ependent Protein Kinase catalytic subunit
DNP	Di-nitrophenol
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
eEF	Eukaryotic Elongation Factors

eIF4E	Eukaryotic Initiation Factor 4E
ER	Endoplasmic Reticulum
eRFs	Eukaryotic Release Factors
ERK1/2	Extracellular-ligand-Regulated Kinase-1 and-2
FAT	FRAP-ATM-TRRAP domain
FATC	FAT Carboxy-terminal homology domain
FKBP	FK506-Binding Protein
FRB	FKBP/Rapamycin Binding domain
GAP	GTPase–Activating Protein
GDP	Guanosine Diphosphate
GEF	GTP Exchange Factor
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor-Binding Protein 2
GSK3	Glycogen Synthase Kinase-3
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate
HEAT	Huntingdon, EF3, A subunit of PP2A and TOR motifs
HEK	Human Embryonic Kidney
HIV-1	Human Immunodeficiency Virus-1
IB	Immuno-Blot
IC_{50}	50% Inhibitory Concentration
IFN-γ	Interferon-γ
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IRS-1	Insulin Receptor Substrate-1
JAK	Janus Kinase
kDa	Kilo Dalton
KIC	α-Ketoisocaproate
Km	Michaelis constant
LDM	Low-Density Microsomes
LPS	Lipopolysaccharide

MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MEFs	Mouse Embryonic Fibroblasts
mTOR	Mammalian Target of Rapamycin
NES	Nuclear Export Signal
NLS	Nuclear Localisation Sequence
PA	Phosphatidic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinase
PIKK	Phosphatidylinositol Kinase Related-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PKI	Protein Kinase A Inhibitor
PKR	Human interferon induced double stranded RNA activated protein kinase
PLD	Phospholipase D
PMA	Phorbol-12-myristate-13-acetate
PP2A	Protein Phosphatase 2A
PtdIns (3, 4, 5)P_3	Phosphatidyl-inositol (3, 4, 5) phosphate
PtdIns (4, 5)P_2	Phosphatidyl-inositol (4, 5) phosphate
PTEN	Phosphatase and Tensin Homologue Deleted on Chromosome Ten
PTP	Mitochondrial Permeability Pores
Raptor	Regulatory Associated protein of mTOR
Rheb	Ras homologue enriched in brain

RMS	Rhabdomyosarcoma
RNAi	RNA interference
RT	Room Temperature
S6K1	70 kDa-ribosomal S6 Kinase
SAP	Sit4-Associated Proteins
SAPK4	Stress-Activated Protein Kinase 4
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser/thr	Serine/Threonine
SH2	Src Homology 2 domains
SHC	Src-Homology-Collagen-like
SOS	Son-Of-Sevenless
STAT	Signal Transducers and Activators of Transcription
TBS	Tris Buffered Saline
TLC	Thin Layer Chromatography
TNFα	Tumour Necrosis Factor α
TOS	mTOR Signalling motif
TSC	Tuberous Sclerosis Complex

Chapter 1: Introduction

1 Introduction

Insulin signalling plays a major role in the regulation of several cellular processes. A key role of insulin signalling is the acute stimulation of protein synthesis in mammalian cells, a process which is part of a complex signalling pathway involving many signalling elements orchestrating endpoint responses that result in cell survival, growth, proliferation and differentiation.

1.1 Initiation of the insulin signalling cascade

Insulin binding to the insulin receptor (IR) activates the intrinsic IR-tyrosine kinase activity, initiating the recruitment of several downstream signalling proteins containing Src homology 2 domains (SH2). These include; the insulin receptor substrate (IRS) family of proteins (White, 1997) and the adaptor protein, Src-homology-collagen-like (SHC) protein (Pelicci *et al.*, 1992). IRS proteins and SHC are phosphorylated by the IR on specific tyrosine residues; activating two major insulin stimulated signalling cascades (Fig. 1.1). Tyrosine-phosphorylated SHC and IRS-1 mediate the recruitment of growth factor receptor-binding protein 2 (Grb2) via its SH2 domains. Grb2 is tightly associated with the Ras-GDP exchange factor son-of-sevenless protein (SOS) via Src homology 3 (SH3) domains. Recruitment of the Grb2-SOS complex activates the small G-protein Ras at the plasma membrane (Takai *et al.*, 2001) by SOS mediated GDP/GTP exchange. Ras-GTP activates Raf-1 triggering the activation of the mitogen-activated protein kinase (MAPK) cascade via MAP/ERK kinase (MEK) and extracellular-ligand-regulated kinase-1 and -2 (ERK1/2) (Proud and Denton, 1997). Tyrosine-phosphorylation of the IRS proteins is also the main mechanism of activating class 1a phosphatidylinositol 3-kinase (PI3K), one of the main starting points of insulin stimulated signalling cascades (Shepherd *et al.*, 1998).

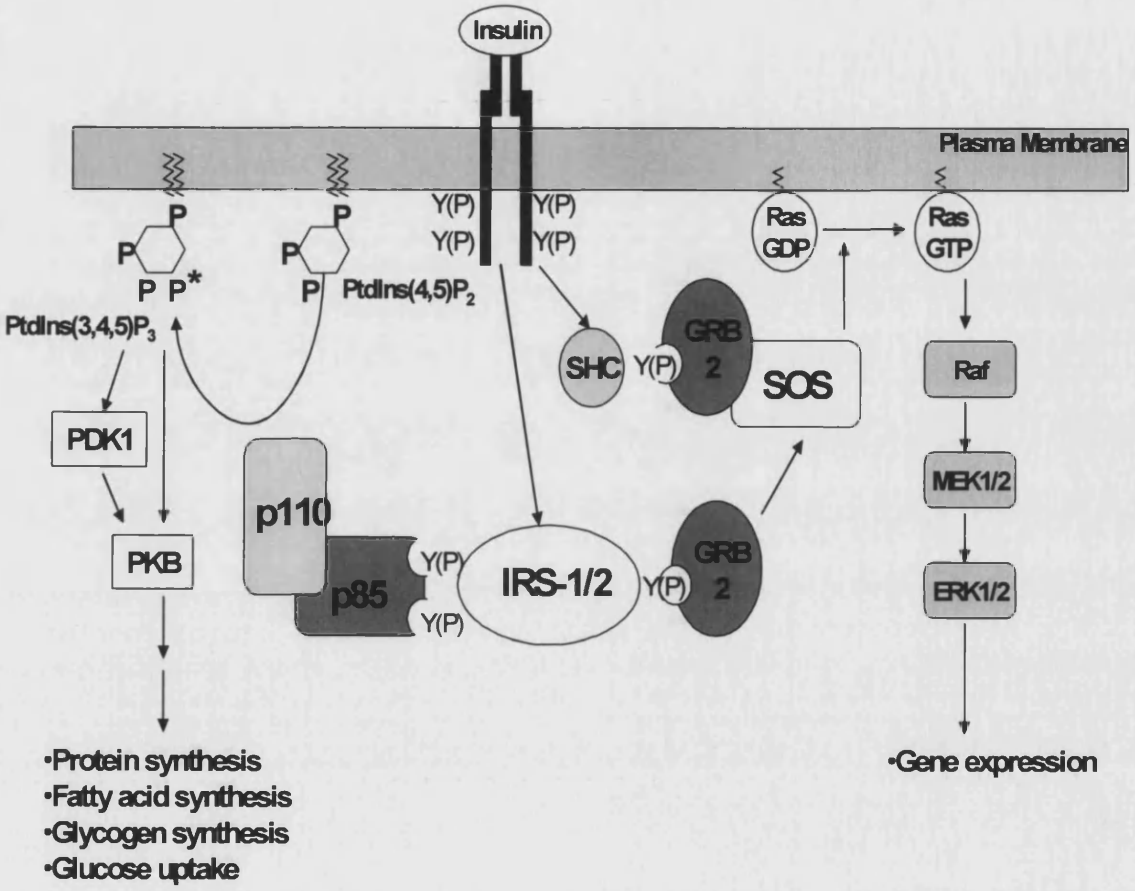

Figure 1.1 Initiation of the insulin signalling cascade

Key events induced by insulin stimulation, as described in the text. Figure modified from Shepherd *et al.*, (1996). 'Y(P)' denotes tyrosine phosphorylation.

Multi-site tyrosine phosphorylation of IRS-1 initiates the recruitment of PI3K to the plasma membrane via the SH2 domain of the p85 subunit. PI3K catalyses the phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PtdIns (4, 5)P_2) on the 3' position of the inositol ring to generate phosphatidylinositol 3, 4, 5-triphosphate (PtdIns (3, 4, 5)P_3). Elevated levels of PtdIns (3, 4, 5)P_3 induces the recruitment of proteins containing the pleckstrin-homology (PH) domain to the plasma membrane, including; phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). PKB recruitment to the plasma membrane initiates its activation by phosphorylation at residues T308 and S473. T308 phosphorylation is mediated by PDK1 (Alessi *et al.*, 1997a) while phosphorylation at S473 may be mediated by a kinase termed PDK2, though the identity of the latter is not yet known (Alessi and Downes, 1998). PKB is an important protein kinase link between IR activation and downstream kinase cascades which mediate endpoint metabolic responses including; glucose uptake, glycogen synthesis, fatty acid synthesis and protein synthesis (Shepherd *et al.*, 1998; Toker and Cantley, 1997).

1.2 Overview of Translation

Insulin can regulate the rate of mRNA translation which can increase the overall rate of protein synthesis by 2-3 fold (Gingras *et al.*, 2001b). Translation is a multi-step process that can be divided into 3 phases: initiation, elongation and termination. Translation initiation is the most complex phase and will be discussed in greater detail than elongation and termination. Many translation factors in the translational machinery are tightly regulated to permit control of global and specific mRNA translation (Dever, 1999). Translation initiation involves several stages that are numerically ordered as shown in Fig. 1.2, but each stage may also occur in parallel (Dever, 1999; Proud and Denton, 1997). Stage 1 involves dissociation of the 80S ribosome into the 60S and 40S subunits, a process which also requires eukaryotic initiation factor (eIF) 3 and eIF1A. In stage 2 a ternary complex is formed between the initiator methionyl-tRNA (Met-tRNA) and GTP bound eIF2 which associates

with the 40S ribosomal subunit together with eIF3 and eIF1A to form the 43S pre-initiation complex. Stage 3 and 4 involves formation and assembly of the trimeric eIF4F complex on mRNA. eIF4F comprises eIF4E, eIF4A and eIF4G. eIF4E recognizes and binds to the 5' 7-methyl-GTP-cap of mRNA to allow cap-dependent translation, eIF4A acts as a RNA helicase involved in uncoiling secondary structure in the 5'-untranslated region (5'UTR) a process that may also involve eIF4B, and eIF4G acts as the protein scaffold which has contacts with both eIF4E and eIF4A. In stage 5, the 43S pre-initiation complex interacts with the eIF4F complex via contacts between eIF4G and eIF3, permitting the 40S ribosome to bind to the mRNA near its 5' end and begin 'scanning' for the initiation AUG codon, which is recognised by Met-tRNA. Finally in stage 6, hydrolysis of ribosome bound eIF2-GTP is stimulated by eIF5 resulting in the release of eIF2-GDP and all the initiation factors from the 40S ribosome. Re-association of the 60S ribosomal subunit to form the 80S initiation complex is then permitted and translation elongation can proceed. This final stage also recycles eIF2-GDP to regenerate eIF2-GTP, a process requiring the guanine nucleotide-exchange factor eIF2B.

Translation initiation is a less energy consuming process than elongation and termination. As such, the translational machinery can be regulated by insulin and nutrient availability. Stage 2 and stage 3/4 of translation initiation are particularly sensitive to these changes. Another mechanism of translational control can be exerted through a specific subset of mRNA. The mRNAs which encode several ribosomal proteins and translation factors contain a distinctive 5' tract of oligopyrimidine (5'TOP) structure, adjacent to the 5'-cap. 5'TOP mRNA are selectively translated in response to mitogenic stimuli (Shah *et al.*, 2000).

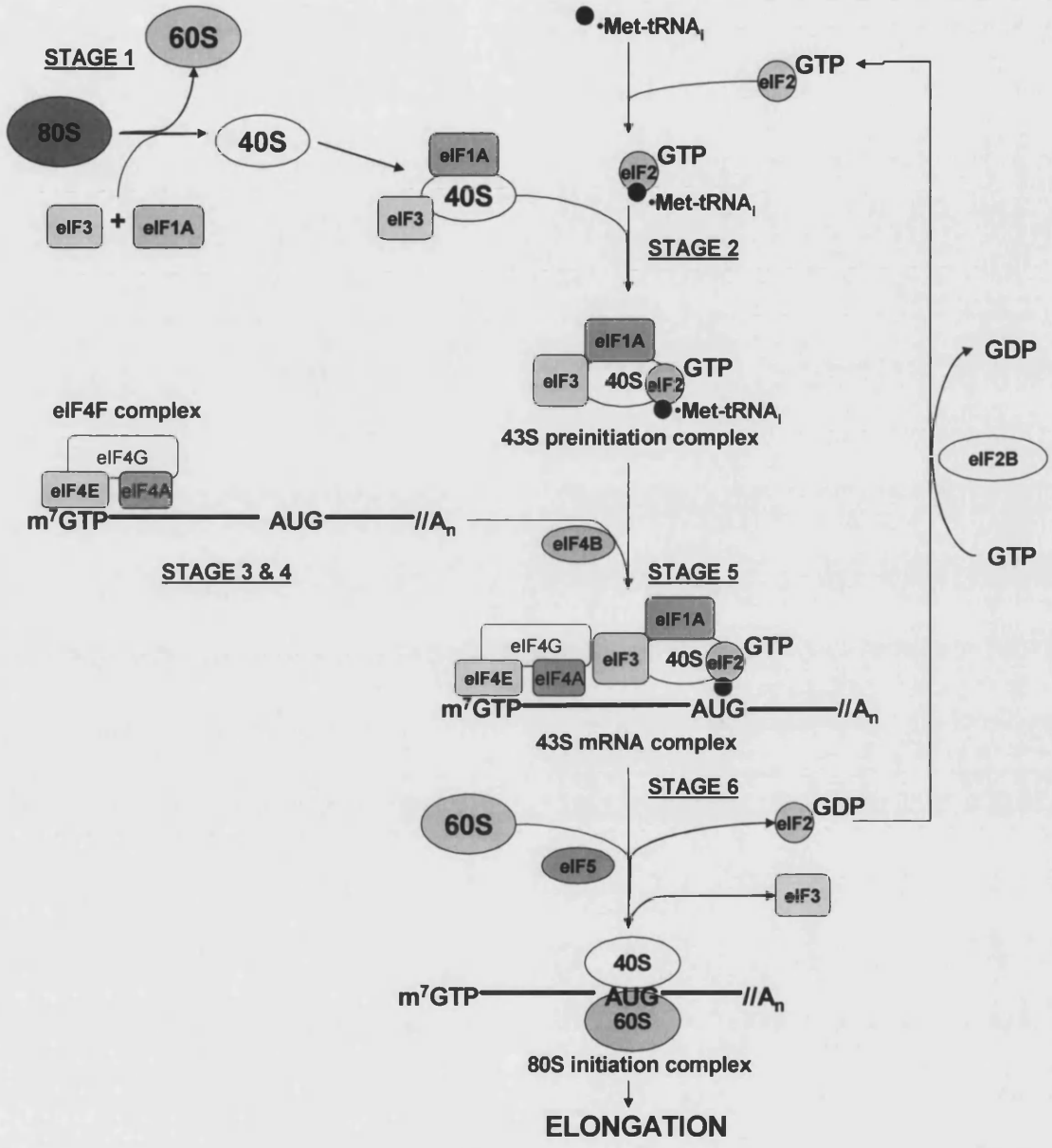

Figure 1.2 Overview of translation initiation

The six key stages of translation initiation are described in detail in the text. Although stages are numerically labelled here, each stage may also occur in parallel. Briefly, stage 1: dissociation of 80S ribosome, stage 2: formation of 43S pre-initiation complex, stage 3 & 4: Cap recognition and eIF4F complex assembly on mRNA, stage 5: 43S mRNA complex formation and ribosomal scanning and stage 6: formation of the 80S initiation complex. Modified from Proud and Denton (1997).

Translation elongation is a highly energy-dependent process that is regulated by eukaryotic elongation factors (eEF) eEF1A, eEF1B (α, β, γ subunits) and eEF2, which control the speed and accuracy of the elongation process. The mechanism of elongation is briefly described here: firstly, recruitment of amino acyl-tRNA to the A-site of the ribosome requires eEF1A-GTP which is subsequently hydrolysed and released; eEF1B acts as a guanine nucleotide-exchange factor (GEF) and mediates GDP/GTP exchange on eEF1A, which is then recycled for amino acyl-tRNA recruitment; the nascent peptide chain transfers to the ribosomal A-site and a peptide bond is formed between the C-terminal of the peptide and the recruited amino acyl-tRNA, forming peptidyl-tRNA; eEF2 and GTP mediate the translocation of the ribosome by one codon along the mRNA, shifting the peptidyl-tRNA into the empty ribosomal P-site following GTP hydrolysis and release of eEF2. The cycle is repeated along the mRNA until a stop codon is reached (Merrick and Nyborg, 2000). The translation elongation factors are also subject to reversible phosphorylation under the regulation of growth factors and nutrient availability (Browne and Proud, 2002).

Polypeptide chain termination occurs when a stop codon (UAA, UAG and UGA) is reached by the A-site of the ribosome initiating the recruitment of eukaryotic release factors (eRFs) to the A-site and catalyzing the hydrolysis of the ester bond between the final amino acid of the nascent peptide chain and the tRNA. Termination also requires GTP hydrolysis mediating the release of the polypeptide chain, the termination factors and the ribosomal subunits are then recycled for translation initiation (Welch *et al.*, 2000).

The process of translation is dependent on both energy and nutrient availability and control of translation is important for cell growth and proliferation. As mentioned above, there are several stages in which translation can be controlled. Moreover, the molecular mechanisms involved in translation initiation have been subject to much analysis. A key signalling pathway involved in the regulation of translation initiation is sensitive to the immunosuppressant rapamycin which specifically inhibits the

mammalian target of rapamycin (mTOR). mTOR is able to integrate growth factor, nutrient and energy signalling and its role is best characterised in the regulation of p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein 1(4E-BP1), a protein which controls eIF4E availability (Gingras *et al.*, 2001b; Proud, 2002a, 2002b, 2004a, 2004b). Both, these downstream effectors exert some control on translation initiation.

It has become increasingly clear that mTOR is a multi-functional and important regulatory protein. Much advancement has been made in understanding the factors which regulate mTOR and how mTOR regulates its downstream effectors. Furthermore novel sensory pathways and proteins have been implicated with the control of mTOR and these will be discussed in subsequent sections.

1.3 The TOR kinase family

The macrolide ester rapamycin was widely used as a potent anti-fungal, anti-neoplastic and immunosuppressant (Abraham and Wiederrecht, 1996; Diggle *et al.*, 1996) and was subsequently found to be a highly specific inhibitor of a cellular protein which lead to identification of the target of rapamycin proteins (TOR).

Two related proteins termed target of rapamycin 1 and 2 (TOR1 and TOR2) (from separate genes) were first identified in the budding yeast *Saccharomyces cerevisiae*. Mutation in either gene confers rapamycin resistance to allow G_1 progression. The TOR proteins share 67% overall identity (Heitman *et al.*, 1991; Zheng *et al.*, 1995). The mammalian homologue was subsequently identified and has ~40% identity to the yeast TOR proteins (Sabatini *et al.*, 1994; Sabers *et al.*, 1995). The mammalian homologue has been termed FRAP (FKBP12-rapamycin associated protein), RAFT (rapamycin and FKBP12 target 1) and mammalian target of rapamycin (mTOR) and will be referred to as mTOR throughout. Additionally the *Drosophila melanogaster* homologue was identified and has 35% and 38% overall amino acid identity to TOR1 and TOR2 respectively, while sharing 56% identity to mTOR (Oldham *et al.*, 2000).

TOR2 has dual function: control of cell cycle-dependent organisation of the actin cytoskeleton by a GTPase switch involving RHO1, RHO2, ROM2 and SAC7 (Schmidt *et al.*, 1997) – this process is not sensitive to rapamycin; and in common with TOR1, signalling activation of translation initiation in response to nutrient availability (Barbet *et al.*, 1996), a function that is preserved from yeast to mammals (Manteuffel *et al.*, 1996). The TOR proteins are therefore implicated in important signalling cascades involving mitogen stimulation and nutrient availability in the regulation of protein synthesis.

1.4 The Mammalian Target of Rapamycin

mTOR is a 290kDa protein of 2549 amino acids which acts as a checkpoint protein in the integration of growth factor signalling and nutrient availability (Gingras *et al.*, 2001b; Proud, 2002a, 2002b, 2004a, 2004b). mTOR has been localised to intracellular membrane fractions that are distributed in the cytoplasm (Sabatini *et al.*, 1999; Withers *et al.*, 1997). Similarly the TOR2 protein is membrane associated and localises on the surface of the yeast vacuole (Cardenas and Heitman, 1995).

1.4.1 The modular domains of mTOR

The TOR proteins possess the same domain architecture and contain several well defined domains as shown in Fig. 1.3.

1.4.1.1 mTOR HEAT domain

The mTOR HEAT domain comprises two blocks which occupy the first 1200 amino acids of the mTOR protein (Andrade and Bork, 1995) and is named after the four functionally characterised proteins containing the structural motif, namely, Huntingtin, Elongation Factor 3 (EF3), Alpha subunit of protein phosphatase 2A & TOR 1 (yeast). The domain consists of consecutive repeating units of a degenerate hydrophobic 37-43 amino acid motif, of which mTOR contains 20 units (Andrade and Bork, 1995). The hydrophobic nature of the HEAT repeats suggests it may act as a scaffold and contribute to protein-protein interaction. Gephryin, a tubulin-binding protein involved in postsynaptic clustering of neuronal glycine receptors (Sabatini *et al.*, 1999), Ubiquilin 1 (Wu *et al.*, 2002) and the recently identified regulatory associated protein of mTOR (Raptor) (Kim *et al.*, 2002) were all found to interact with the HEAT domain of mTOR.

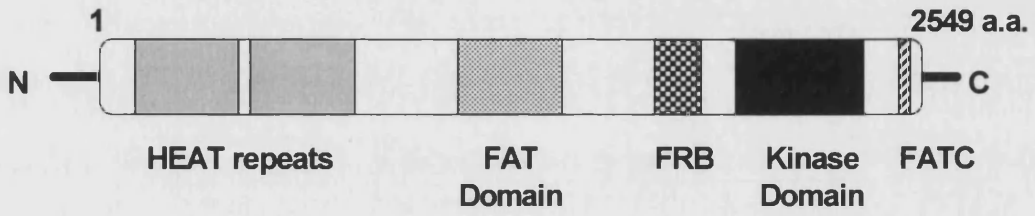

Figure 1.3 Domain architecture of mTOR

The TOR family of proteins possess the domain structure represented in this generic figure. The domains are described in the text.

1.4.1.2 mTOR FKBP/rapamycin binding domain (FRB)

The cytosolic receptor FK506 binding protein (FKBP-12), a prolyl isomerase, binds to rapamycin which complexes with the FKBP/rapamycin binding domain (FRB) of mTOR (Sabers *et al.*, 1995). The FRB domain spans residues 2025-2114 (Chen *et al.*, 1995) and the FKBP-12-rapamycin complex binds solely to the FRB domain of mTOR in mammalian cells (Sabatini *et al.*, 1994). Rapamycin is a highly specific inhibitor of mTOR (Davies *et al.*, 2000) and has been a valuable tool in dissecting pathways which converge or diverge from mTOR. The crystal structure of the ternary FKBP12-rapamycin complex interacting with the FRB domain of mTOR has been solved, revealing that rapamycin interacts extensively with both protein partners while limiting actual protein-protein interaction (Choi *et al.*, 1996). Additionally, evidence indicates that the FRB domain is required for mTOR kinase activity (Vilella-Bach *et al.*, 1999). A point mutation at S2035 to aspartate, glutamate, threonine, or isoleucine prevents the binding of the rapamycin/FKBP12 complex producing a rapamycin resistant mutant in mTOR (Chen *et al.*, 1995; Hara *et al.*, 1997). Corresponding rapamycin resistant mutations were also identified in yeast (Lorenz and Heitman, 1995). However, the S2035I mutant can affect the substrate preference of mTOR (Lawrence *et al.*, 2004; McMahon *et al.*, 2002).

1.4.1.3 mTOR catalytic domain

The TOR proteins are members of a family of phosphatidylinositol kinase related-kinases (PIKK) that contain a domain which has homology to the catalytic domain of PI-3 kinase. This family also includes DNA dependent protein kinase catalytic subunit (DNA-PK_{cs}) and ataxia telangiectasia mutated (ATM) (Abraham, 1996). The C-terminal catalytic domain of mTOR exhibits autokinase activity (Brown *et al.*, 1995) but lacks lipid kinase activity (Brown *et al.*, 1995); however, mTOR and TOR2 have been isolated with phosphatidylinositol-4 (PI-4) kinase activity but this activity was not inhibited by binding of the FKBP12/rapamycin complex so it is unclear whether the PI-4

kinase activity is intrinsic or associated via another kinase (Cardenas and Heitman, 1995; Sabatini *et al.*, 1995). In contrast, TOR1 has been isolated with protein kinase activity towards 4E-BP1 (Alarcon *et al.*, 1999), much-like the serine/threonine (ser/thr) protein kinase activity isolated with mTOR (Brunn *et al.*, 1997b). mTAb1 is an antibody raised against a peptide spanning residues 2433-2450 of the mTOR catalytic domain and can activate mTOR protein kinase activity *in vitro* (Brunn *et al.*, 1997b). The substrates of mTOR will be discussed in subsequent sections.

1.4.1.3.1 mTOR autokinase activity and regulation by phosphorylation

mTOR possesses serine-specific autokinase activity (Brown *et al.*, 1995; Peterson *et al.*, 2000; Withers *et al.*, 1997) which is sensitive to the PI3K inhibitors wortmannin and LY294002 (Brunn *et al.*, 1996; Peterson *et al.*, 2000; Withers *et al.*, 1997). The concentration of wortmannin required for 50% inhibition (IC_{50}) of mTOR auto-phosphorylation is ~200nM *in vitro* and ~300nM *in vivo*. mTOR is 100 fold less sensitive to wortmannin than the phosphoinositide activity of PI3K (IC_{50} ~ 2-5nM); LY294002 inhibits mTOR autokinase activity *in vivo* and *in vitro* (IC_{50} 5μM) at a similar concentration needed to inhibit PI3K activity (~3μM) (Brunn *et al.*, 1996). S2481 has been reported as a site of auto-phosphorylation *in vivo* and *in vitro* which is sensitive to wortmannin but not to rapamycin, amino acid starvation or serum withdrawal (Peterson *et al.*, 2000), conditions which affect phosphorylation of the mTOR effectors S6K1 and 4E-BP1 (Section 1.72). Thus the autokinase activity of mTOR S2481 may not reflect mTOR activity for other substrates. However, c-Abl a non-receptor-tyrosine kinase activated by DNA damage, was found to negatively regulate mTOR. c-Abl can associate and phosphorylate mTOR *in vivo* and *in vitro* to prevent mTOR auto-phosphorylation (Kumar *et al.*, 2000b). The functional significance of auto-phosphorylation on mTOR catalytic activity is therefore unclear. Insulin stimulation of mTOR may be dependent on activation of PI3K and PKB (Gingras *et al.*, 1998; Scott *et al.*, 1998; Takata *et al.*, 1999). Furthermore, mTOR is also phosphorylated at S2448 at the C-terminus of the catalytic domain (Bolster *et al.*, 2002; Navé *et al.*, 1999; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000). Phosphorylation at this

site is wortmannin sensitive but rapamycin insensitive and phosphorylation is mediated by PKB. *In vivo* phosphorylation of mTOR-S2448 is stimulated by insulin and modulated by nutrient availability (Bolster *et al.*, 2002; Navé *et al.*, 1999; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000). Thus, mTOR is a target of PKB and is phosphorylated in a linear pathway in response to mitogenic stimuli as depicted in Fig. 1.1. However, additional factors such as amino acid availability may also regulate mTOR activity.

1.4.1.4 mTOR FAT and FATC domain

Apart from homology to the lipid kinase domain, all PIKK family members possess a FRAP, ATM and TRRAP (FAT) domain which is always accompanied by the FAT-carboxy-terminal homology domain (FATC) at the extreme C-terminus. Neither the FAT nor FATC domains are common to PI3K or PI4K (Alarcon *et al.*, 1999; Bosotti *et al.*, 2000). The functions of the FAT and FATC domain are unclear though it has been speculated that they interact together or are important for protein-protein interaction or modulation of kinase activity (Bosotti *et al.*, 2000). In yeast, the FAT domain has been described as a 'toxic effector' domain because it inhibited growth when over-expressed; however, the mechanism of conferring toxicity is undefined (Alarcon *et al.*, 1999).

1.5 Targets of mTOR kinase activity

Several downstream pathways have been implicated in the mTOR signalling pathway by use of the specific mTOR inhibitor, rapamycin.

1.5.1 eIF4E binding protein (4E-BP1)

eIF4E is the 5'cap recognition component of the eIF4F complex that interacts with the 43S pre-initiation complex required for translation initiation (Dever, 1999; Proud and Denton, 1997) (Section 1.2). Under translationally repressive conditions eIF4E is tightly associated with the eIF4E-binding protein 1 (4E-BP1, also known as phosphorylated heat- and acid- stable protein, PHAS-I) which prevents eIF4E contribution to the eIF4F complex. There are three 4E-BPs (4E-BP1, 4E-BP2 and 4E-BP3) whereby multi-site ser/thr phosphorylation dictates 4E-BP association with eIF4E; 4E-BP1 is the best characterised (Gingras *et al.*, 1999b). Upon mitogenic stimulation 4E-BP1 becomes hyper-phosphorylated, causing it to dissociate from eIF4E to permit Cap-dependent translation (Diggle *et al.*, 1996; Herbert *et al.*, 2000; Manteuffel *et al.*, 1996). In contrast, nutrient deprivation and rapamycin causes the rapid dephosphorylation of 4E-BP1 to promote 4E-BP1 association with eIF4E (Beretta *et al.*, 1996; Fadden *et al.*, 1997; Hara *et al.*, 1998). 4E-BP1 is phosphorylated on at least five ser/thr-proline (S/T-P) directed sites, namely T37, T46, S65, T70, S83 (Fadden *et al.*, 1997) and at S112 (a ser-glutamine site) (Heesom *et al.*, 1998); numbered according to the human sequence of 4E-BP1, minus 1 for numbering to rat sequence. Each site has varied sensitivity to serum-stimulation and inhibitors like wortmannin and rapamycin (Brunn *et al.*, 1997a; Fadden *et al.*, 1997; Gingras *et al.*, 1998; Heesom and Denton, 1999; Manteuffel *et al.*, 1996). The precise order of phosphorylation has been contested; however the sites are considered to be phosphorylated in a hierarchal system (Gingras *et al.*, 2001a).

1.5.1.1 Phosphorylation of 4E-BP1

The hierarchal phosphorylation begins with the 'priming phosphorylation' at T37 and T46 which does not cause 4E-BP1 to dissociate from eIF4E. These two sites are not sensitive to serum deprivation and are only mildly rapamycin sensitive (Burnett *et al.*, 1998; Gingras *et al.*, 1999a, 2001a). T70 is phosphorylated next which then allows phosphorylation of S65. Both these phosphorylation sites are sensitive to serum withdrawal and rapamycin (Gingras *et al.*, 1999a, 2001a; Mothe-Satney et al., 2000a). Phosphorylation at S65 and T70 is required for release of eIF4E from 4E-BP1 and prevention of their re-association (Gingras *et al.*, 1999a, 2001a; Heesom and Denton, 1999; Mothe-Satney *et al.*, 2000a). Recently, an additional phosphorylation site was identified at S101 which was constitutively phosphorylated and rapamycin insensitive. S101 may be required for S65 phosphorylation (Wang *et al.*, 2003). S83 is constitutively phosphorylated independently of the 'priming phosphorylation' and is not sensitive to insulin, amino acids or rapamycin (Mothe-Satney *et al.*, 2000b). Phosphorylation at S112 is rapamycin-insensitive but there is conflicting evidence as to whether S112 is sensitive to insulin (Heesom *et al.*, 1998; Wang *et al.*, 2003).

Several kinases have been shown to phosphorylate the S/T-P sites including MAPK (Haystead *et al.*, 1994; Lin *et al.*, 1994), casein kinase 2 (CK2) (Lin *et al.*, 1994) and mTOR (Burnett *et al.*, 1998; Gingras *et al.*, 1999a, 2001a; Heesom and Denton, 1999; Mothe-Satney et al., 2000a). However, *in vivo*, MAPK activity does not seem to be sufficient for 4E-BP1 phosphorylation and dissociation from eIF4E (Azpiazu *et al.*, 1996; Diggle *et al.*, 1996). However, rapamycin treatment does attenuate insulin stimulated phosphorylation of 4E-BP1 (Azpiazu *et al.*, 1996). This supports a role for mTOR in growth factor regulation of 4E-BP1. Several groups have shown *in vitro* phosphorylation of T37/T46 (Burnett *et al.*, 1998; Gingras *et al.*, 1999a, 2001a; Heesom and Denton, 1999). mTOR can also phosphorylate the S65 and T70 sites *in vitro* with use of the activating mTAb1 antibody (Brunn *et al.*, 1997b; Mothe-Satney *et al.*, 2000a), however, an mTOR associated kinase was also credited with phosphorylation of S65 and T70 (Heesom and Denton, 1999). Whether mTOR is responsible for the *in vivo*

phosphorylationof the sites it phosphorylates *in vitro* remains to be determined; nevertheless, the sensitivity to rapamycin at these phosphorylation sites indicates that mTOR is closely involved in the regulation of 4E-BP1 phosphorylation and translation initiation (Fig. 1.4). Caspase cleavage of 4E-BP1 truncates the protein by twenty four amino acids and although the 4E-BP1 phosphorylation sites are intact, insulin stimulated phosphorylation is reduced, leading to identification of the RAIP motif (Tee and Proud, 2002). The RAIP motif is named after the amino acids in the motif. RAIP is located at the N-terminus of 4E-BP1 and is required for mTOR-dependent regulation (Tee and Proud, 2002).

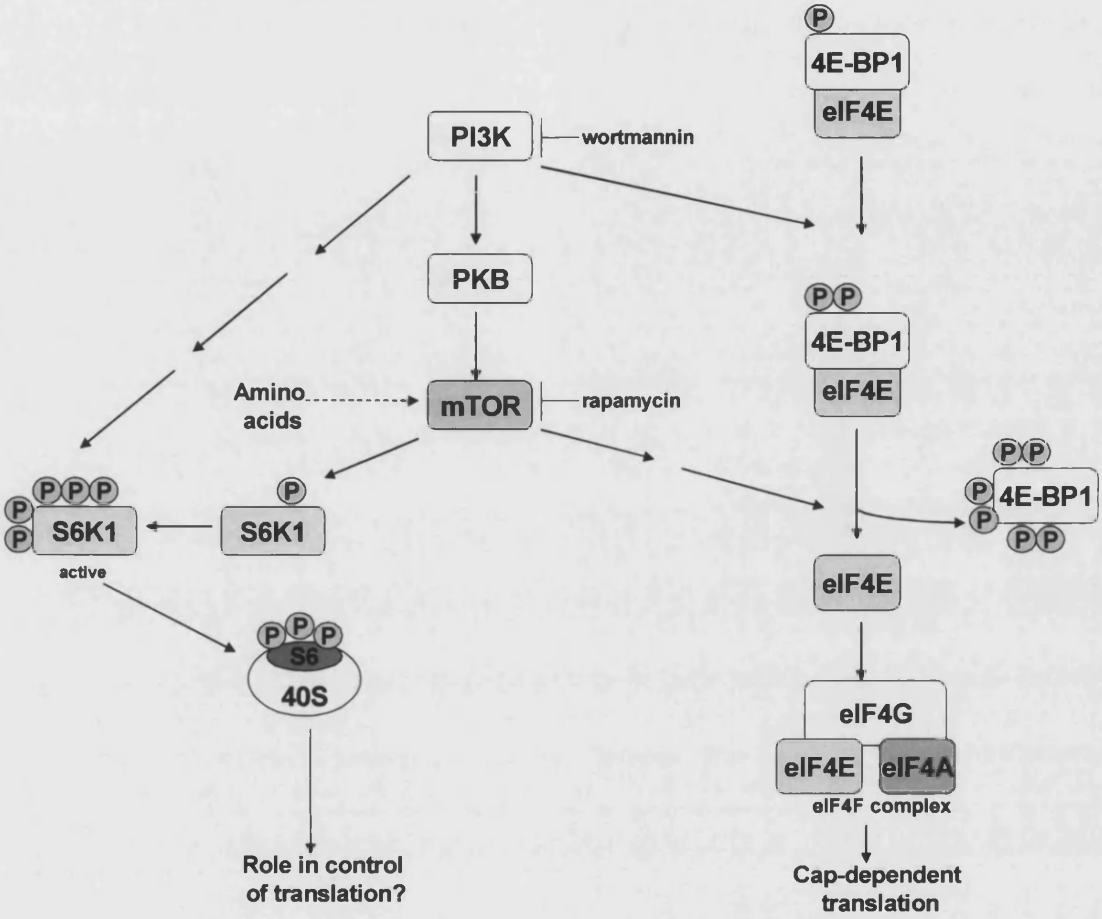

Figure 1.4 Connections between mTOR and control of translation initiation

Mitogenic stimulation activates signalling cascades through PI3K which stimulates PKB mediated phosphorylation of mTOR, which may cause mTOR activation. Two signalling pathways diverge from mTOR both of which are positively regulated by mTOR and affect translation initiation. Multi-site phosphorylation of 4E-BP1 is mediated by a wortmannin and rapamycin sensitive pathway which initiates the release of eIF4E; this permits formation of the eIF4F complex which is required for 5'cap dependent translation. mTOR signalling also mediates phosphorylation of S6K1 which also requires wortmannin sensitive signalling to activate. Active S6K1 phosphorylates the S6 protein of the 40S ribosomal subunit; however, the role of S6K1 in translation is unclear (Pende *et al.*, 2004). Figure modified from Proud, (2004). mTOR and its downstream effectors are also sensitive to changes in amino acid availability. mTOR may also be regulated by amino acids as indicated (e.g. Hara *et al.*, 1998).

1.5.2 S6 Kinases

The S6 kinases (S6K) phosphorylate the S6 protein of the 40S ribosome to stimulate translation of a specific subset of mRNAs, namely 5'TOP containing transcripts which encode ribosomal proteins and translational machinery; factors which contribute to cell growth and proliferation (Dufner and Thomas, 1999; Jeffries *et al.*, 1997; Kawasame *et al.*, 1998; Kimball, 2002; Loreni *et al.*, 2000). However, recent evidence suggests that S6K1 phosphorylation of the S6 protein is insufficient for 5' TOP mRNA translation in response to amino acids (Tang *et al.*, 2001). Furthermore, in S6K1(-/-)/S6K2(-/-) cells 5'TOP mRNAs are still modulated by mitogens in a rapamycin-dependent manner, suggesting the involvement of an alternative rapamycin-sensitive kinase (Pende *et al.*, 2004). Nevertheless, S6K1 is involved in the regulation of translation and its activation is sensitive to rapamycin; moreover it is a major target of mTOR signalling (Chung *et al.*, 1992; Hara *et al.*, 1998; Kuo *et al.*, 1992; Shigemitsu *et al.*, 1999; Weng *et al.*, 1998; Xu *et al.*, 1998a).

Two isoforms of S6K1 (or p70 S6 kinase α), denoted S6K1αI (also known as p70α1 and p85 S6 kinase) and S6K1αII (also known as p70α2 and p70 S6 kinase), are generated from a single gene by alternative mRNA splicing and use of translational start sites (Groves *et al.*, 1999). The S6K1αI isoform has a 23 amino acid nuclear localisation sequence (NLS) at the N-terminus which targets it exclusively to the nucleus, but is otherwise identical to S6K1αII which is expressed in the cytoplasm (Groves *et al.*, 1999) (Fig. 1.5). Both isoforms are collectively known as S6K1 and have identical phosphorylation sites. The sites mentioned herein will refer to S6K1αII (p70 S6 kinase); plus 23 amino acids for numbering to S6K1αI (p85 S6 kinase). A novel isoform of S6K has been identified, termed S6K2 (or p70 S6 kinase β) which also has two variants generated from a single gene by alternative translational start sites (Gout *et al.*, 1998). The variants are denoted S6K2βI (or p70β1) and S6K2βII (or p70β2) (Gout *et al.*, 1998) (Fig. 1.5). S6K1αI and S6K2βII possess 70% identity and 85% similarity at the protein level (Minami *et al.*, 2001).

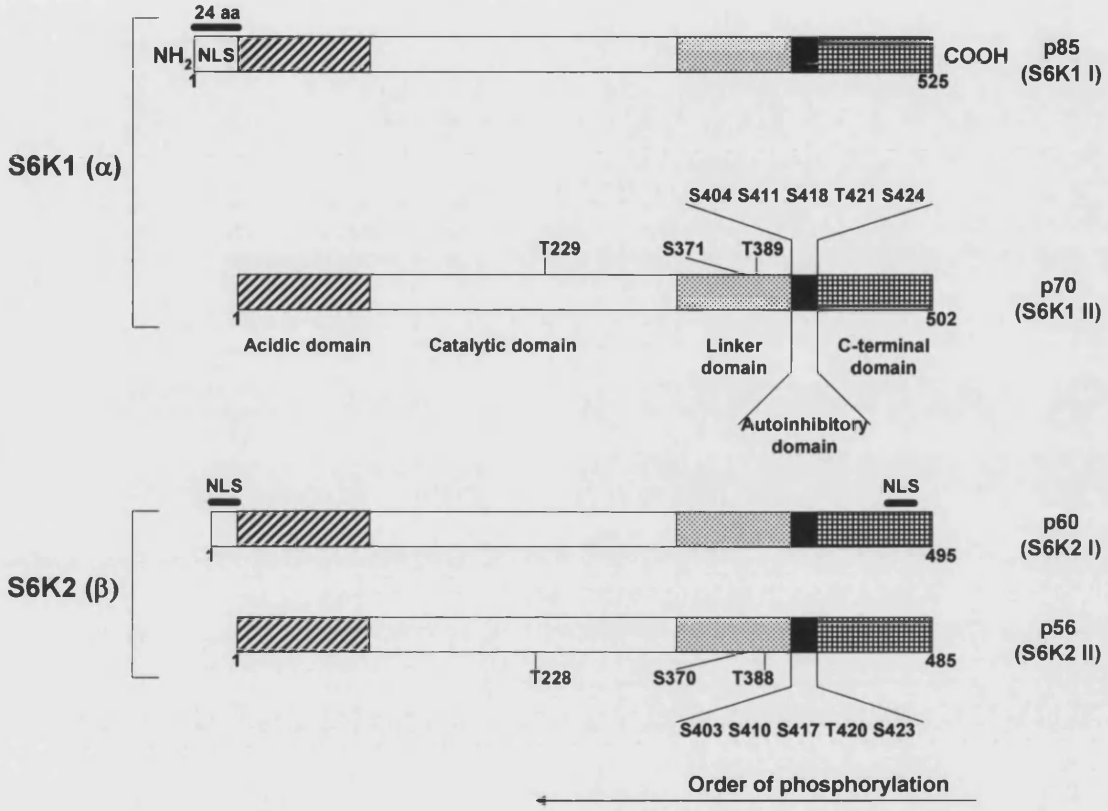

Figure 1.5 Schematic representation of the S6 kinases

S6K1 and S6K2 and their splice variants are shown with their alternative nomenclature (Minami *et al.*, 2001). The domains are as indicated and the phosphorylation sites are shown with the corresponding amino acid. Phosphorylation sites are numbered according to the short form (p70 and p56 for S6K1 and S6K2 respectively); the arrow indicates the order of phosphorylation. S6K1αI has an N-terminal nuclear localisation signal (NLS) but is otherwise identical to S6K1αII. Similarly, S6K2βII is identical to S6K2βI through residues 14-495 but lacks the NLS located at both the -N and -C termini.

1.5.2.1 Regulation of S6K1 by phosphorylation

Mitogenic stimulation activates S6K1 by mediating a complex series of ser/thr phosphorylations associated with phosphorylation at eight sites: T229 in the catalytic domain; S371 and T389 in the linker domain; and S404, S411, S418, T421, S424 in the auto-inhibitory domain which are in a S/T-P motif (Dufner and Thomas, 1999). Phosphorylation of S6K1 can be mediated by several kinases which include: MAPK, atypical PKCs, PKB and mTOR (Dufner and Thomas, 1999), though the precise mechanisms and pathways involved still require clarification. S6K1 activation requires hierarchal phosphorylation beginning with the S/T-P sites in the autoinhibitory domain. This is proposed to facilitate phosphorylation of T389 which disrupts interaction between the -N and -C terminus to allow phosphorylation of T229 (Dufner and Thomas, 1999; Volarević *et al.*, 2000). Phosphorylation of S6K1 is sensitive to rapamycin and wortmannin which indicates that mTOR and PI3K signalling is important for its activation. PDK1 was identified as phosphorylating S6K1 *in vitro* at T229 (Alessi *et al.*, 1997b; Pullen *et al.*, 1998) and when PDK1 was over-expressed, phosphorylation was also induced at T389 *in vivo* (Balendran *et al.*, 1999). Activation was determined to be dependent on co-operative phosphorylation at T229 and T389 (Alessi *et al.*, 1997b).

Aside from mitogenic activation, S6K1 is also sensitive to nutrient availability, particularly amino acid levels and it is regulated through mTOR as determined through use of rapamycin (Chung *et al.*, 1992; Hara *et al.*, 1998; Kuo *et al.*, 1992; Shigemitsu *et al.*, 1999; Weng *et al.*, 1998; Xu *et al.*, 1998a). The T229 and T389 S6K1 phosphorylation sites are sensitive to rapamycin (Weng *et al.*, 1998) and mTOR was subsequently found to directly phosphorylate the T389 site *in vitro* on bacterially expressed S6K1 (Burnett *et al.*, 1998) and on full length native S6K1. Phosphorylation of T389 was required for kinase activation (Isotani *et al.*, 1999) implicating mTOR in the direct regulation of S6K1. Another mTOR phosphorylation site exists in the S6K1 linker domain; S371 is regulated by mTOR *in vivo* and phosphorylated by mTOR *in vitro*. Phosphorylation at S371 modulates T389 phosphorylation though the mechanism is as yet unresolved (Saitoh *et al.*, 2002). S6K2 activation also requires mTOR kinase

activity (Park *et al.*, 2002); however, S6K2 is less sensitive to rapamycin and amino acid availability (Gout *et al.*, 1998; Minami *et al.*, 2001). Figure 1.4 shows mTOR regulation of S6K1 in translation initiation.

Many protein kinases recognise their substrate by identification of specific sequence motifs (Pinna and Ruzzene, 1996). As mentioned earlier, mTOR phosphorylates 4E-BP1 at S/T-P directed sites; however, the S/T-P sites in the autoinhibitory domain of S6K1 are not phosphorylated by mTOR. Although the S371 phosphorylation site is in an S-P motif, the rapamycin sensitive mTOR phosphorylation site T389 is in an FTY motif suggesting that mTOR kinase activity is either less stringent than other kinases for substrate selectivity or that other mechanisms are in place for regulation of S6K1 and/or 4E-BP1 e.g. mTOR regulation of a phosphatase.

1.6 Additional targets of mTOR

1.6.1 mTOR and Protein Phosphatase 2A (PP2A)

A common mechanism employed to regulate enzyme activity is reversible substrate phosphorylation. Protein phosphatase 2A (PP2A) is a major cellular ser/thr phosphatase involved in a wide range of processes which include metabolism, transcription and translation (Janssens and Goris, 2001; Millward *et al.*, 1999; Wera and Hemmings, 1995). PP2A exists as a heterotrimer (A, B and C subunits) with a core heterodimer, consisting of a 36 kDa catalytic subunit (PP2Ac or C subunit) and a 65 kDa scaffolding subunit (PR 65/A or A subunit), which binds to one of 15 identified regulatory B subunits (Zolnierowicz, 2000). The A subunit contains 15 HEAT repeats for which the crystal structure has been solved (Groves *et al.*, 1999).

There has been speculation that mTOR acts by restraining phosphatase activity towards the mTOR effectors S6K1 and 4E-BP1. In support of this hypothesis, osmotic stress and rapamycin induced down-regulation of S6K1 activity via a calyculin A-sensitive phosphatase (Parrott and Templeton, 1999). Similarly, activation of S6K1 by amino acids is also inhibited by the phosphatase inhibitors okadaic acid and calyculin-A (Krause *et al.*, 2002). Moreover, nutrient deprivation increased PP2A activity toward 4E-BP1 and this was enhanced by rapamycin treatment (Peterson *et al.*, 1999). PP2A was also found to interact with full length S6K1 but not with a truncated S6K1 which was resistant to rapamycin and nutrient deprivation and also resistant to de-phosphorylation. Additionally, mTOR was shown to phosphorylate PP2A *in vitro* with greater efficiency than towards GST-4EBP1 (Peterson *et al.*, 1999), though the precise phosphorylation sites were not determined. Phosphorylation of PP2A-C is associated with inactivation of phosphatase activity (Janssens and Goris, 2001), suggesting that mTOR can act as a negative regulator of PP2A.

1.6.1.1 Model for mTOR control of PP2A

The interest toward PP2A within the mTOR signalling pathway arose by comparison to the equivalent TOR signalling pathway in *S. cerevisiae*. The yeast PP6 and PP2A homologue Sit4 can bind the regulatory Sit4-associated proteins (SAP) as well as Tap42 (DiComo and Arndt, 1996; Duvel and Broach, 2004). Nutrients promote the association of Tap42 with Sit4 and the yeast PP2A-C subunit, independently of SAP or the PP2A-A and B subunits respectively, acting in a positive regulatory manner for translation initiation and cell cycle progression. In contrast nutrient deprivation and rapamycin inhibits growth by dissociation of Tap42 (DiComo and Arndt, 1996). Tap42 is a phospho-protein and TOR is able to directly phosphorylate Tap42 and improve it as a competitor for association with Sit4 and PP2A-C (Jiang and Broach, 1999). Tap42 dephosphorylation is mediated by activated trimeric PP2A (A, B and C subunits).

It is possible that the yeast PP2A/Tap42 regulatory system exists in mammalian cells (Gingras *et al.*, 2001b; Goldberg, 1999). A mammalian homologue to Tap42 has been identified. α4 is a phospho-protein identified by its association with the immunoglobulin (Ig) receptor-related MB-1 protein complex. α4 has 24% identity and 37% similarity to yeast Tap42 (Inui *et al.*, 1995; Kuwahara *et al.*, 1994). The cellular and biochemical functions of α4 are not well characterised but it has been shown to associate consistently with the N terminus of PP4, PP6 (Chen *et al.*, 1998; Nanahoshi et al., 1999) and PP2A catalytic subunit (Chen *et al.*, 1998; Murata *et al.*, 1997) and modulate phosphatase activity. Binding of α4 to PP2A-C can be disrupted by rapamycin in COS7 (Murata *et al.*, 1997) and Jurkat cells (Inui *et al.*, 1998), which also decreases phosphatase activity *in vitro*. Conflicting reports show constitutive association between PP2A/α4 which is rapamycin-insensitive in HEK293 (Nanahoshi *et al.*, 1998) and Jurkat cells (Chen *et al.*, 1998). Moreover, α4 has negative regulatory effects on PP2A-C phosphatase activity toward 4E-BP1 that had been pre-phosphorylated by mTOR; however the phosphatase activity was not sensitive to rapamycin (Nanahoshi *et al.*, 1998). In contrast association of α4 augmented PP2A-C

phosphatase activity toward myelin basic protein (MBP) and rapamycin inhibited phosphatase activity by disrupting PP2A/α4 association (Inui *et al.*, 1998). Similarly, over-expression of α4 reduced phosphorylation of eEF2 but not of S6K1 or 4E-BP1 (Chung *et al.*, 1999).

The phosphatase activity of PP2A/α4 may be cell-type specific and substrate selective. Nevertheless, those studies which demonstrate rapamycin sensitivity of PP2A/α4 phosphatase activity implicate an mTOR-dependent pathway. A study in rat skeletal muscle showed that insulin inactivated PP2A, a process inhibited by wortmannin and rapamycin (Begum and Ragolia, 1996), suggesting deactivation of PP2A via a PI3K/mTOR dependent pathway. Furthermore, α4 has been shown to mediate B-cell receptor (BCR) signalling via an mTOR-dependent pathway; loss of α4 caused a decrease in cell growth and S6K1 signalling while increasing rapamycin sensitivity (Inui *et al.*, 2002). A model of mTOR regulation of PP2A via a rapamycin sensitive pathway is depicted in Fig. 1.6.

Though the yeast Sit4/Tap42, PP2A/Tap42 system is seemingly conserved in mammalian cells, as yet there are no reports of the direct phosphorylation of α4 by mTOR; furthermore, the substrates of PP2A/α4 phosphatase activity and its interaction and interplay with the mTOR signalling pathway needs further clarification.

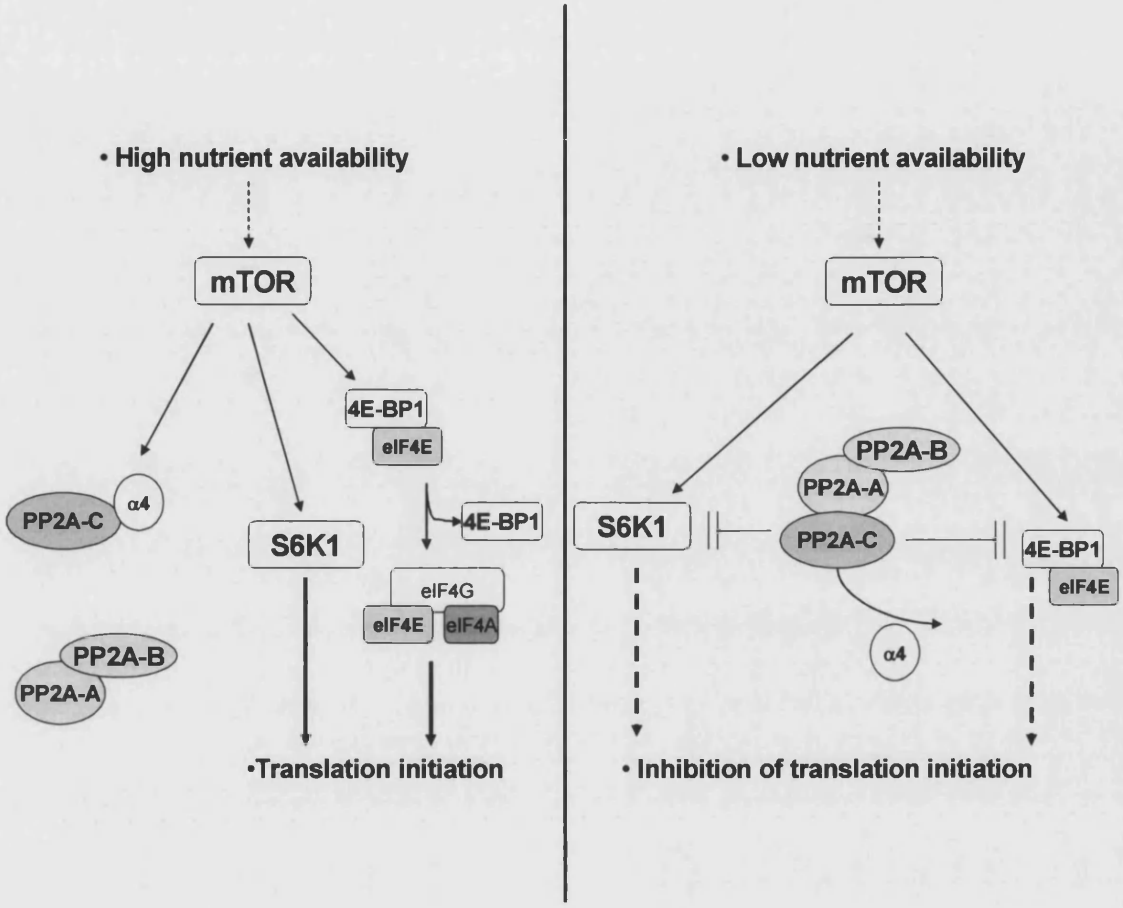

Figure 1.6 Model of mTOR regulation of PP2A via a rapamycin sensitive pathway in response to nutrient availability

Figure 1.6 is a simplified representation of the interaction of mTOR and PP2A/α4. During high nutrient availability, mTOR signalling to α4 promotes its association with PP2A-C and inhibits phosphatase activity toward S6K1 and 4E-BP1; translation initiation proceeds. During low nutrient availability, reduced signalling through mTOR causes the dissociation of α4 from PP2A-C and the PP2A trimeric complex is formed activating phosphatase activity toward S6K1 and 4E-BP1; translation initiation is inhibited.

1.6.2 mTOR regulation of eEF2

As mentioned in Section 1.2, eEF2 is involved in the translocation step of translation elongation and is a 90kD protein that is able to bind GTP. eEF2 is negatively regulated by phosphorylation at T56 by eEF2–kinase, formerly known as calcium/calmodulin-dependent kinase (Ca/CaM-kinase III) (Browne and Proud, 2002; Proud, 2004a). Insulin stimulates a decrease in the phosphorylation of eEF2 to promote elongation which is concomitant with inhibited eEF2-kinase activity in many cell types including Chinese hamster ovary expressing human insulin receptor (CHO-IR) (Redpath *et al.*, 1996) and mouse embryonic fibroblasts (MEFs) (Wang *et al.*, 2001). Insulin causes dephosphorylation of eEF2, requiring the presence of both amino acids and glucose (Campbell *et al.*, 1999). Amino acid withdrawal increases phosphorylation of eEF2 to inhibit elongation (Wang *et al.*, 1998). Rapamycin blocks insulin stimulation of eEF2, implicating the mTOR signalling pathway (Redpath *et al.*, 1996). S6K1 and also $p90^{RSK1}$ were found to inactivate eEF2-kinase at low Ca^{2+} concentrations (1-5μM) by phosphorylating a single residue at S366 (of the human eEF2 kinase sequence) in the C-terminus of the catalytic domain (Wang *et al.*, 2001). Additionally, eEF2 kinase can be phosphorylated *in vitro* at S359 by stress-activated protein kinase 4 (SAPK4), causing eEF2 kinase inactivation (Knebel *et al.*, 2001). S359 can also be phosphorylated *in vivo* in a rapamycin-sensitive manner, though SAPK4 is not a rapamycin sensitive kinase (Knebel *et al.*, 2001). Thus control of eEF2-kinase can be mediated by an mTOR-independent pathway, via MAPK, and by mTOR-dependent signalling, via S6K1 and possibly by an mTOR-regulated kinase. More recently, a novel mTOR-regulated phosphorylation site was identified in eEF2 kinase, located at the N-terminus, adjacent to the calmodulin (CAM) binding domain at S78 (Browne and Proud, 2004). Phosphorylation at S78 is stimulated by insulin in a rapamycin sensitive manner and is also modulated by amino acids. It appears that S78 phosphorylation inhibits binding of CAM and thus inhibits eEF2 kinase activity; however, the mTOR-dependent kinase responsible for S78 phosphorylation is not S6K1 (Browne and Proud, 2004), and the kinase remains to be identified.

1.6.3 mTOR regulation of other translation initiation factors

Apart from controlling the availability of the translation initiation factor eIF4E through control of 4E-BP1, there is evidence that mTOR controls additional members of the translational machinery. eIF4G, the scaffold element of the eIF4F complex (Fig. 1.2) exists as two homologues (eIF4GI and eIF4GII) and is a phospho-protein. Three serum-stimulated phosphorylation sites were identified in the eIF4GI hinge region at S1108, S1148 and S1192 (Raught *et al.*, 2000). Phosphorylation at these sites is wortmannin and rapamycin sensitive (Raught *et al.*, 2000). Interestingly, truncation of the N-terminus of eIF4GI confers resistance to wortmannin and rapamycin, and phosphorylation at S1108, S1148 and S1192 becomes constitutive. This indicates that eIF4GI phosphorylation is modulated through a PI3K- and mTOR- dependent signalling pathway through the C-terminal portion of the protein. The kinase responsible for phosphorylation remains elusive as neither mTOR nor S6K1 can phosphorylate eIFGI *in vitro* (Raught *et al.*, 2000). Additionally, eIF4B is reported to be controlled through an mTOR-dependent signalling pathway (Gingras *et al.*, 2004). S6K1 is able to phosphorylate eIF4B at a serum-responsive and rapamycin sensitive phosphorylation site *in vitro* (Gingras *et al.*, 2004). This provides further evidence that the mTOR signalling pathway is important for modulating translation initiation.

1.6.4 mTOR regulation of IRS-1

Insulin stimulation mediates activation of the insulin signalling cascade via the insulin receptor substrate (IRS) proteins and IRS-1 is the most well characterised IRS protein (White, 1997). Multi-site tyrosine phosphorylation of IRS-1 is associated with activation of the insulin signalling cascade (White, 1997) while a feedback mechanism involving ser/thr phosphorylation of IRS-1 is responsible for the degradation of IRS-1 (Cengal and Freund, 1999; DeFea and Roth, 1997a, 1997b; Li and DeFea, 1999; Paz *et al.*, 1997). This appears to initiate IRS-1 degradation by the proteasome and downregulate insulin action (Haruta *et al.*, 2000). Ser/thr phosphorylation of IRS-1 has

also been implicated in the development of insulin resistance; tumour necrosis factor α (TNFα) is a pro-inflammatary cytokine that increases ser/thr phosphorylation and prevents insulin receptor mediated tyrosine phosphorylation of IRS-1. TNFα is commonly over-expressed in models of obesity and insulin resistance (Marchand-Brustel *et al.*, 2003; White, 1997).

Evidence suggests an additional role for mTOR in the negative feedback regulation of IRS-1. PDGF signalling is implicated in downregulation of insulin signalling via the negative regulation of IRS-1, a process which may involve mTOR (Li and DeFea, 1999; Ricort *et al.*, 1997; Staubs *et al.*, 1998). Insulin-induced redistribution of IRS-1 from low-density microsomes (LDM) to the cytosol is inhibited by rapamycin and sensitive to amino acid availability (Takano *et al.*, 2001). Additionally, amino acid treatment of muscle cells activates the mTOR/S6K1 pathway while concomitantly decreasing IRS-1 associated PI3K signalling, a process also sensitive to rapamycin (Tremblay and Marette, 2001).

Several kinases have been shown to phosphorylate serine residues on IRS-1 *in vitro* which inhibits IR-tyrosine-phosphorylation of IRS-1. These include: MAPK (DeFea and Roth, 1997), PKCζ (Ravichandron *et al.*, 2001), GSK3 (glycogen synthase kinase-3) (Eldar-Finkelman and Krebs, 1997), S6K1 (Hartman *et al.*, 2001) and mTOR (Hartman *et al.*, 2001). Furthermore, serine phosphorylation of IRS-1 has been shown to be both wortmannin and rapamycin sensitive which suggests that IRS-1 degradation can occur via an mTOR dependent pathway which is downstream of PI3K but independent of the Ras/MAPK pathway (Hartley and Cooper, 2002; Hartman *et al.*, 2001; Haruta *et al.*, 2000; Li and DeFea, 1999). mTOR and S6K1 are both capable of phosphorylating ser residues on IRS-1 *in vitro* (between residues 511-722) (Hartman *et al.*, 2001). Moreover, mTOR was found to constitutively associate with IRS-1 and TNFα was able to activate mTOR which specifically phosphorylated S636 and S639 of IRS-1 (Ozes *et al.*, 2001). This suggests a direct role for mTOR in the negative regulation of IRS-1. There is evidence that mTOR regulates IRS-1 degradation indirectly by negatively regulating PP2A activity (Hartley and Cooper, 2002). Nevertheless, it seems the mTOR

pathway is implicated in modulating IRS-1 signalling and mTOR may be a component in the onset of insulin resistance.

1.6.5 mTOR regulation of PKC

Ca^{2+} phospholipid activated protein kinase C (PKC) has been implicated in many cellular processes including apoptosis, cell growth and proliferation. There are 10 PKC isozymes which can be split into 3 classes: conventional PKCs (α, βI, βII and γ) which are stimulated by diacyglycerol, phosphatidylserine and are dependent on Ca^{2+}; novel PKCs (δ, ε, η/L and θ) which are stimulated by diacyglycerol and phosphatidylserine but are not Ca^{2+} dependent; atypical PKCs (ζ and ι/λ) which are stimulated by phosphatidylserine (Newton, 2001). PKCs possess a modular structure comprising a regulatory and catalytic domain; three ordered phosphorylation steps in the catalytic domain are required for activation (Newton, 2001). PDK1 is able to phosphorylate PKCδ and PKCζ (Good et al., 1998) and is implicated in activation of other PKC isozymes (Newton, 2001). mTOR may positively regulate the novel PKCs (Parekh *et al.*, 1999). Serum-dependent phosphorylation of PKCδ and PKCε at the C-terminal hydrophobic residue S662 and S729 respectively is inhibited by rapamycin and amino acid deprivation (Parekh *et al.*, 1999). Conditions which modulated the rapamycin sensitive phosphorylation sites did not affect PDK1 mediated phosphorylation of PKCδ which suggests that mTOR mediated phosphorylation is distinct from that of PDK1 and implicates the novel PKCs (δ and ε) in the mTOR pathway. Constitutive association between PKCδ and mTOR has been reported whereby PKCδ potentiates mTOR mediated phosphorylation of 4E-BP1 thus stimulating cap-dependent translation (Kumar *et al.*, 2000a). mTOR was not demonstrated to directly phosphorylate PKCδ; however the S662 phosphorylation site is similar to the mTOR mediated S6K1 T389 phosphorylation site in being flanked by bulky hydrophobic residues. mTOR may therefore modulate phosphorylation of PKCδ directly or via inhibition of a phosphatase.

1.6.6 mTOR regulation of STATs

Cytokine signalling is mediated by the JAK/STAT pathway (Janus kinase/signal transducers and activators of transcription). Upon cytokine binding, receptor associated JAKs are activated which mediate phosphorylation of specific tyrosine residues on the receptor, initiating recruitment of the STAT family of transcription factors (Kisseleva *et al.*, 2002). STATs are a family of seven structurally and functionally related proteins which can be phosphorylated by JAKs on a single conserved tyrosine residue, mediating STAT dissociation from receptor, STAT dimerisation and translocation to the nucleus (Kisseleva *et al.*, 2002; Rawlings *et al.*, 2004). However, maximal STAT activation requires both tyrosine and serine phosphorylation (S727 of STAT1 and STAT3) (Wen *et al.*, 1995), thus implicating a serine kinase. mTOR has been implicated in the regulation of STAT1 (Kristof *et al.*, 2003) and STAT3 (Yokogami *et al.*, 1999). The cytokine ciliary neurotrophic factor (CNTF) stimulates STAT3 phosphorylation at S727 in neuroblastoma cells in a rapamycin sensitive manner and mTOR was shown to phosphorylate a STAT3 peptide containing the S727 phosphorylation site (Yokogami *et al.*, 1999). HEK293 cells transfected with a reporter that had a STAT3-responsive promoter, demonstrated decreased activation of the reporter construct when treated with rapamycin. The decrease in transcriptional activity was similar to that observed with the STAT3 point mutation S727A (Yokogami *et al.*, 1999); this demonstrated that mTOR-dependent phosphorylation of STAT3 was required for its activation. However, in lung epithelial adenocarcinoma cells (A549) treated with a combination of the cytokine interferon-γ (IFN-γ) and lipopolysaccharide (LPS), STAT1 phosphorylation at S727 was sensitive to LY294002 but not wortmannin or rapamycin (Kristof *et al.*, 2003). LY294002 was used at a concentration (100μM) which inhibited PI3K and mTOR signalling as demonstrated by reduced S6K1 T389 phosphorylation and mTOR autophosphorylation (S2481) but not phosphorylation of the PKB mediated mTOR S2448 site (Kristof *et al.*, 2003). Over-expression of mTOR increased basal S727 phosphorylation of STAT1 in IFN-γ/LPS-stimulated cells which was insensitive to rapamycin treatment; this implicates mTOR in the regulation of STAT1 but not via a PKB-dependent or rapamycin-sensitive pathway. Further, mTOR, PKCδ and STAT1

were shown to be recruited into a macromolecular complex *in vivo* in the presence of IFN-γ/LPS (Kristof *et al.*, 2003). As mentioned above, constitutive association between PKCδ and mTOR has been reported (Kumar *et al.*, 2000a). Since STAT1 phosphorylation is rapamycin insensitive, it is possible that mTOR mediates association between the two and PKCδ phosphorylates S727 of STAT1 to regulate transcription in a rapamycin insensitive manner (Uddin *et al.*, 2002). It seems likely that various cytokines mediate different effects through mTOR but the present evidence suggests that in addition to regulation of translation, mTOR may also be involved in the regulation of transcription activation.

1.7 Regulation of mTOR

As discussed mTOR has been implicated in the regulation of several pathways; however, the regulation of mTOR itself has also been subject to intense investigation. Apart from growth factor stimulation and amino acid sensing there are many novel factors which have recently been implicated in the regulation of mTOR or of factors in the mTOR regulatory pathway.

1.7.1 The role of Raptor association with mTOR

The kinase activity of mTOR has been difficult to assess. Previous reports indicated that mTOR *in vitro* kinase activity, but not mTOR autokinase activity, was sensitive to the detergent NP40 (Nishiuma *et al.*, 1998). This suggested the loss of a stabilising mTOR-associated protein during isolation of mTOR. An mTOR binding partner has been identified in which the interaction is salt, detergent and rapamycin sensitive. The 150kD regulatory associated protein of mTOR (Raptor) was isolated and found to be highly conserved in most eukaryotes including *D. melanogaster* and *S. cerevisiae* (Hara *et al.*, 2002; Kim *et al.*, 2002). Raptor homologues all contain the novel Raptor N-terminal conserved (RNC) domain which comprises 3 blocks with 67-79% sequence identity, followed by a region of three HEAT repeats, ending with seven WD40 repeats at the C-

terminus. Raptor not only binds mTOR but also its downstream effectors S6K1 and 4E-BP1, an association that is detergent insensitive (Hara *et al.*, 2002). This may occur via the mTOR signalling motif (TOS) (Schalm and Blenis, 2002). TOS is a conserved five amino acid motif identified as a minimal requirement for mTOR mediated signalling and has been located at the N-terminus of the S6 kinases (FDIDL) and in the C-terminus of 4E-BP1 (FEMDI) (Schalm and Blenis, 2002), both major downstream targets of mTOR. Raptor can bind S6K1 and 4E-BP1 through their TOS motif. Mutation of the TOS motif in 4E-BP1 inhibits its phosphorylation by mTOR *in vitro* (Nojima *et al.*, 2003). Thus, the TOS motif may be a requirement for mTOR signalling by mediating interaction of Raptor-mTOR with mTOR target proteins. Raptor seems to be an essential factor for mTOR kinase activity towards 4E-BP1 (Hara *et al.*, 2002) but not towards S6K1α in vitro (Hara *et al.*, 2002; Kim *et al.*, 2002) and is necessary for the positive regulation of cell size and growth (Kim *et al.*, 2002). A model has been proposed (Kim and Sabatini, 2004; Kim *et al.*, 2002) whereby Raptor forms a complex with mTOR via two interactions, one which is 'constitutive' and the other 'nutrient sensitive'. The 'constitutive' interaction is proposed to play a positive regulatory role, existing irrespective of the nutrient status of the cell. The 'nutrient sensitive' interaction is proposed to have negative regulatory effects since nutrient poor conditions increases the stability of the 'nutrient sensitive' interaction which decreases mTOR in vitro kinase activity. Conversely, a nutrient rich condition disrupts the complex to increase mTOR activity. The mechanism which regulates the switch between the two proposed interactions is yet to be identified. However, the mechanism proposed by Kim *et al.*, (2002) is not consistent with the studies performed by Hara *et al.*, (2002), which again may arise from differences in isolation procedures. Data from Kim *et al.*, (2002) suggest that mitochondrial metabolism or oxidative stress may play a part in the regulation of the Raptor-mTOR complex, specifically via intracellular levels of ATP, to which mTOR may be sensitive (Section 1.7.5). The present evidence suggests that Raptor functions as an important scaffold between mTOR and both 4E-BP1 and S6K. While the mechanisms involved in the regulation and interaction of Raptor with mTOR await clarification, further investigation should shed light on other proposed mTOR interacting proteins.

1.7.2 Amino acid regulation of mTOR and its downstream effectors

Amino acid deprivation of CHO-IR (Campbell *et al.*, 1999; Hara *et al.*, 1998; Shigemitsu *et al.*, 1999), HEK293 (Hara *et al.*, 1998) and Jurkat cells (Iiboshi *et al.*, 1999) results in rapid deactivation of S6K1 and de-phosphorylation of 4E-BP1, with cells becoming unresponsive to insulin stimulation; re-addition of amino acids restores S6K1 activity and 4E-BP1 phosphorylation and cells regain responsiveness to agonists (Campbell *et al.*, 1999; Hara *et al.*, 1998). These effects are specific to amino acid withdrawal as omissions of glucose or vitamins do not affect S6K1 activity (Campbell *et al.*, 1999; Hara *et al.*, 1998) but phosphorylation of 4E-BP1 can be dependent on glucose availability (Patel *et al.*, 2001). Amino acid withdrawal also increases eEF2 phosphorylation in CHO-K1 cells to inhibit translation (Wang *et al.*, 1998). This indicates that amino acid availability exerts control over translation but this may be cell type specific. In hepatocyte H4IIE cells, withdrawal of amino acids does not abolish insulin stimulation of S6K1 and phosphorylation of 4E-BP1 (Shigemitsu *et al.*, 1999). Nevertheless, the effects of amino acid deprivation are the same as rapamycin treatment, where re-addition of amino acids could not overcome effects of rapamycin (Hara *et al.*, 1998), indicating a role for mTOR in the amino acid regulation of 4E-BP1 and S6K1. Rhabdomyosarcoma Rh30 cells stably expressing mTOR, containing the rapamycin resistant S2035I mutation, supported S6K1 activity in the presence of rapamycin when amino acids were re-introduced after amino acid starvation (Iiboshi *et al.*, 1999). Moreover, an S6K1 deletion mutant Δ2-46/ΔCT104 (Weng *et al.*, 1995), sensitive to wortmannin but resistant to rapamycin, was also resistant to amino acid deprivation (Hara *et al.*, 1998). However, mTOR fully inhibited *in situ*, as judged by inhibition of S6K1, retains *in vitro* autokinase and protein kinase activity (Dennis *et al.*, 2001; Hara *et al.*, 1998), so whether mTOR directly phosphorylates 4E-BP1 or S6K1 *in vivo* is unclear. Nevertheless, mTOR is involved in the regulation of S6K1 and 4E-BP1 as amino acid deprivation does not affect PI3K, PKB or MAPK activity in CHO-IR cells

(Hara *et al.*, 1998; Kimball *et al.*, 1999; Patel *et al.*, 2001; Wang *et al.*, 1998). Similarly in Jurkat cells, activation of S6K1 and phosphorylation of 4E-BP1 is dependent on amino acid availability but other ser/thr kinases (PKB and $p90^{rsk}$) are less affected (Iiboshi *et al.*, 1999). Use of 3-methyladenine, an inhibitor of autophagy (protein breakdown), inhibited insulin stimulation of S6K1 and 4E-BP1 in amino acid deprived H4IIE cells but did not inhibit insulin stimulated tyrosine phosphorylation, activation of PI3K or MAPK (Shigemitsu *et al.*, 1999). This suggests that amino acid signalling may not be propagated through growth factor signalling cascades. However, the PI3K inhibitor wortmannin can partially inhibit amino acid activation of S6K1 without inhibiting PKB activation (Iiboshi *et al.*, 1999; Patti *et al.*, 1998; Wang *et al.*, 1998; Xu *et al.*, 1998b); in these cases wortmannin was used at 100nM which is lower than the IC_{50} required for inhibition of mTOR (~200nM). Therefore, in addition to mTOR regulation by amino acids, PI3K may also be involved in regulation of S6K1 in response to amino acids but not via PKB.

1.7.2.1 Branched chain amino acids and mTOR signalling

Withdrawing individual amino acids from cells incubated complete with 1× amino acids (D-PBS containing all the amino acids at 1× standard concentration minus an individual amino acid) inhibits S6K1 with varied potency (Hara *et al.*, 1998). Withdrawal of arginine or leucine caused 70-90% inhibition; lysine or tyrosine caused 30-50% inhibition in CHO-IR cells (Hara *et al.*, 1998). This demonstrates differential sensitivity of the mTOR pathway to individual amino acids. Re-addition of individual amino acids at the standard 1× concentration (D-PBS containing the individual amino acid at 1× standard concentration) did not stimulate S6K1 activation (Hara *et al.*, 1998) but re-addition at 4 or 5× the standard concentration of leucine (in buffer) did stimulate S6K1 activity but with less potency than the complete amino acid mixture in CHO-K1 (Campbell *et al.*, 1999; Wang *et al.*, 1998), L6 myoblasts (Kimball *et al.*, 1999), hepatocytes (Patti *et al.*, 1998) and adipocytes (Lynch *et al.*, 2000). Similarly, leucine, valine and isoleucine induce phosphorylation of 4E-BP1 and S6K1 to the same level as complete amino acids in the RINm5F pancreatic β cell-line (Xu *et al.*, 1998a) but only

leucine was effective at stimulating S6K1 phosphorylation at a physiological concentration (Xu *et al.*, 1998a). Recently, leucine was reported to stimulate production of the hormone leptin in rat adipocytes via an mTOR-dependent pathway as determined by use of rapamycin (Roh *et al.*, 2003). Glutamine was also identified as a nutrient regulator of cardiac gene expression mediated through both PKA and mTOR signalling pathways (Xia *et al.*, 2003). So although the complete amino acid mixture is optimal for stimulating the mTOR pathway, the individual effects of leucine and other branched chain amino acids (BCAA) at stimulating mTOR signalling suggest they have an important regulatory role.

The importance of leucine and other BCAA e.g. isoleucine and valine, has been extended to whole animal studies. In rat skeletal muscle, only leucine was able to stimulate protein synthesis; furthermore it was the most effective at increasing phosphorylation of 4E-BP1 and S6K1 (T389) in a rapamycin sensitive manner (Anthony *et al.*, 2000). Similarly, in rat hepatocytes, BCAA induced increased phosphorylation of 4E-BP1 and S6K1 (T389) and again leucine was most potent (Anthony *et al.*, 2001; Krause, 2002). Though BCAA did not induce a difference in total protein synthesis rates in hepatocytes, leucine was effective in promoting the association of ribosomal protein mRNA with polysomes in the liver; this correlates to S6K1 phosphorylation and is distinct from total protein synthesis rates (Anthony *et al.*, 2001). This suggests tissue specific regulation of mTOR signalling by leucine. In support of this, chronic leucine/norleucine supplementation induces a tissue specific increase in the rate of protein synthesis in adipose tissue, liver and skeletal muscle but not heart or kidney (Lynch *et al.*, 2002). These findings indicate that *in vivo*, leucine can stimulate tissue/cell type specific translation initiation via a rapamycin sensitive pathway and leucine is important for translation of ribosomal mRNA. However, global protein synthesis is most likely to require additional factors such as complete amino acids.

In addition, leucine can influence pathways independent of mTOR. Glucose uptake in soleus muscle can be stimulated by leucine in a rapamycin insensitive manner (Nishitani *et al.*, 2002). In C2C12 myotubes withdrawal of leucine promoted autophagy but did

not affect S6 phosphorylation, suggesting leucine regulation of translation via an mTOR-independent pathway in myotubes (Mordier *et al.*, 2000).

1.7.2.2 mTOR detection of amino acid levels

How mTOR senses changes in amino acid availability is uncertain. tRNA aminoacylation was a mechanism considered for amino acid regulation of mTOR and its downstream effectors. In Jurkat cells, the activation of S6K1 and phosphorylation of 4E-BP1 is dependent on amino acid availability and is inhibited by rapamycin (Iiboshi *et al.*, 1999). Treatment of Jurkat cells with amino acid alcohols inhibits corresponding tRNA synthetases by preventing aminoacyl-tRNA formation, whereby a reduction of intracellular aminoacyl-tRNA levels leads to inhibition of S6K1 (Iiboshi *et al.*, 1999). However, in freshly isolated rat adipocytes, the regulation of S6K1 and 4E-BP1 is sensitive to rapamycin but not to amino acid alcohols such as $_{L}$-leucinol or $_{L}$-histinol (Pham *et al.*, 2000). Therefore, aminoacylated tRNA regulation of mTOR may be cell line or cell type dependent. In HEK293 cells, amino acid deprivation inhibits S6K1 activity but does not affect total amounts of aminoacylated tRNA (Dennis *et al.*, 2001). This suggests that mTOR is regulated by intracellular pools of amino acids rather than charged tRNA. Moreover, inhibition of protein synthesis in CHO-K1 cells with cycloheximide, emetine or anisomycin causes an increase in intracellular levels of amino acids and permits insulin stimulated activation of S6K1 and phosphorylation of 4E-BP1, even after amino acid deprivation (Beugnet *et al.*, 2003). However, inhibition of autophagy prevents insulin activation of S6K1 and phosphorylation of 4E-BP1 in the absence of amino acids (Beugnet *et al.*, 2003; Shigemitsu *et al.*, 1999). This would be consistent with mTOR regulation by intracellular levels of amino acids; however, amino-acylated tRNA may still be involved. The mechanism by which mTOR can sense changes in amino acid availability remains to be determined.

1.7.2.3 Leucine regulation of mTOR

In addition to speculation regarding the mode of amino acid signalling to mTOR, the essential amino acid leucine has been of particular interest because of all the amino acids, it is the most potent at stimulating S6K1 and 4E-BP1 phosphorylation (see above). The mechanism of leucine signalling has also been investigated. The method by which leucine exerts its effects on mTOR is unclear as there is no evidence of a 'leucine receptor'. Mitochondrial metabolism of leucine has been considered as having signalling potential to the mTOR pathway. mTOR has been found to associate with outer mitochondrial membranes (Desai *et al.*, 2002), so its physical location would be ideal for mitochondrial signals. Leucine is metabolised in mitochondria by oxidative decarboxylation. Firstly, in a reversible step, leucine is converted to α-ketoisocaproate (KIC) by branched chain aminotransferase (BCAT). Secondly, KIC is oxidised and decarboxylated by the multi-enzyme complex branched-chain α-keto acid dehydrogenase (BCKD) to form NADH and isovaleryl-CoA which can be further metabolised to acetyl-CoA for entry into the Krebs cycle (Voet and Voet, 1995). BCKD-kinase phosphorylates S293 of the E1α-subunit of BCKD to inactivate it (Zhao *et al.*, 1994). In the RINm5F pancreatic β-cell line, leucine but not isoleucine or valine stimulates phosphorylation of S6K1 in a rapamycin and wortmannin sensitive manner (Patti *et al.*, 1998; Xu *et al.*, 2001). The leucine metabolite KIC is also effective in activating S6K1 (Xu *et al.*, 2001) and stimulating phosphorylation of 4E-BP1 (Fox *et al.*, 1998) in several different cell types. Inhibition of mitochondrial function with azide inhibits leucine activation of S6K1 and this was not a cytotoxic effect because inhibition was reversible after removal of azide (Xu *et al.*, 2001). This suggests that in β-cells, leucine may stimulate S6K1 phosphorylation via the mTOR signalling pathway by acting as mitochondrial fuel. In contrast, Lynch *et al.*, (2003) assessed the role of leucine metabolism in adipose tissue by using a phospho-S293 antibody to assess the activity of BCKD. In nutrient deprived adipose tissue, BCKD was highly phosphorylated and inactive but when rats were orally administered a diet rich in leucine, circulating levels of leucine were accordingly raised and BCKD was dephosphorylated (active) in adipose tissue. Therefore, BCKD is also dependent on

changes in leucine availability. However, much higher doses of leucine were required to elicit BCKD dephosphorylation compared to S6K1 T389 phosphorylation in adipose tissue. This implies that BCKD regulates leucine metabolism but this may not be required for stimulation of the mTOR pathway and suggests that leucine itself is the most effective at stimulating the mTOR signalling pathway.

1.7.3 Cytoplasmic nuclear shuttling of mTOR

mTOR is associated with membrane fractions distributed in the cytoplasm (Sabatini et al., 1999; Withers et al., 1997). mTOR has been located at the endoplasmic reticulum (ER) and Golgi apparatus (Drenan *et al.*, 2004) and with the outer mitochondrial membrane (Desai *et al.*, 2002). Localisation to these particular compartments presumably aids mTOR function. However, mTOR is found to be predominantly located in the nucleus of cells expressing the HIV-1 envelope glycoprotein (Castedo *et al.*, 2001) and in human malignant cell lines, human fibroblasts and mouse myoblasts but was found to be excluded from the nucleus in HEK293 cells (Zhang *et al.*, 2002). No identifiable nuclear import/export sequence is located in mTOR yet treatment of cells with leptomycin B (LMB), a specific inhibitor of nuclear export receptor Crm1, specifically retains mTOR in the nuclear fraction and inhibits activation of S6K1 and phosphorylation of 4E-BP1. In addition, mTOR is reported to shuttle between the nucleus and the cytoplasm without affecting mTOR kinase activity (Kim and Chen, 2000). Addition of a nuclear localisation sequence (NLS) to mTOR enhances activation of nuclear localised S6K2 (Park *et al.*, 2002) and also enhances activation of S6K1 and phosphorylation of 4E-BP1 which are cytoplasmic (Chen, 2004). Conversely, addition of a nuclear export signal (NES) reduces activation of S6K2 and S6K1 and reduces phosphorylation of 4E-BP1. This implies that nuclear localisation of mTOR may convey some regulatory mechanism. So far, the upstream signalling elements required for nuclear shuttling of mTOR are unknown and shuttling may be cell type specific (Chen, 2004). The importance of mTOR shuttling is unclear but may implicate mTOR in the regulation of nuclear proteins or in relaying nuclear signals to cytoplasmic proteins.

1.7.4 Phosphatidic acid mediated mTOR signalling

Levels of the lipid second messenger phosphatidic acid (PA) are rapidly increased upon mitogenic stimulation via activation of phospholipase D (PLD) and have been linked to mTOR signalling (Chen, 2004; Chen and Fang, 2002; Fang *et al.*, 2001). Extracellular PA is rapidly incorporated into the cell to mediate cellular signalling and was shown to stimulate activation of S6K1 and phosphorylation of 4E-BP1 in a rapamycin and amino acid sensitive manner (Fang *et al.*, 2001). Moreover, serum stimulation rapidly increased cellular levels of PA which was concomitant to activation of S6K2 (Park *et al.*, 2002), S6K1 and 4E-BP1 phosphorylation (Fang *et al.*, 2001). Addition of the alcohol 1-butanol inhibits PA production and inhibits the phosphorylation of S6K1 and 4E-BP1; inhibition did not appear to be mediated by reduced signalling through PKB as PKB was not inhibited by 1-butanol (Fang *et al.*, 2001). The stimulatory effects of PA may therefore be dependent on the amino acid sensory mechanism of mTOR but independent of PI3K signalling; this was confirmed with use of an S6K1 mutant resistant to rapamycin and sensitive to wortmannin (Weng *et al.*, 1995) which was not inhibited by 1-butanol. PA did have direct electrostatic interaction with the FRB domain of mTOR which was fully competed by FKBP/rapamycin (Fang *et al.*, 2001); the interaction is not important for mTOR activity because PA did not affect mTOR kinase activity *in vivo* or *in vitro* (Chen, 2004). The stimulatory effects of PA are a novel input into mTOR regulation and though mTOR does have direct interaction with PA the signalling potential mediated by PA requires further examination.

1.7.5 mTOR regulation by ATP levels

mTOR has been described as a sensory protein which detects cellular levels of ATP (Dennis *et al.*, 2001). The glycolytic inhibitor 2-deoxy glucose causes a decrease in intracellular levels of ATP which induces inhibition of mTOR-mediated S6K1 activation and 4E-BP1 phosphorylation but not PKB activation (Dennis *et al.*, 2001), suggesting a

specific ATP sensory system within the mTOR signalling pathway. As described above, mTOR is regulated by intracellular amino acids and regulation is via a mechanism independent of detection of intracellular ATP levels because ATP depletion does not affect cellular amino acid pools and vice versa (Dennis *et al.*, 2001). mTOR has a high Km (Michaelis constant) for ATP at ~1mM, which is considerably higher than most other protein kinases. Dennis *et al.*, (2001) used 100mM 2-deoxy glucose to elicit the described effects, an extremely high concentration which translates to an extreme depletion of cellular ATP that would have multiple metabolic effects *in vivo;* mTOR may therefore be a sensory molecule for intracellular ATP levels only during extreme ATP depletion. Sensitivity to ATP levels via an mTOR dependent signalling pathway has been demonstrated *in vivo;* ethionine treatment of rats induces rapid depletion of hepatic ATP levels which causes de-phosphorylation of 4E-BP1 and S6K1 (Yoshizawa *et al.*, 2002). Additionally, mTOR has been localised to the outer mitochondrial membrane and this may allow it to detect changes in ATP levels through association with components of mitochondrial permeability pores (PTP), which include adenine nucleotide transporters (ANT) that transport ATP generated in mitochondria to the cytosol (Desai *et al.*, 2002). Therefore, an ATP sensory mechanism may exist in mTOR that occurs indirectly via an intermediate ATP sensing protein, possibly AMP-activated protein kinase (AMPK).

1.7.5.1 Interplay between mTOR and AMPK

AMPK is an established energy sensing/signalling protein that responds to the cellular ATP/AMP ratio of the cell (Carling, 2004; Hardie *et al.*, 1998). AMPK is a heterotrimeric complex comprising a catalytic (α) subunit and two regulatory subunits (β and γ) and is structurally and functionally related to a protein kinase complex in *S. cerivisiae* termed SNF1 (sucrose non-fermenting). Increasing cellular AMP levels activates AMPK allosterically via phosphorylation of the α-catalytic subunit (T172) by AMPK-kinase (AMPKK). AMPK activation promotes the production of ATP by stimulating glucose uptake and fatty acid oxidation while inhibiting energy consuming biosynthetic pathways (Hardie *et al.*, 1998; Kemp *et al.*, 1999). 5-aminoimidazole-4-

carboxamideribonucleoside (AICAR) is a specific activator of AMPK (Corton *et al.*, 1995); AICAR injection of rats significantly decreases skeletal muscle protein synthesis via 4E-BP1 and S6K1 (Bolster *et al.*, 2002). Activation of AMPK with AICAR also abrogates amino acid induced activation of S6K1, a process mediated through mTOR (Dubbelhuis and Meijer, 2002; Krause *et al.*, 2002). AICAR may have cellular effects aside from AMPK activation *in vivo* (Kemp *et al.*, 1999) but similar studies in which AMPK was activated by glucose withdrawal or 2-deoxyglucose substitution also inhibited S6K1 activity by an mTOR-dependent signalling pathway (Inoki *et al.*, 2003b; Kimura *et al.*, 2003). This supports the theory that AMPK signals energy changes to mTOR which in turn mediates signals to its downstream effectors.

1.7.6 TSC

The tuberous sclerosis complex (TSC) is an inherited human genetic disorder, occurring in 1:6000 newborns. TSC is an autosomal dominant disorder characterised by benign tumour development (hamartomas) in a variety of organs including most commonly the brain, kidney and heart (Young and Povey, 1998). Mutations in either the *TSC1* or *TSC2* tumour suppressor genes cause the TSC phenotype (Young and Povey, 1998). The *TSC1* gene is located on chromosome 9q34 (Slegtenhorst *et al.*, 1997) and encodes the 130kDa protein TSC1 (also known as hamartin) which contains a potential N-terminal transmembrane domain and a predicted coiled-coil domain but no obvious catalytic domain (Slegtenhorst *et al.*, 1997). TSC1 is also involved in adhesion and Rho-dependent signalling for actin fibre stress formation (Lamb *et al.*, 2000). The *TSC2* gene is located on chromosome 16p13 (Consortium, 1993) and encodes the 200kDa protein TSC2 (also known as tuberin) which also has a coiled-coil domain and a carboxy terminal which shares homology to the catalytic domain of murine GTPase –activating protein (GAP) and human GAP3 (Consortium, 1993). TSC2 demonstrates *in vitro* GAP activity toward Rap1 (Wienecke *et al.*, 1995) and Rab5 (Xiao *et al.*, 1997) and may also have a role in endo/exocytosis (Kleymenova *et al.*, 2001; Xiao *et al.*, 1997)). TSC1 and TSC2 interact to form a functional complex (herein, TSC denotes TSC1-TSC2 complex) (Potter *et al.*, 2001), attributed to occur in a number of ways: via the TSC1 coiled coil

domain (Slegtenhorst *et al.*, 1998); through the N-terminus of both proteins (Hodges *et al.*, 2001); by tyrosine phosphorylation of TSC2 (Aicher *et al.*, 2001); and/or by phosphorylation of both proteins (Nellist *et al.*, 2001). The TSC1 and TSC2 association occurs rapidly after protein synthesis, co-localising the complex into the cytoplasmic fraction (Nellist *et al.*, 1999). Both TSC1 and TSC2 are required for reciprocal stabilization; uncomplexed TSC2 is heavily ubiquinated and targeted for proteasome-dependent degradation which is prevented by TSC1 association (Benvenuto *et al.*, 2000); in return TSC2 acts as a chaperone to TSC1 to prevent TSC1 self-aggregation (Nellist *et al.*, 1999; Nellist *et al.*, 2001). Missense or point mutations of either gene, as found in TSC patients, can abrogate TSC function and/or interaction (Aicher *et al.*, 2001; Hodges *et al.*, 2001; Nellist *et al.*, 2001) and thus their tumour suppressor function.

1.7.6.1 TSC and the mTOR signalling pathway

In *Drosophila*, mutation of either *dTsc1* or *dTsc2* increases cell size and proliferation, whereas over-expression of either gene reduces cell size and proliferation, suggesting a negative regulatory role for TSC (Tapon *et al.*, 2001). Epistatic studies in *Drosophila* showed that TSC functioned in parallel to the insulin signalling pathway, converging at dAkt (dPKB) and upstream of dS6K (Gao and Pan, 2001; Potter *et al.*, 2001) and in parallel or upstream of dTOR (Gao *et al.*, 2002). Signalling in mammalian cells is predicted to be the same as that in *Drosophila*. Thus far, biochemical and bioinformatic techniques have identified TSC2 as a physiological substrate of PKB primarily at S939 and T1462 (Dan *et al.*, 2002; Manning *et al.*, 2002). Phosphorylation of these sites does not affect association with TSC1 (Dan *et al.*, 2002; Manning *et al.*, 2002) but may promote degradation of existing complexes (Dan *et al.*, 2002). The analogous PKB phosphorylation in *Drosophila* S924 and T1518 (Manning *et al.*, 2002) does disrupt TSC complex formation and the sub-cellular localization of the complex (Potter *et al.*, 2002). TSC2 can also be phosphorylated by AMPK at T1227 and S1345 (Inoki *et al.*, 2003b); activation of AMPK mediates phosphorylation of TSC2 and decreases signalling through the mTOR pathway.

Loss of TSC function results in the constitutive activation of S6K1 (Gao *et al.*, 2002; Inoki *et al.*, 2002; Jaeschke *et al.*, 2002; Kwiatkowski *et al.*, 2002; Zhang *et al.*, 2003a) which remains sensitive to rapamycin (Gao *et al.*, 2002; Kwiatkowski *et al.*, 2002; Zhang *et al.*, 2003a). Over-expression of TSC inhibits phosphorylation at the T389 site of S6K1 (Inoki *et al.*, 2002) but not at the P421 or S424 sites suggesting a negative regulatory role for TSC which is specific to the T389 rapamycin sensitive site. Co-expression of TSC inhibits insulin-stimulated phosphorylation of 4E-BP1 leading to repression of Cap-dependent translation (Tee *et al.*, 2002). Evidence is consistent with TSC negatively regulating the mTOR pathway. dTOR was found to interact strongly with dTsc2 (Gao *et al.*, 2002) but there is no evidence of physical interaction between mTOR and TSC (Gao *et al.*, 2002; Zhang *et al.*, 2003a). Instead TSC was found to interact with 14-3-3γ, a phospho-serine/threonine-binding protein (Zhang *et al.*, 2003a).

In CHO-IR cells amino acid deprivation induces rapid dephosphorylation and deactivation of S6K1 (Hara *et al.*, 1998). This was also observed in mouse embryonic fibroblasts (MEFs) that were TSC2-null and p53-null, in which amino acid withdrawal reduces S6K1 activity (Zhang *et al.*, 2003a). In insect S2 cells, amino acid withdrawal causes a 90% reduction in S6K1 activity after 15min and activity was abolished after 30min (Gao *et al.*, 2002). However, in contrast to Zhang *et al.*, (2003), S6K1 activity was resistant to amino acid starvation when *TSC1* and *TSC2* expression was inhibited in S2 cells by RNA interference (RNAi) (Gao *et al.*, 2002). S6K1 activity was only reduced by 39% after 30min and even after 6 hours amino acid starvation there was residual S6K1 activity and S6K1 activity remained rapamycin sensitive. This implies that TSC is not only a negative regulator of S6K1 but is also a component of amino acid signalling, given that resistance to amino acid starvation is a phenotype associated with loss of TSC. The conflicting evidence between Zhang *et al.*, (2003) and Gao *et al.*, (2002) may be accounted for by the use of different cell types and the method used to knock-out *TSC2* expression. Though evidence places mTOR as a mediator between the TSC complex and S6K1 (Inoki *et al.*, 2002; Tee *et al.*, 2002), the use of an alternative rapamycin resistant S6K1 mutant (Dennis *et al.*, 2001) in a study by Jaeschke *et al.*,

(2002) suggested that TSC inhibits S6K1 via PI3K but independently of mTOR. Nevertheless, TSC may operate in both an mTOR-dependent and mTOR-independent pathway and may play a part in integrating metabolic and nutrient signals to mTOR.

1.7.6.2 TSC2 GAP activity may regulate mTOR

As mentioned above, TSC2 displays GAP (GTPase-activating protein) homology (Consortium, 1993), demonstrating *in vitro* GAP activity toward Rap1 (Wienecke *et al.*, 1995) and Rab5 (Xiao *et al.*, 1997). TSC also possesses GAP activity toward the small G-protein, Ras homologue enriched in brain (Rheb) (Yamagata *et al.*, 1994), which is related to the Ras, Rap and Ral super family of small GTP binding proteins (Reuther and Der, 2000). GAPs down-regulate activated small GTP-bound proteins by accelerating GTP hydrolysis to produce the inactive GDP bound form (Donavan *et al.*, 2002). Evidence suggests that the GAP activity of TSC2 is specific for Rheb and this may contribute to regulation of mTOR signalling (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Li *et al.*, 2004; Tee *et al.*, 2003). Studies in *Drosophila* implicate Rheb in the insulin signalling cascade downstream of dTSC and upstream of dTOR and dS6K (Saucedo *et al.*, 2003; Stocker *et al.*, 2003; Zhang *et al.*, 2003b). Moreover, Rheb-GTP may be a positive regulator of the mTOR signalling pathway (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003) and contribute to the amino acid and energy sensing capabilities of mTOR. Over-expression of Rheb can promote S6K1 phosphorylation even in the absence of amino acids (Inoki *et al.*, 2003a; Tee *et al.*, 2003) or during energy depletion (Inoki *et al.*, 2003a). Furthermore, Rheb is membrane associated by farnesylation and this feature seems to be important for activation of mTOR signalling (Tee *et al.*, 2003), possibly through co-localisation of Rheb and mTOR at membrane surfaces as mTOR is also membrane associated (Sabatini *et al.*, 1999; Withers *et al.*, 1997). Overall, the GAP activity of TSC2 in complex with TSC1 negatively regulates the positive signalling instigated through Rheb and mTOR (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003). A putative model for TSC contribution to the mTOR-dependent signalling pathway is shown in Fig. 1.7 (see Tee *et al.*, 2003).

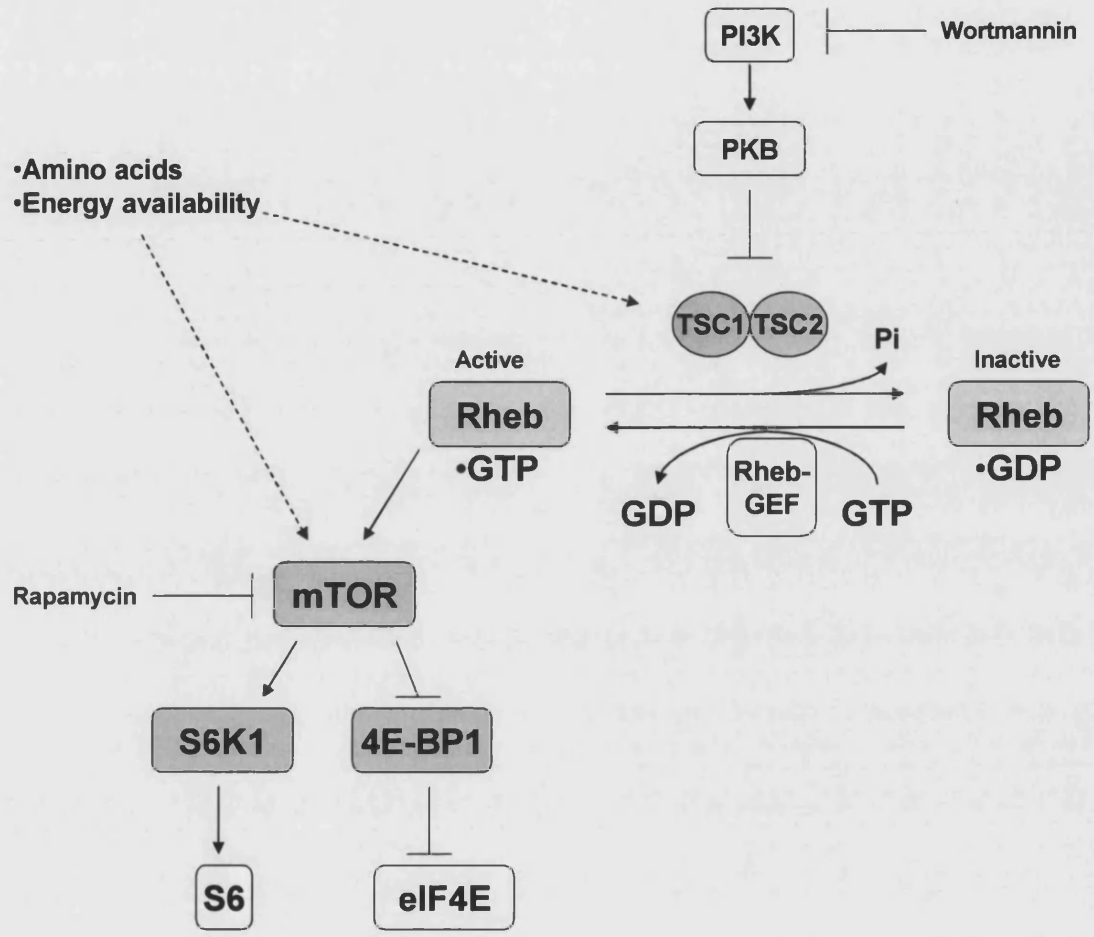

Figure 1.7 Putative model for TSC signalling via the mTOR-dependent signalling pathway

TSC GAP activity hydrolyses Rheb-GTP to the inactive GDP bound state and reduces signalling through mTOR. Upon activation of the PI3K signalling, PKB phosphorylates and inactivates the TSC GAP activity, and a Rheb-GEF (GTP exchange factor) activates Rheb by GDP/GTP exchange. Rheb-GTP can activate the mTOR signalling pathway through activation of S6K1 and phosphorylation of 4E-BP1. Schematic modified from Tee *et al.*, 2003. mTOR and TSC may also have additional sensory capabilities for nutrient and energy availability.

1.8 Role for mTOR in oncogenesis

The checkpoint properties of mTOR in the regulation of protein synthesis also extend to control of cellular growth and cell cycle progression, processes which are dependent on the energy and nutrient status of the cell. Therefore, it is not surprising that mTOR is also implicated in the control of cell cycle regulatory proteins, indicating that mTOR may be involved in tumorigenesis and be a viable target for cancer therapy.

1.8.1 mTOR and regulation of cell growth

In *Drosophila*, dTOR is important for regulating growth during larval development by integrating growth factor signalling and nutrient availability (Oldham *et al.*, 2000; Zhang *et al.*, 2000). dTOR mutants have a cellular phenotype normally associated with amino acid deprivation where cells are smaller than wildtype dTOR cells (Oldham *et al.*, 2000; Zhang *et al.*, 2000) and growth is arrested at the second instar larval stage (Zhang *et al.*, 2000). Lack of dPTEN (*Drosophila orthologue*: Phosphatase and tensin homolog deleted on chromosome 10), a tumour suppressor, increases cell growth (Huang *et al.*, 1999). Cells null for both dPTEN and dTOR were of reduced cell size and accumulated in the G_1 phase, a phenotype indistinguishable from cells null for dTOR alone (Zhang *et al.*, 2000). This indicates that growth stimulation by the PI3K pathway requires dTOR (Zhang *et al.*, 2000). Similarly, mTOR is required for embryonic development and cell proliferation in a mouse model (Hentges *et al.*, 2001). A mutation of mTOR in a mouse model (termed flat top) inhibited phosphorylation of S6K1 and 4E-BP1 but did not cause a reduction in cell size, as observed in *Drosophila*. In contrast, in U2OS osteosarcoma cells, rapamycin reduces cell size especially during the G_1 phase (Fingar *et al.*, 2002) but expression of a rapamycin resistant mTOR mutant (S2035I) rescues cells from reduced cell size. Thus, mTOR regulation of cell size may be cell-type dependent. Aside from regulating cell growth via S6K1 and 4E-BP1 (Fingar *et al.*, 2002; Hentges *et al.*, 2001), another mechanism may be through regulation of nutrient uptake. In the interleukin-3 dependent cell line FL5.12, growth factor withdrawal reduces cell size and causes downregulation of cell surface receptors for glucose and amino acids; over-expression of

activated PKB maintains these cell surface receptors via an mTOR dependent pathway as determined by use of rapamycin (Edinger and Thompson, 2002).

1.8.2 mTOR and cell cycle progression

The downstream effector S6K1 has also implicated mTOR in the control of cell cycle progression. In cancerous pancreatic β-cell lines, S6K1 is constitutively phosphorylated but treatment by rapamycin inhibited S6K1 phosphorylation and induced G_0-G_1 arrest (Shah *et al.*, 2001). Moreover, $S6K1^{-/-}$ murine embryonic stem cells are characterised by retarded proliferation that is sensitive to rapamycin and having an elevated level of cells in G_0-G_1 arrest (Kawasame *et al.*, 1998). This implies that mTOR/S6K1 signalling is important for G_1 to S phase progression. Similarly, in yeast, TOR is required for activating Cap-dependent translation and G_1 progression in response to nutrients by regulating yeast G_1-cyclin CLN3 (Barbet *et al.*, 1996). In mammalian cells, mTOR can also modulate translation of mRNAs which encode proteins involved in cell cycle progression. These include: cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors. cdks are ser/thr kinases that regulate the cell cycle and are activated when complexed with cyclins, which are sequentially expressed during the G_1 phase in response to growth factors (Pines, 1993). Possible cdk/cyclin combinations include: D-type cyclins complexed with cdk4 or cdk2 and cyclin E in complex with cdk2 (Pines, 1993). Cip/Kip (kinase inhibitor family) is a class of cyclin-dependent kinase inhibitors that include $p21^{Waf\text{-}1}$ and $p27^{Kip1}$ which are down-regulated by mitogenic stimulation and can bind and inactivate cyclin/cdk2 complexes (Dai and Grant, 2003; Ho and Dowdy, 2002). The G_2 to M transition is mediated by phosphorylation of the cdk2 complex and this is inhibited by rapamycin which arrests cells in transition without affecting $p21^{Waf\text{-}1}$ and $p27^{Kip1}$ availability (Shah *et al.*, 2001). In contrast, in D10 T-cells stimulated with interleukin-2 (IL-2), $p27^{Kip1}$ was eliminated. Rapamycin prevented $p27^{Kip1}$ elimination making it available for association with cyclin E/cdk2 complexes and inhibition of cell cycle progression (Nourse *et al.*, 1994). Similarly in 3T3 fibroblasts, rapamycin increased association of $p27^{Kip1}$ with cdk2 (Hashemolhosseini *et al.*, 1998). Furthermore, rapamycin inhibits G_1 to S phase cell

cycle progression by destabilising and promoting degradation of cyclin D1 mRNA and protein. Inhibition of mitogen induced cyclin D1 expression causes a decrease in cdk4 activity due to deficiency of cyclin D1 (Hashemolhosseini *et al.*, 1998). This indicates that mTOR is involved in cell cycle progression, not only through S6K1 and 4E-BP1 but also through regulation of cell cycle proteins.

1.8.3 mTOR and cell survival

p53 is a tumour suppressor which regulates cell cycle progression and apoptosis; inactivation of p53 leads to tumorigenesis (Kaelin, 1999; Vousden, 2000). p53 primarily acts as a transcription factor and exerts control over specific mRNAs which include: the cyclin dependent kinase inhibitor $p21^{Waf1}$ (Gorospe *et al.*, 1998) and cyclin dependent kinase 4 (cdk4) (Miller *et al.*, 2000). Activation of p53 leads to a rapid decrease in translation initiation, correlating with an increase in 4E-BP1 de-phosphorylation, inhibition of S6K1 and increased expression of $p21^{Waf1}$ which inhibits cell cycle progression (Horton *et al.*, 2002). p53 suppression of mTOR signalling may occur via c-Abl, a growth inhibitory non-receptor tyrosine kinase which is activated by DNA damage (Kharbanda *et al.*, 1997; Kharbanda *et al.*, 1995). c-Abl binds to p53 *in vitro* and enhances p53 dependent transcription (Goga *et al.*, 1995). Mutation of c-Abl renders it unable to bind p53 and p53 transcriptional activity is not enhanced; moreover cell growth is not suppressed (Goga *et al.*, 1995). c-Abl can also bind to the C-terminal domain of mTOR and mediate tyr-phosphorylation of mTOR *in vivo* and *in vitro* (Kumar *et al.*, 2000b). Tyrosine phosphorylated mTOR demonstrated reduced activity towards both S6K1 and 4E-BP1. Furthermore c-Abl also negatively regulates PI3K (Yuan *et al.*, 1997). Therefore, c-Abl activation could negatively regulate PI3K and translation via mTOR to induce G1 arrest in response to DNA damage.

Rhabdomyosarcoma (RMS) cells lack functional p53 and rapamycin treatment of RMS cells induces G1 arrest and apoptosis, implicating mTOR in apoptosis (Hosoi *et al.*, 1999; Huang *et al.*, 2001). This indicates rapamycin induced apoptosis is independent of p53 because expression of a rapamycin resistant form of mTOR (S2035I) can rescue

cells from apoptosis, demonstrating the importance of mTOR in cell survival (Hosoi *et al.*, 1999). However, in syncytia (fusion) of cells expressing the human immunodeficiency virus-1 (HIV-1) encoded envelope glycoprotein complex (Env) and cells expressing CD4/CXCR4, apoptosis is induced via mTOR and p53 (Castedo *et al.*, 2001; Castedo *et al.*, 2002). mTOR could associate with p53 from syncytial cells but not single cells; furthermore, delineation of the apoptotic pathway in syncytial cells showed that the initial step leading to apoptosis involved translocation of mTOR to the nucleus where it mediates p53 phosphorylation at S15 (Castedo *et al.*, 2001; Castedo *et al.*, 2002). Apoptosis of syncytial cells was inhibited by rapamycin (Castedo *et al.*, 2001; Castedo *et al.*, 2002), suggesting that mTOR regulation of apoptosis is dependent on p53. The involvement of mTOR in these apoptotic pathways requires further investigation.

1.8.4 mTOR and Cancer

The phosphoinositide 3' phosphatase, PTEN (Phosphatase and tensin homolog deleted on chromosome 10) is a tumour suppressor which is mutated in a variety of cancers (Cantley and Neel, 1999; Li *et al.*, 1997). It functions as a negative regulator of PI3K/PKB signalling and loss of PTEN function results in increased levels of PtdIns (3, 4, 5)P_3 and PtdIns-3, 4-P_2 (Stambolic *et al.*, 1998) corresponding to enhanced activation of PKB (Maehama and Dixon, 1999; Mayo and Donner, 2002). In *Drosophila*, PI3K signalling is important for growth and development (Leevers *et al.*, 1996). Loss of PTEN increases cell growth and over-expression of PTEN inhibits cell growth in *Drosophila* (Huang *et al.*, 1999) and mouse models (Stambolic *et al.*, 1998).

As established earlier, mTOR/S6K1 signalling is involved in progression to the S phase of the cell cycle (Shah *et al.*, 2001) and mTOR is also involved in tumours with activated PI3K signalling. In chicken embryo fibroblasts (CEF) transformed by class 1a PI3K and PKB, constitutive phosphorylation of S6K1 and 4E-BP1 is observed (Aoki *et al.*, 2001). The transforming activities of PI3K and PKB are specifically sensitive to rapamycin as other oncoproteins such as Src and Abl, were not affected by rapamycin.

However, rapamycin did enhance the transforming activity of the Myc oncogene (Aoki *et al.*, 2001). Similarly in human neuroblastoma cells, rapamycin potently inhibited mTOR dependent cell proliferation but did not inhibit Myc oncogene expression or the MAPK pathway (Misawa *et al.*, 2003). Inhibition of mTOR by rapamycin can therefore specifically inhibit the oncogenic properties induced by PI3K and PKB transformation. In the heterozygote $PTEN^{+/-}$ tumour mouse model, PTEN expression is reduced or absent and accompanied by increased phosphorylation of PKB and S6K1 (Podsypanina *et al.*, 2001), suggesting that the mTOR pathway is up-regulated in $PTEN^{+/-}$ tumours. The ester derivative of rapamycin, cell cycle inhibitor-779 (CCI-779), reduces neoplasia and normalises S6K1 activity without affecting PKB activation (Podsypanina *et al.*, 2001). Similarly, in human tumour cells deficient for PTEN ($PTEN^{-/-}$), 4E-BP1 is highly phosphorylated and S6K1 is constitutively activated; both are sensitive to CCI-779 (Neshat *et al.*, 2001). PKB is also constitutively phosphorylated in the $PTEN^{-/-}$ cells but is insensitive to CCI-779. In addition, $PTEN^{-/-}$ cells had enhanced sensitivity to CCI-779 compared to the wildtype $PTEN^{+/+}$ cells. However, when wildtype $PTEN^{+/+}$ cells were transfected with activated PKB and transferred to severe combined immunodeficient (SCID) mice, tumour growth rate increased but tumours were highly sensitive to CCI-779, compared to $PTEN^{+/+}$ cells lacking activated PKB (Neshat *et al.*, 2001). This supports mTOR being downstream of PI3K/PKB as previously demonstrated (Navé *et al.*, 1999; Sekulić *et al.*, 2000) and suggests that deregulation of mTOR signalling may contribute to tumours arising from loss of PTEN and enhanced PI3K and PKB signalling.

1.8.5 Rapamycin therapy

As mTOR is involved in activated PI3K (Aoki *et al.*, 2001) and loss of PTEN mediated oncogenesis (Neshat *et al.*, 2001; Podsypanina *et al.*, 2001), it is an attractive target for chemotherapy because its inhibition can specifically govern translation which is a requirement for cell growth. Moreover, mTOR is specifically inhibited by rapamycin and the high specificity of inhibition makes mTOR a good target for cancer therapy (Davies *et al.*, 2000). Rapamycin was discovered as an antifungal and

immunosuppressant which also had potential as an anti-cancer agent (Abraham and Wiederrecht, 1996; Dilling *et al.*, 1994). Rapamycin is a potent inhibitor in the growth of childhood rhabdomyosarcoma (RMS) cells (IC_{50} ~4000 ng/ml) via inhibition of the insulin-like growth factor-1 (IGF-1) receptor signalling pathway, a requirement for signalling in RMS cells (Dilling *et al.*, 1994; Hosoi *et al.*, 1998). Though rapamycin was effective at inhibiting RMS and some neuroblastoma and glioblastoma cell lines, S6K1 remained inactive in some rapamycin resistant cancer cell lines, suggesting redundancy or inactivation of S6K1 via an mTOR independent pathway (Hosoi *et al.*, 1998). Nevertheless, rapamycin or analogues of rapamycin may provide therapeutic efficacy as anti-cancer agents. Cell cycle inhibitor-779 (CCI-779) by Wyeth-Aycrst (PA, USA) and RAD001 by Novartis AG (Basel, Switzerland) are derivatives of rapamycin with anti-cancer properties and improved pharmaceutical qualities e.g. stability in solution. CCI-779 has demonstrated inhibition of tumour growth *in vivo* (Neshat *et al.*, 2001; Podsypanina *et al.*, 2001) and has been used in pre-clinical trials for chemotherapy of breast cancer (Yu *et al.*, 2001). Currently, both CCI-779 and RAD001 are in phase I-III clinical trials and show efficacy in tumour regression (Hidalgo and Rowinsky, 2000; Houghton and Huang, 2004; Huang and Houghton, 2003). So far, only mild side effects were reported from phase I trials of CCI-779 (Hidalgo and Rowinsky, 2000)

Aim of study

Protein translation involves complex signalling networks which have implicated mTOR both directly and indirectly. Rapamycin has increased our understanding of pathways which involve mTOR and it is now clear that mTOR plays an important role in regulating cellular homeostasis in response to mitogens, energy and nutrient availability. Although mTOR is a convergence point for insulin signalling and amino acid sensing, the mechanisms by which mTOR is regulated are still not well understood.

The current studies were aimed at characterisation of a novel mTOR phosphorylation site and understanding the role of phosphorylation in the regulation of mTOR. Further, we examined the effects of other signal transduction pathways which have been linked to mTOR signalling.

An outline of the studies performed is summarized below:

I. Identification and characterisation of a novel mTOR phosphorylation site and assessment of pathways which lead to mTOR phosphorylation.

II. Investigation of the role of mTOR phosphorylation by mutational analysis and whether mTOR regulation is via phosphorylation of mutually exclusive phosphorylation sites.

III. Investigation of the involvement of TSC in upstream signalling to mTOR

Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 General Reagents

Tissue culture media, phosphate-buffered saline, trypsin, and fetal calf serum, were obtained from Sigma. Phosphoinositides, agarose conjugated protein A and agarose conjugated anti-mouse IgG were purchased from Sigma. [γ-32P]ATP (specific activity: 10mCi/ml) was from NEN. Protein-G agarose was purchased from Santa Cruz. PD98059 was purchased from Cell Signalling Technologies. Rapamycin, puromycin and okadaic acid were obtained from Calbiochem. Activated recombinant ERK1 and CK2 was from Upstate Biotechnology. DNA sequencing was performed and peptides were generated by the Wolfson Institute for Biomedical Research (University College London). All chemicals were from Sigma unless stated otherwise and sources of other reagents or facilities are stated in the text.

2.1.2 Antibodies

Lab generated antibodies included: R27 mTOR antibody raised against the HEAT domain (Withers *et al.*, 1997) and phospho-mTOR –S2448 and corresponding de-phospho-S2448 (Navé *et al.*, 1999). Other antibodies used were as follows: - monoclonal FLAG-M2 and HA-tag antibody was purchased from Sigma. Polyclonal total mTOR antibody, phospho-mTOR -S2442 and -T2446 and corresponding de-phospho-antibodies were generated as described in Methods. αTOR antibody raised against the C-terminal portion of mTOR (a.a. 2433-2450) (Heesom and Denton, 1999) and total 4E-BP1 were kindly provided by Prof. R. Denton (University of Bristol, UK). hTOR, a mouse monoclonal antibody raised against a peptide corresponding to residues

230-240 of human mTOR was purchased from Oncogene (CN Biosciences) and Anti-FRAP polyclonal antibody raised against a peptide corresponding to residues 2524-2538 in rat mTOR that is conserved in the human sequence was from Calbiochem. Phospho-AMPK (T172), total AMPK, phospho-PKB (S473) and phospho-S6K (T389) antibodies were purchased from Cell Signalling Technologies. Total S6K and PKC-ζ antibodies were purchased from Santa Cruz. Phospho-ERK and total ERK was purchased from New England Biolabs (Beverly, MA, USA). Total PKB antibody against the C-terminus of PKB was kindly provided by Dr D. Alessi (University of Dundee). Phospho-4E-BP1 at positions T37, S65 or T70 antibodies (equivalent to rat numbering T36, S64 and T69 respectively) were kindly provided by Dr J. C. Lawrence, Jr. (University of Virginia, USA)

2.1.3 Additional gift reagents

The following reagents were also kindly provided through collaboration:

- Activated recombinant S6K1, Glu-Glu tagged S6K1αII and Glu-Glu tag antibody were provided by Dr Ivan Gout (Ludwig Institute for Cancer Research, University College London, UK).
- Recombinant AMPK, AMPK resin and H2K lysates were provided by Dr David Carling (Cellular Stress Group, MRC Clinical Sciences Centre, Imperial School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK).
- Control and $TSC2^{-/-}$ MEFs were provided by Dr Richard Lamb (Cancer Research UK Centre for Cell and Molecular Biology, The Institute of Cancer Research, 237 Fulham Road, London).
- Min6 extracts were provided by Dr Guy Rutter (Department of Biochemistry, School of Medical Sciences, University of Bristol, BS8 1TD).
- HA tagged human 4E-BP1 in the vector pACTAG-2 was a gift from Dr A.-C. Gingras (McGill University, Canada).

2.2 Methods

2.2.1 Nucleic acid manipulation

2.2.1.1 Polymerase Chain Reaction

PCR amplifications were performed in 50µl reaction volume containing 1× Cloned PfuTurbo DNA polymerase reaction buffer (20mM Tris-HCl, pH 8.8, 2mM MgSO4, 10mM KCl, 10mM (NH4)2 SO4, 0.1% Triton X-100, 1mg/ml nuclease-free BSA), 200µM each dNTP, 10ng template DNA, 20pmol primers and 2.5U Pfu Turbo polymerase (all purchased from Stratagene). Amplifications were performed in a Techne Progene thermo-cycler. Cycling parameters were as follows: A single 2min denaturing step at 95°C followed by 30 cycles of 30s denaturation at 95°C, 30s annealing at 5°C below the lowest primer melting temperature and 1min per kb of the target extension at 72°C. Annealing temperatures were calculated for each primer using the equation:

Tm (°C) = 2(NA+NT) + 4(NG+NC), where N equals the number of primer adenine (A), thymine (T), guanine (G), or cytosine (C) bases.

PCR products were resolved on a 1.5% agarose gel to check for yield and specificity of amplification. The amplified DNA was concentrated by ethanol precipitation and further purified by agarose gel electrophoresis, excision of the DNA band and extraction of DNA using a QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.2.1.2 Ethanol precipitation of DNA

0.1 volumes of 3M sodium acetate (pH 5.2) was added to DNA samples which were then precipitated with 3 volumes of ice cold 100% ethanol, then centrifuged at 14,000*g* for 15min at 4°C. The supernatant was discarded and the DNA pellet was flushed with 200µl ice cold 70% ethanol, then centrifuged at 14,000*g* for 15min at

4°C. The supernatant was discarded and the DNA pellet was air dried then re-suspended in deionised water (ddH_2O) or appropriate buffer.

2.2.1.3 Restriction enzyme digestion of DNA

All restriction enzymes and reaction buffers used were purchased from New England Biolabs (NEB). Restriction digestions were performed in a reaction volume of 20µl containing 0.5µg of plasmid DNA and 10U of enzyme. Reactions were incubated at 37°C for one hour.

2.2.1.4 Electrophoresis of DNA fragments

Agarose (Gibco/BRL) was dissolved in 1× TAE buffer (40mM Tris-acetate, 1mM EDTA) by heating and ethidum bromide at a concentration of 1µg/ml was added when cooled, prior to the gel being cast. DNA fragments of less than 1Kb in size were resolved on 1% (w/v) agarose gels; fragments of greater than 1Kb were resolved on 0.7% (w/v) agarose gels. 6× gel loading buffer (0.25% (w/v) bromophenol blue, 30% glycerol dissolved in water) was added to DNA samples, which were electrophoresed in 1× TAE buffer at 60mA. For the determination of the fragment size, 1Kb DNA ladder (Gibco/ BRL) was electrophoresed simultaneously. DNA bands were visualised by illuminating the gel on a UV light box.

2.2.1.5 Ligation of DNA fragments

Ligations were performed in a 10µl reaction volume containing 1× ligation buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 25µg/ml BSA) and 1µl T4 DNA ligase. 100ng total DNA was used containing a 3-fold molar excess of insert DNA relative to plasmid DNA. Reactions were incubated at 16°C for 16h.

2.2.1.6 Transformation of competent *Escherichia coli* cells

Competent cells were purchased from Stratagene: XL1-Blue competent cells were used for plasmid DNA and XL10-Gold ultra-competent cells for ligation products. Transformation of cells was achieved by mixing either 100ng plasmid DNA or 2µl of ligation reaction with 100µl of freshly thawed competent cells and heat-pulsing at 42°C for 45s, followed by 2min incubation on ice. Cells were allowed to recover by adding 0.9ml of NZY^+ broth (per litre of LB: 10g tryptone, 5g yeast extract, 10g NaCl made up to 1l with ddH_2O, pH 7.5 and autoclaved. For NZY^+ broth, LB was supplemented with a final concentration of 125mM $MgCl_2$, 125mM $MgSO_4$ and 20mM sterile glucose). Cells were shaken for 1h at 37°C at 220rpm then spread on agar plates containing 100µg/ml ampicillin. Per litre LB: 20g agar was added, pH 7, and autoclaved. Filter-sterilised ampicillin was added to cooled LB-agar at a final concentration of 100µg/ml, prior to pouring into plates; plates were incubated overnight at 37°C.

2.2.2 Propagation and purification of plasmid DNA

2.2.2.1 Miniprep plasmid purification

This procedure was used predominantly to screen bacterial colonies for recombinant DNA plasmids. 5ml LB supplemented with ampicillin (100µg/ml), inoculated with a single colony isolated from a freshly streaked ampicillin plate, was incubated with shaking at 37°C overnight and cells harvested by centrifugation at 4000*g* for 5min. The cell pellet was further processed using a QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.2.2.2 Maxiprep plasmid purification

This method was applied to produce plasmid DNA which was predominantly used for transfections. A 5ml starter culture was inoculated with a single colony isolated from a freshly streaked agar plate (containing 100µg/ml ampicillin) and incubated with shaking for 8h at 37 °C. The starter culture was used to inoculate a 200ml

culture and incubated overnight at 37°C with shaking. Cells were harvested by centrifugation at 10,000*g* for 15min at 4°C. Pelleted cells were further processed using a QIAfilter TM Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. Purified plasmid DNA was suitable for downstream applications, such as DNA sequencing, *in vitro* transcription/translation or transfection experiments.

2.2.2.3 Determination of DNA concentration

DNA concentration was determined spectrophotometrically by measuring OD at 260nm. Concentration was then calculated from the equation:

$A_{OD260} \times 50 \times$dilution factor = concentration of double stranded DNA (μg/ml)

2.2.3 Plasmid Constructs

2.2.3.1 Generation of point mutations by site-directed mutagenesis

Point mutations were generated by site directed mutagenesis using the QuikChange™ Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primers containing the desired mutation were used in a PCR reaction to generate nicked circular plasmid DNA bearing the desired mutation. PCR reactions were performed as described in Section 2.2.1.1, using a range of plasmid concentrations (5, 10, 20, 50ng of plasmid DNA template). Following amplification, the parental methylated DNA was digested with 10U of Dpn I for 1h at 37°C. After digestion with Dpn I, 1μl of the PCR reaction was used to transform Epicurian Coli® XL1-Blue supercompetent cells (Stratagene). Transformed cells were then selected on agar plates containing 100μg/ml ampicillin. Plasmid DNA was purified from colonies (Section 2.2.2.1) and sequenced.

2.2.3.2 Construction of rapamycin resistant mTOR mutants

Wildtype mTOR in N-terminal FLAG-tagged PBJ5.1 (Peterson *et al.*, 2000) was first digested with SacI which cleaves within the coding sequence of mTOR. This

removed a 3.8Kb fragment to create a smaller wildtype mTOR plasmid termed ΔmTOR (~8Kb) to improve efficiency of mutagenesis. A series of point mutations were generated within the ΔmTOR construct to convert each of the following sites: S2442; T2446 and S2448 into either alanine or glutamic acid. The aim was to reconstitute the point mutations into a rapamycin resistant (S2035T) sequence rather than the wild-type sequence. Each ΔmTOR construct containing a point mutated sequence was digested with NotI and BspEI. Full length mTOR containing the rapamycin resistant S2035T mutation (termed M1) was also digested with NotI and BspEI and the fragment containing the rapamycin resistance mutation was ligated into ΔTOR in place of the wild-type sequence (Fig. 2.1). The accuracy of the mutations was verified by DNA sequencing.

2.2.3.3 Construction of GST-mTOR-HEAT chimera

The region between amino acids 668 and 939 of the mTOR sequence corresponding to the HEAT domain was amplified by PCR to incorporate a 5' EcoRI site and a 3' XhoI restriction site. The HEAT-mTOR fragment and the pGEX4T-1 vector (Pharmacia) were digested with EcoRI and XhoI, then separated and purified from a 1% agarose gel. The HEAT-mTOR fragment was ligated into pGEX4T-1 and the accuracy of construction was verified by DNA sequencing.

2.2.3.4 Construction of His-tag α4 chimera

α4 construct in pUC18 (Inui *et al.*, 1995) was cloned into a modified pTri-ex vector (Novagen), cloning out the α4 STOP codon to incorporate the pTri-ex HSV and His tag at the C-terminus. Positive clones were selected and sequenced; a correct clone was selected to transform XL-1 blue bacterial cells in an attempt to produce recombinant α4.

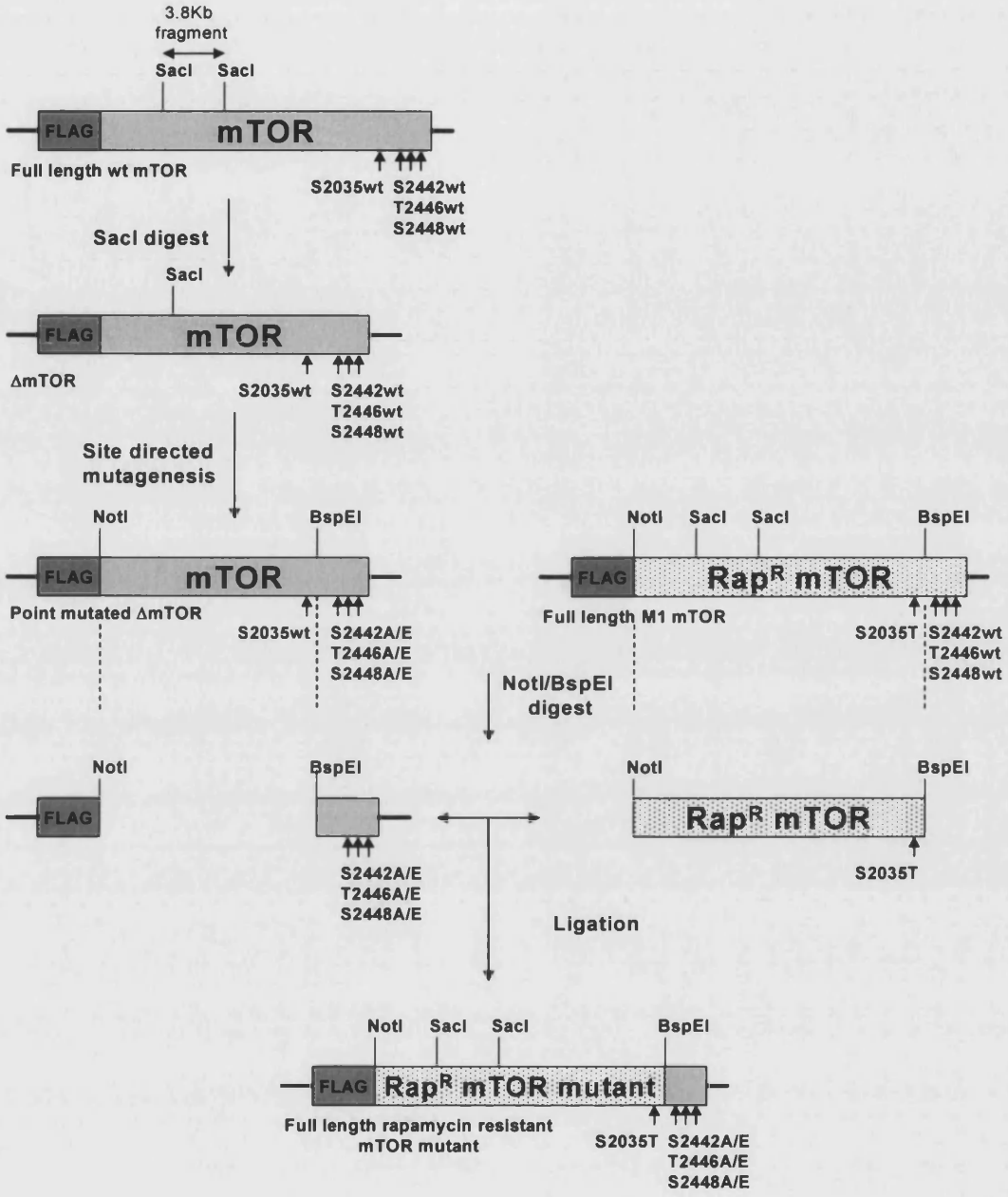

Figure 2.1 Schematic representation of the construction of mTOR rapamycin resistant mutants

Wildtype FLAG-tagged mTOR in pBJ5.1 was mutated at sites S2442, T2446 and S2448 into A or E, then reconstituted to full length with the rapamycin resistant mutant as detailed in the figure above and described in the text. 'Rap^R' denotes rapamycin resistance and 'wt' denotes wild-type.

2.2.4 Cell Culture

All procedures were carried out in a laminar flow hood using aseptic technique. All surfaces were disinfected with 70% ethanol before and after any manipulation. Medium for mammalian cell culture was pre-warmed to 37°C before use.

2.2.4.1 Cryo-preservation of cells

80% confluent cell monolayers were trypsinised and 10ml of culture medium containing 10% foetal calf serum (FCS) was added. The cell suspensions were transferred to a sterile 15ml tube and spun at 150*g* for 5min at 4°C. The supernatant was aspirated and the cell pellet re-suspended in ice-cold freezing medium (culture medium containing 20% FCS and 10% DMSO). 1ml aliquots of the suspension were transferred to sterile cryo-vials, which were frozen slowly in an insulated polystyrene box at -80°C overnight. The following day, cells were transferred to liquid nitrogen for long-term storage.

2.2.4.1.1 To revive frozen cells

Aliquots were removed from storage and thawed quickly in a 37°C water bath before transfer to a tissue culture flask containing pre-warmed culture medium (containing 10% FCS). Following overnight incubation in a 37°C humidified incubator with 5% CO_2, the medium was changed to remove traces of the cryo-preservation medium.

2.2.4.2 Mammalian cell culture

2.2.4.2.1 Maintenance of HEK 293, MEFs and CHO-IR cells

Human embryonic kidney (HEK 293) and control mouse embryonic fibroblasts (MEFs) [$p53^{+/+}$ $TSC2^{+/+}$] and $TSC2^{-/-}$ cells [$p53^{-/-}$ $TSC2^{-/-}$] were cultured in Dulbecco's Modified Eagle's Essential Medium (DMEM) and chinese hamster ovary cells stably expressing the human insulin receptor (CHO-IR) cells were cultured in nutrient medium F12 (Ham). Medium was supplemented with 10% fetal calf serum

and 1% antibiotic-antimycotic solution (100×: 10,000U/ml penicillin, 10mg/ml streptomycin sulfate, 25μg/ml amphotericin B); this will be referred to as complete media. Cells were grown at 37°C in a humidified incubator with 5% CO_2. For further propagation, cells were trypsynised when 80-90% confluent; culture medium was removed and the monolayer briefly rinsed with phosphate buffered saline (PBS) (without calcium or magnesium). Trypsin/EDTA solution was added to just cover the monolayer then incubated at 37°C for 2min. The culture vessel was tapped gently to dislodge cells, which were then re-suspended in medium and plated at a density of approximately 60% of confluence.

2.2.4.2.2 Calcium Phosphate transfection

HEK 293 cells were transfected using the calcium phosphate precipitation method (Invitrogen). Briefly, the medium was changed 1h prior to transfection to remove traces of trypsin. For co-transfections: 5μg of mTOR and 3μg of S6K1 or 5μg of mTOR and 5μg 4E-BP1 plasmid DNA were co-transfected per 6cm tissue culture dish; or 10μg mTOR plasmid DNA was transfected per 10cm tissue culture dish as indicated. 500μl 2X HBS (280mM NaCl, 50mM HEPES, 750mM NaH_2PO_4, 750mM Na_2HPO_4, pH 7.0) was added to a solution containing plasmid DNA and 200mM $CaCl_2$, in 500μl ddH_20. The mix was left to stand for 30min at room temperature to form the precipitate. The precipitate was added drop-wise to the cell monolayer. Following an 18h incubation in a 37°C incubator maintained with 5% CO_2, the cells were washed twice with PBS (without calcium or magnesium) and fresh complete medium was added. Protein expression of the transfected DNA was found to be optimal if cells were allowed to recover in this medium for 24h.

2.2.4.2.3 LipofectAmine transfection

CHO-IR cells were transfected using LipofectAMINE (Invitrogen). Briefly, 90% confluent CHO-IR cells were trypsinised and re-plated at a dilution of 1:10 in 6cm plates and grown overnight. Cell monolayers were rinsed the following day with PBS and 2ml fresh F12 medium (without FCS) was added. For co-transfections, 5μg of mTOR and 3μg of S6K1, or 5μg of mTOR and 5μg 4E-BP1plasmid DNA (per

6cm tissue culture dish) and 5-10µl LipofectAMINE reagent were each diluted in 250µl F12 medium (without FCS). The media containing DNA and LipofectAMINE were mixed together (500µl total) and following a 30min incubation at room temperature, the mix was added dropwise to the monolayer. Cells were incubated with transfection mixture for 5 hours before the addition of 2.5ml F12 containing 20% FCS and 1% antibiotic-antimycotic solution (final 10% serum including transfection mix). Cells were incubated overnight in a 37°C incubator maintained with 5% CO_2, then washed twice with PBS (without calcium or magnesium) and complete medium was added as required. Protein expression from the transfected plasmids was found to be optimal 36h post-transfection, as determined by transfection with green fluorescent protein (GFP).

2.2.5 Large scale protein purification

2.2.5.1 Ribosomal purification

Six littermate rats, each weighted at 200-220g, were obtained and maintained at UCL Animal House. These were starved for 24 hours with water only, then anaesthetized by subcutaneous injection with Pentobarbitol 35mg/kg, prior to liver extraction. The livers were extracted and washed several times in ice-cold buffer A (20mM Tris-HCl, 100mM KCl, 5mM $MgCl_2$ 1mM DTT, 200mM sucrose with a cocktail of protease inhibitors), weighed, then homogenised with a Teflon homogeniser in 2× weight volumes of buffer A. The homogenate was centrifuged at 10,000*g* for 30min at 4°C in a Sorvall centrifuge. The supernatent was centrifuged at 10,000*g* for 15min at 4°C then filtered through a nylon membrane before application to a sucrose gradient.

Six Ti45 tubes were used to prepare discontinuous sucrose gradients comprising 10ml buffer B (buffer A with 1.6M sucrose and 0.5M KCl), overlaid with 10ml buffer C (buffer A with 0.7M sucrose). These gradients were topped with the filtered liver extracts and balanced weight for weight to within 0.01g for ultracentrifugation at 38,000*g* overnight at 4°C in a Beckman ultracentrifuge with a Ti45 rotor.

Supernatents were aspirated and the pellets re-suspended in a total of 5ml buffer D (buffer A with 0.5M KCl) then incubated at 37°C for 30min, after which 0.65 volumes of ethanol and a final concentration of 10mM $MgCl_2$ was added. This was centrifuged at 15,000*g* for 2min at room temperature. The buffer D wash and centrifugation step was repeated and the pellet re-suspended in buffer E (buffer A with 0.7M KCl and 2mM puromycin) then incubated at 37°C for 30min, after which 0.65 volumes of ethanol was added and centrifuged at 13,000*g* for 2min at room temperature. The pellet was re-suspended in buffer E without puromycin then incubated at 37°C for 30min, after which 0.65 volumes of ethanol was added and centrifuged at 13,000*g* for 2min at room temperature. The pellet was then re-suspended in storage buffer (20mM MOPS, 1mM EGTA, 1mM DTT) and the protein concentration measured.

2.2.6 Immunological Methods

2.2.6.1 Generation of mTOR HEAT domain antibodies, phospho-specific S2442 and phospho-specific T2446 antibodies

Polyclonal antibody against the HEAT domain of mTOR (as described in Withers *et al*, 1997) was generated by immunising rabbits with a chimeric protein, corresponding to the region between amino acids 668 and 939 of the mTOR sequence fused to glutathione-S-transferase (GST). Phospho-mTOR S2442 was raised in rabbit using the phospho-peptide CGNKRS*(P)RTRT, corresponding to amino acids G2438-T2446 and phospho-mTOR T2446 was raised in rabbit using the phospho-peptide CSRTRT*(P)DSYS, corresponding to amino acids S2442-S2450 of the human mTOR sequence, with *(P) representing the phosphorylated residue. Peptides were conjugated to keyhole limpet hemocyanin (KLH) using m-maleimidobenzoyl-N-hydroxysuccinimide ester according to protocols described elsewhere (Harlow and Lane, 1988). All immunisations were carried out by Eurogentec (Belgium).

Total immunoglobulins from the HEAT mTOR antiserum were purified by passing through an affinity column made by coupling the GST-mTOR-HEAT chimera to

Actigel (Sterogene). The flow-through was re-run through the column, and then the column was washed twice with PBS (0.5M NaCl) and once with PBS.

Affinity columns for the purification of the phospho-S2442 and phospho-T2446 antiserum were made by coupling the relative peptides to SulfoLink resin (Pierce) according to the manufacturer's instructions. The antiserum was purified by affinity chromatography, first through a column of the corresponding dephospho-peptide to remove clones reacting with non-phosphorylated -S2442 or –T2446. The flow-through was further purified by passing through a second column made of the phospho-peptide coupled to SulfoLink. Bound immunoglobulins from both the dephospho-peptide (termed dephospho–S2442 or dephospho–T2446 antibodies) and phospho-peptide (termed phospho–S2442 or phospho–T2446 antibodies) columns were eluted.

In all cases, bound immunoglobulins were eluted with 100mM glycine pH 2.5, 150mM NaCl. The eluate was immediately neutralised by addition of 1M Tris pH 8.0 then concentrated using spin filters and dialysed against PBS. Polyethyleneglycol was added at 50% final concentration to the purified antibodies and the final preparation was aliquoted and stored at –20°C. The purified antibody preparation was characterised by immuno-blotting lysates of cell extracts.

2.2.6.2 Expression and purification of GST fusion proteins

pGEX4T-1 plasmid containing the region between amino acids 668 and 939 of the mTOR sequence was constructed as described above. The construct was used to transform competent *E.coli* XL1-Blue cells. After selection on ampicillin-containing agar plates, a single colony was used to inoculate a 5ml starter culture in LB medium containing 100μg/ml ampicillin. This was grown overnight with shaking at 37°C and the next day used to inoculate a 500ml culture in LB medium supplemented with 100μg/ml ampicillin. The culture was grown at 37°C with shaking until it reached an A_{OD600} of 0.6. Expression of the plasmid was induced by addition of IPTG to a final concentration of 0.2mM, followed by incubation overnight at 20°C with shaking. Bacterial cells were pelleted at 10,000*g*, 4°C, for 10 minutes. Pelleted cells were

resuspended in 10 volumes (v/w cell pellet) ice-cold lysis buffer (50mM Tris-HCl pH 7.5, 1% Triton X-100 and 1mM PMSF). The suspension was sonicated on ice by applying five rounds of 10s pulses, with 10s intervals between pulses. The lysate was centrifuged at 10,000*g* for 15 minutes to remove cell debris. Protein was isolated from the lysate by rotating with Glutathione-Sepharose 4B (Pharmacia) in a 15ml Falcon tube at 4°C. Sepharose beads with bound GST fusion proteins were washed three times in lysis buffer, followed by three washes with 50mM Tris-HCl pH 8.0 and then bound GST fusion proteins were eluted by incubating with 10mM reduced glutathione. The eluate was concentrated using Vivaspin-20 filters (Sartorius), dialysed three times against PBS, aliquoted then snap-frozen in liquid nitrogen and stored at -80°C.

2.2.6.3 Protein Assay

Determination of protein concentration was performed using the BCA method (Pierce) according to the manufacturer's instructions. BSA was used as a standard.

2.2.6.4 Immunoprecipitation

Cell monolayers were washed once with ice cold D-PBS and lysed at 4°C in lysis buffer A [50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20mM NaF, 20mM β-glycerophosphate, 1mM EDTA, 6mM EGTA, 1% (v/v) NP40, 1mM DTT, 5mM benzamidine, 1mM PMSF, 0.25mM Na_3VO_4, 2μg/ml aprotinin, 1μg/ml pepstatin, 1μg/ml leupeptin]. 0.5ml of lysis buffer was used per 10cm dish, 0.3ml of lysis buffer was used per 6cm dish. Lysates were clarified from insoluble material by centrifugation at 14,000*g* for 10min at 4°C. Immunoprecipitations were performed from the detergent-soluble fraction using the indicated antibodies at the appropriate dilution. Lysates were incubated with the antibody for 2h at 4°C under rotation followed by the addition of protein A- or protein G-agarose beads (30μl of 1:2 slurry), then incubated for a further 45min under rotation. Immune complexes were washed three times with "high salt" buffer, followed by washes with kinase assay buffer and further processed as described for each assay.

2.2.6.5 Tris-glycine-SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970). Vertical slab gels were prepared using either a Hoeffer gel apparatus or BioRad mini gel apparatus. Samples were denatured by addition of electrophoresis sample buffer (4x concentrated: 50% glycerol, 10% SDS, 500mM DTT, 500mM Tris-HCl pH 6.8) and heating at 95°C for 5min. Electrophoresis was performed under constant voltage of 200V for 3 hours in the Hoeffer gel system or 150V for 90min for the BioRad apparatus. Gels were then either processed for Western blotting, stained with Coomassie blue or fixed, dried and exposed to autoradiographic film.

2.2.6.6 Tris-tricine-SDS-polyacrylamide gel electrophoresis

This system is suitable for resolving polypeptides in the range from 5 to 20 kDa. This is achieved by use of tricine rather than glycine in the electrophoresis buffer, and lowering the pH of the gel. Here, it was used for the electrophoretic analysis of low molecular weight proteins or peptides. Gels were prepared according to the method of Schagger and von Jagow (Schagger and Jagow, 1987). The BioRad mini gel apparatus was used. 15% resolving gels were made up containing 12% glycerol, 15% acrylamide-0.5% bisacrylamide, 0.3% SDS, 0.1% ammonium persulphate, 0.2% TEMED in 0.125M Tris-HCl pH 8.45. Stacking gels were made to a final concentration of 4% acrylamide in 0.1M Tris-HCl pH 8.45, 0.1% ammonium persulphate, 0.05% TEMED. The anode buffer consisted of 200mM Tris-HCl pH 8.9 and the cathode buffer of 100mM Tris, 100mM Tricine and 0.1% SDS. Electrophoresis was performed under constant voltage of 80V. Following electrophoresis the gels were processed for western blotting, stained with Coomassie or fixed, dried and exposed to a phosphorimager screen.

2.2.6.7 Coomassie staining of acrylamide gels

Following electrophoresis, the gel was submerged in a solution of 40% (v/v) methanol, 10% (v/v) acetic acid, 0.3% (w/v) Coomassie brilliant blue R25O and

incubated under constant agitation for 1h at room temperature. Protein bands were visualised by washing the gel in destaining solution (20% (v/v) methanol, 10% (v/v) glacial acetic acid). Several changes of the destaining solution were required to completely destain the background.

2.2.6.8 Autoradiographic exposure of acrylamide gels

Following electrophoresis, the gel was submerged in fixing solution (20% (v/v) ethanol, 10% (v/v) glacial acetic acid) and incubated under constant agitation for 1h at room temperature in order to fix proteins into the gel. The gel was dried under vacuum then exposed to a phosphorimager screen. Images of radiolabelled protein bands were acquired using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software.

2.2.6.9 Western blotting and immunodetection of transferred proteins

Proteins separated by electrophoresis, as described in Section 2.2.6.5 and 2.2.6.6, were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore) using a Bio-Rad Trans-Blot Cell. The membrane was soaked in methanol briefly and then rinsed in water for 2min. Both gel and membrane were equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) for 5min. The transfer stack was assembled immersed in transfer buffer. To assemble the transfer stack a foam pad was placed on one side of the transfer cassette and one sheet of 3MM filter paper was placed on top. The gel was then placed on top of the filter paper and the membrane on top of the gel. One sheet of 3MM filter paper was then placed on top of the membrane and a foam pad was put on top of the filter paper to complete the stack. The transfer cassette was closed and placed in the transfer tank containing transfer buffer such that the gel faced the cathode. The transfer was run at 20V overnight for large gels or 100V for 1hour for small gels. Following transfer to PVDF membranes, the membrane was first incubated in blocking buffer (5% (w/v) dried skimmed milk in 0.1% (v/v) Tween 20 in phosphate buffered saline (PBS-T)) for 1 hour at room temperature to saturate the binding sites on the membrane. For

immunoblotting using phosphospecific antibodies, bovine serum albumin (BSA) and Tris-buffered saline (TBS) substituted the use of skimmed milk and PBS, respectively, throughout the procedure.

The primary antibody was then added at the appropriate dilution in 20ml of 1% (w/v) dried milk powder in TBS-T and incubated with the membrane on a rocking platform at room temperature for 1h or at 4°C overnight. The membrane was then washed with TBS-T (three times for 5min). The secondary antibody, conjugated to horseradish peroxidase (HRP), was diluted at 1:2000 in 20ml of 1% milk in PBS-T and incubated with the membrane for 45min at room temperature. The membrane was then washed in TBS-T (three times for 5min). Antibody-antigen complexes were detected by Enhanced Chemiluminescence (KPL) according to the manufacturer's instructions. Images were captured by a Fuji LAS-1000 Luminescent Image Analyser and analysed with Fuji Image Gauge software.

2.2.7 Kinase assays

2.2.7.1 Peptide assays

Confluent CHO-IR cells were serum-starved overnight with F12 (Ham) containing 1% antibiotic-antimycotic solution. Cells were stimulated with 100nM insulin for 10min then lysed in PKB lysis buffer [50mM HEPES, 0.2mM EDTA, 2.2mM EGTA, 100mM KCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1mM DTT, 10μg/ml aprotinin, 10μg/ml leupeptin, 20nM okadaic acid]. Following lysate clarification, supernatants were pooled and equal volumes were immuno-precipitated with anti-C-terminal PKB (provided by Dr D. Alessi, Protein Phosphorylation Unit, University of Dundee) for 2h. Immuno-complexes were then incubated with Protein G-agarose (Santa Cruz) for 45min. Cells stimulated with 1μM PMA for 10 min were lysed as described above then immunoprecipitated with anti-PKCζ (Santa Cruz) with protein A-agarose. Immunoprecipitates were washed and assayed as described in (Moule *et al.*, 1997). The reactions with recombinant enzyme (AMPK, S6K1) and immunoprecipitates were incubated with 1mM peptide (peptides were generated by the Wolfson Institute for Biomedical Research (UCL), see Table 3.1), and reactions

were initiated with: 1μM PKI, 20 mM $MgCl_2$, 100 μM unlabelled ATP, 5μCi/reaction [γ-^{32}P] ATP in assay buffer [20mM MOPS (pH 7.0), 1mM EDTA, 1mM EGTA, 0.01% Triton X-100, 5% (v/v) glycerol] (AMPK samples also contained 1mM AMP) in a final reaction volume of 30μl. Reactions were incubated at 30°C for 15min with agitation then stopped by spotting all the reaction mixture onto p81 grade phospho-cellulose paper (Whatman), and washed five times in 75mM ortho-phosphoric acid. Papers were dried and transferred to scintillation vials for liquid scintillation counting (Beckman).

2.2.7.2 PI 3-kinase lipid kinase assays

Cell extracts were lysed and processed as described in Section 2.2.6.4. p85, Py-99 or IRS-1 were used for immunoprecipitations then assayed for PI 3-kinase lipid kinase activity. Reactions were performed in a final volume of 50μl in a buffer containing 50mM HEPES pH 7.4, 100mM NaCl, 1mM DTT, 5mM $MgCl_2$, 100μM ATP (plus 0.1μCi of [γ-^{32}P] ATP/assay) using 200μg/ml phosphatidylinositol or phosphatidylinositol-4,5-bisphosphate as a substrate. Reactions were initiated by adding a mix of the ATP and Mg^{2+} and incubated at 25°C for 20min. Reactions were terminated by the addition of 100μl 0.1M HCl. 200μl of a 1:1 (v/v) mixture of chloroform and methanol was added. The mixture was vortexed and the phases separated by centrifugation at 10,000*g* for 2min. The aqueous phase was discarded and the lower organic phase was washed with 80μl of a 1:1 (v/v) mixture of methanol and 1M HCl. After centrifugation at 10,000*g* for 2min, the aqueous phase was discarded and the lower organic phase was vacuum dried. The dried lipids were re-suspended in 30μl of chloroform: methanol (4:1) and spotted on to thin layer Silica Gel-60 plates (Merck), pre-treated with 1% oxalic acid, 1mM EDTA in water: methanol (6:4). TLC plates were developed in chloroform: methanol: 4M ammonia (9:7:4) for analysis of phosphatidylinositol-3-phosphate; or n-propanol: 2M acetic acid (13: 7) for analysis of phosphatidylinositol-3,4,5-trisphosphate. After completion of the separation, the plates were dried and exposed to phosphorimager film. Images of radio-labelled lipid products were acquired using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software.

2.2.7.3 mTOR autokinase assay

Cells were lysed in lysis buffer containing 10mM Tris-HCl pH 7.6, 5mM EDTA, 50mM NaCl, 50mM NaF, 30mM sodium pyrophosphate, 1% NP40, 100μM sodium vanadate, 1mM PMSF, 10μg/ml aprotinin and 10μg/ml leupeptin. Lysates were immunoprecipitated with mTOR antibody as specified then washed four times with lysis buffer, once with wash buffer [0.5M LiCl, 50mM Tris pH 7.6 and 1mM DTT] and then once with reaction buffer [25mM HEPES pH 7.7, 50mM KCl, 10mM $MgCl_2$, 0.1% NP40, 20% glycerol, 1mM DTT]. Reactions were initiated by the addition of 100μM ATP plus 0.3μCi/reaction [γ-^{32}P] ATP in assay buffer in a final reaction volume of 30μl. Reactions were incubated at 30°C for 30min with agitation and stopped with 10μl 4× sample buffer then separated by 7.5% SDS-PAGE. The gel was fixed, dried and exposed to phosphorimager film. Images were acquired using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software.

2.2.7.4 S6K1 assay

Cells were lysed as described in Section 2.2.6.4. 50% of the lysate was used to immunoprecipitate transfected S6K1 with Glu-Glu antibody. The immunoprecipitates were washed twice in lysis buffer A (Section 2.2.6.4) containing 0.5M NaCl and three times with 20mM MOPS (pH 7.4), 10mM β-glycerophosphate, 1mM DTT and 1mM PMSF. 20μg of 80S ribosomes were added to each immunoprecipitate and mixed gently. Reactions were initiated by the addition of 2.5μM PKI, 50mM $MgCl_2$, 10μM ATP plus 0.3μCi/reaction [γ-^{32}P] ATP in assay buffer [50mM MOPS (pH 7.4), 2mM EGTA, 10mM β-glycerophosphate and 1mM DTT] in a final reaction volume of 30μl. Reactions were incubated at 30°C for 30min with agitation then stopped by addition of 4× sample buffer; samples were then boiled at 95°C for 5min and proteins were separated by 10% SDS-PAGE. The gel was cut at the 46kDa marker, the upper half containing 70kDa S6K1 was transferred to PVDF membrane to blot total S6K and the lower half was fixed, dried and exposed to a phosphorimager screen. Images of radiolabelled protein bands

were acquired using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software. Activity was normalised to total S6K determined by immuno-blot.

2.2.7.5 4E-BP1 assay

Two methods were used for comparison (Brunn *et al.*, 1997a; Mothe-Satney *et al.*, 2000a).

The first method was as described in Brunn *et al.*, (1997a). Briefly, cells were lysed in lysis buffer containing 50mM β-glycerophosphate, 1.5mM EGTA, 1% Triton X-100, 1mM DTT, 20nM okadaic acid, 0.2mM PMSF, 5μg/ml aprotinin, 5μg/ml pepstatin and 10μg/ml leupeptin. mTOR was immunoprecipitated then washed twice with TGN buffer [50mM Tris-HCl, 50mM β-glycerophosphate, 100mM NaCl, 10% glycerol, 20nM okadaic acid, 5μg/ml aprotinin, 5μg/ml pepstatin and 10μg/ml leupeptin, 600μM PMSF], once with high salt buffer [100mM Tris-HCl pH 7.4, 500mM LiCl] then twice with kinase buffer [10mM HEPES pH 7.4, 50mM β-glycerophosphate, 50mM NaCl, 20nM okadaic acid, 5μg/ml aprotinin, 5μg/ml pepstatin and 10μg/ml leupeptin, 600μM PMSF]. The reaction was started with kinase assay buffer containing 10mM $MnCl_2$, 200μM ATP plus 0.3μCi/reaction [γ-^{32}P] ATP and 0.5μg recombinant 4E-BP1 (purchased from Upstate Biotechnology) in a final reaction volume of 30μl. The reaction was incubated at 30°C for 30min with agitation and stopped with 10μl 4× sample buffer, then separated by 12% tris/tricine SDS-PAGE. The gel was fixed, dried and exposed to a phosphorimager screen.

The second method was as described in Mothe-Satney *et al.*, (2000a). Briefly, cell monolayers were rinsed twice in buffer 1 [150mM NaCl, 50mM Tris-HCl pH 7.4], then lysed in lysis buffer 2 [50mM NaF, 1mM EDTA, 1mM EGTA, 0.5% Triton X-100, 10mM potassium phosphate , 50mM β-glycerophosphate, 2.5mM $MgCl_2$, 1mM DTT, 10nM okadaic acid, 0.5mM PMSF, 10μg/ml aprotinin, 10μg/ml pepstatin]. mTOR was immunoprecipitated then washed twice with buffer 2, twice with buffer 2 [containing 0.5M NaCl], twice with buffer 3 [1mM EDTA, 1mM EGTA, 50mM Tris-HCl pH 7.4] and twice with buffer 4 [50mM NaCl, 0.1mM EGTA, 1mM DTT, 20nM okadaic acid, 10mM HEPES, 50mM β-glycerophosphate]. The reaction was

started with kinase assay buffer containing 10mM $MnCl_2$, 100μM ATP plus 0.6μCi/reaction [γ-^{32}P] ATP and 0.5μg recombinant 4E-BP1 (purchased from Upstate Biotechnology) in a final reaction volume of 30μl. The reaction was incubated at 30°C for 30min with agitation then stopped with 10μl 4× sample buffer and separated by 12% tris/tricine SDS-PAGE. The gel was fixed, dried and exposed to a phosphorimager screen.

Chapter 3: Results

3 Identification and characterisation of a novel mTOR phosphorylation site

3.1 Summary

A key regulator of translation is the mammalian target of rapamycin (mTOR) which is dually regulated by growth factors and nutrient availability. A previous study from this lab, subsequently confirmed by others, identified S2448 as a nutrient regulated phosphorylation site located at the C-terminus of the mTOR catalytic domain. Studies have shown that insulin stimulated phosphorylation of S2448 via PKB and phosphorylation was abrogated by nutrient deprivation. Here, a novel nutrient responsive mTOR phosphorylation site was identified at T2446. In contrast to S2448 phosphorylation; T2446 is de-phosphorylated when CHO-IR cells are insulin stimulated and phosphorylated when cells are nutrient deprived. Similarly, the AMP-activated kinase (AMPK) is activated under conditions of nutrient deprivation and was hypothesised to be involved in the regulation of T2446 phosphorylation. Subsequent studies in cultured murine myotubes treated with 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or di-nitrophenol (DNP) showed activation of AMPK and a concomitant increase in mTOR T2446 phosphorylation, paralleled by a decrease in S6K1 phosphorylation. *In vitro* kinase assays were employed to assess which kinases were capable of phosphorylating mTOR peptides based on the sequence of amino acids 2440-2551, this incorporating both the T2446 and S2448 phosphorylation sites. Data showed that both PKB and AMPK are able to differentially phosphorylate sites in this region, PKB phosphorylation of the phospho-mimic T2446E peptide was restricted. Conversely, AMPK mediated phosphorylation was reduced with the phospho-S2448 peptide. Interestingly PKCζ mediated phosphorylation was completely inhibited with the S2448A and phospho-S2448 peptide. These data suggest that T2446 and S2448 phosphorylation is mutually exclusive and their differential phosphorylation may be

a mechanism to integrate signals from growth factors and nutrient status to control the regulation of protein translation.

3.2 Introduction

Though the domains of mTOR have been defined and interacting proteins, such as Raptor, have been identified it remains unclear exactly how these factors interact and how mTOR itself is regulated. A region of mTOR which is of regulatory interest is the catalytic domain at the C-terminus. The antibody mTAb1 is directed to the C-terminus of mTOR (residues 2433-2450) (Brunn *et al.*, 1997b) and can bind to and activate mTOR protein kinase activity towards 4E-BP1; additionally deletion of the C-terminus residues 2430-2450 significantly increases the basal protein activity (Sekulić *et al.*, 2000). PKB was found to directly phosphorylate the C-terminus of mTOR suggesting regulatory importance (Burgering and Coffer, 1995; Gingras *et al.*, 1998; Scott *et al.*, 1998). Navé *et al.*, (1999) and subsequently others (Bolster *et al.*, 2002; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000) developed a phospho-specific antibody that identified S2448 as a phosphorylation site in mTOR. Phosphorylation at S2448 was mediated by PKB and confirmed to be wortmannin-sensitive but rapamycin-insensitive (Navé *et al.*, 1999). Phosphorylation of S2448 increased with insulin stimulation and was directly related to amino acid availability and regulated by amino acid status (Navé *et al.*, 1999; Reynolds *et al.*, 2002). Evidence also shows that mTOR can act independently of amino acid availability by responding as a sensor of cellular ATP levels (Dennis *et al.*, 2001). More recently it has been suggested that the nutrient effect on mTOR might be via AMPK which is a well established energy sensing/signalling protein that is responsive to the ATP/AMP ratio of the cell (Hardie *et al.*, 1998). Increasing cellular AMP activates AMPK allosterically by phosphorylation of the α-catalytic subunit (T172) by AMPK-kinase (AMPKK) which leads to preservation of ATP levels by increasing glucose uptake and fatty acid oxidation (Hardie *et al.*, 1998). It has been shown that the AMPK activator AICAR significantly decreased skeletal muscle protein synthesis via 4E-BP1 and S6K1: both downstream targets of mTOR. These events were accompanied by down-regulation of PKB activity and subsequently S2448 phosphorylation (Bolster *et al.*, 2002). mTOR S2448 phosphorylation is hypothesised to be a positive

regulatory site since phosphorylation is mediated by mitogenic stimuli (Bolster *et al.*, 2002; Navé *et al.*, 1999; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000) which results in increased protein synthesis. Since activation of AMPK results in reduced signalling through mTOR and its downstream targets (Bolster *et al.*, 2002; Kimura *et al.*, 2003), S2448 phosphorylation might be important for controlling the activity and function of mTOR and the energy sensing protein AMPK may play a role in this process.

The attenuation of S2448 phosphorylation by nutrient deprivation (Navé *et al.*, 1999) raised the possibility that this may be due to phosphorylation of an adjacent site which prevented insulin stimulated phosphorylation via PKB. We hypothesised that the vicinity of S2448 had potential to include other regulatory phosphorylation events since the catalytic domain of mTOR which contains S2448 is 100% conserved in all mammalian sequences for mTOR. Scansite analysis (Obenauer *et al.*, 2003) showed that this region contained a number of serine (S) and threonine (T) residues which may be phosphorylation sites. The most evident site of interest was T2446 which not only overlapped the S2448 site but had a loose consensus to the PKB phosphorylation motif R-X-R-Y-Z-S/T-hyd; where X is any small amino acid, Y and Z are any amino acid and 'hyd' is a hydrophobic amino acid (Alessi *et al.*, 1996). Another proximal site of interest was S2442 which is in a SXXR motif that is recognised by human interferon induced double stranded RNA activated protein kinase (PKR), a member of a family of structurally related ser/thr kinases that have effects on protein translation via S51 phosphorylation of eIF2α. PKR is a negative regulator of cell growth, activated by human interferon and viral double-stranded RNA (Proud, 1995) and although S2442 falls in a PKR motif, studies show that recognition of S51 in eIF2α is determined by flanking sequences (Kawagishi-Kobayashi *et al.*, 1997) which are not present in the mTOR sequence. Nevertheless S2442 was of interest because members of the same enzyme family are regulated by availability of a limiting factor e.g. haem regulated eIF2α kinase (HRI) and the yeast general control nondepressible 2 enzyme (GCN2) which are regulated by levels of haem and amino acids respectively (Wek, 1994).

In this study, we investigated whether S2442 and T2446 were *in vivo* phosphorylation sites. There was no evidence that S2442 was phosphorylated *in vivo* with the reagents generated; however, we identified T2446 to be a novel mTOR phosphorylation site. T2446 phosphorylation is attenuated by growth factor stimulation and activated by amino acid deprivation and factors which also activate AMPK. Further evidence is provided that phosphorylation at T2446 and S2448 are mutually exclusive, indicating that these sites may act antagonistically to integrate the counteracting signals of growth factors and nutrient deprivation.

3.3 Results

3.3.1 Characterisation of mTOR antibody

Total mTOR protein blots were performed with R27, an antiserum produced by Withers *et al.*, (1997) against residues 668-939 of mTOR incorporating the HEAT domain of mTOR. A new polyclonal mTOR antibody was raised against the same region as described by Withers *et al.*, (1997) and affinity purified as described in Materials and Methods (Section 2.2.6.1). The purified antibody was tested against FLAG immunoprecipitates of mTOR (from HEK cells transfected with FLAG-tagged wild-type mTOR) and HEK whole cell lysate. The new antibody reacted with an identical band at the correct molecular weight as the existing mTOR antibody (R27) (Fig. 3.1A). The new mTOR antibody was identified as 'total mTOR' and was used in subsequent experiments. The new total mTOR antibody was further tested with another cell line. Min6 (a mouse β-cell line) whole cell lysates were blotted with various mTOR antibodies which were specific to different regions of mTOR (Fig. 3.1B). The antibodies included:- 'total mTOR' (Fig. 3.1A); R27 antibody (Withers *et al.*, 1997); αTOR, raised against the C-terminal portion of mTOR (residues 2433-2450) (Heesom and Denton, 1999); hTOR, a mouse monoclonal antibody raised against a peptide corresponding to residues 230-240 of human mTOR (Hosoi et al., 1998) (Oncogene); de-phospho-T2446 antibody (eluted from the fraction of immunoglobulin retained from the de-phospho peptide column during phospho-T2446 antibody purification - see Materials and Methods, Section 2.2.6.1) and de-phospho-S2448 antibody, eluted from the de-phospho peptide column during

phospho-S2448 purification from Navé *et al.*, (1998). hTOR did not react with a band corresponding to mTOR which may be due to lack of sensitivity. Although the cell line was derived from mouse, the hTOR antibody is raised to a region (residues 230-240) conserved between human and mouse. Total mTOR, R27 and αTOR all identified a doublet corresponding to mTOR from the Min6 cell line; interestingly de-phospho-T2446 antibody reacted to the lower band of the doublet while de-phospho-S2448 reacted to the upper band of the doublet. The mTOR doublet identified by the various antibodies suggests that mTOR may have *in vivo* modifications which cause different migrating forms.

3.3.2 Characterisation of phospho-T2446 and phospho-S2442 mTOR antibody

Based on the hypothesis that S2442 and T2446 are potential phosphorylation sites adjacent to the existing mTOR S2448 phosphorylation site antisera were generated to specific phospho-peptides containing phosphorylation at the S2442 or T2446 site. The phospho-T2446 and phospho-S2442 antibody were affinity purified as described in Materials and Methods (Section 2.2.6.1) and used to study the *in vivo* phosphorylation states of T2446 and S2442 in CHO-IR cell lysates which had been nutrient fed or deprived. The phospho-T2446 antibody reacted with a band which showed differential reactivity to the nutrient status of the cell and was the same size as that detected by total mTOR; reactivity of phospho-T2446 was fully competed by the corresponding phospho-peptide (Fig. 3.2A). The phospho-S2442 antibody detected no signal from the membrane despite confirming the presence of mTOR with total mTOR antibody (Fig. 3.2B). CHO-IR and HEK cell lysates from different treatments were subsequently used to test the phospho-S2442 antibody in western blots though no signal was detected despite being able to blot total mTOR protein (data not shown). It is possible that S2442 is not phosphorylated *in vivo* or that functional antiserum was not produced. For this reason use of the phospho-S2442 antibody was abandoned.

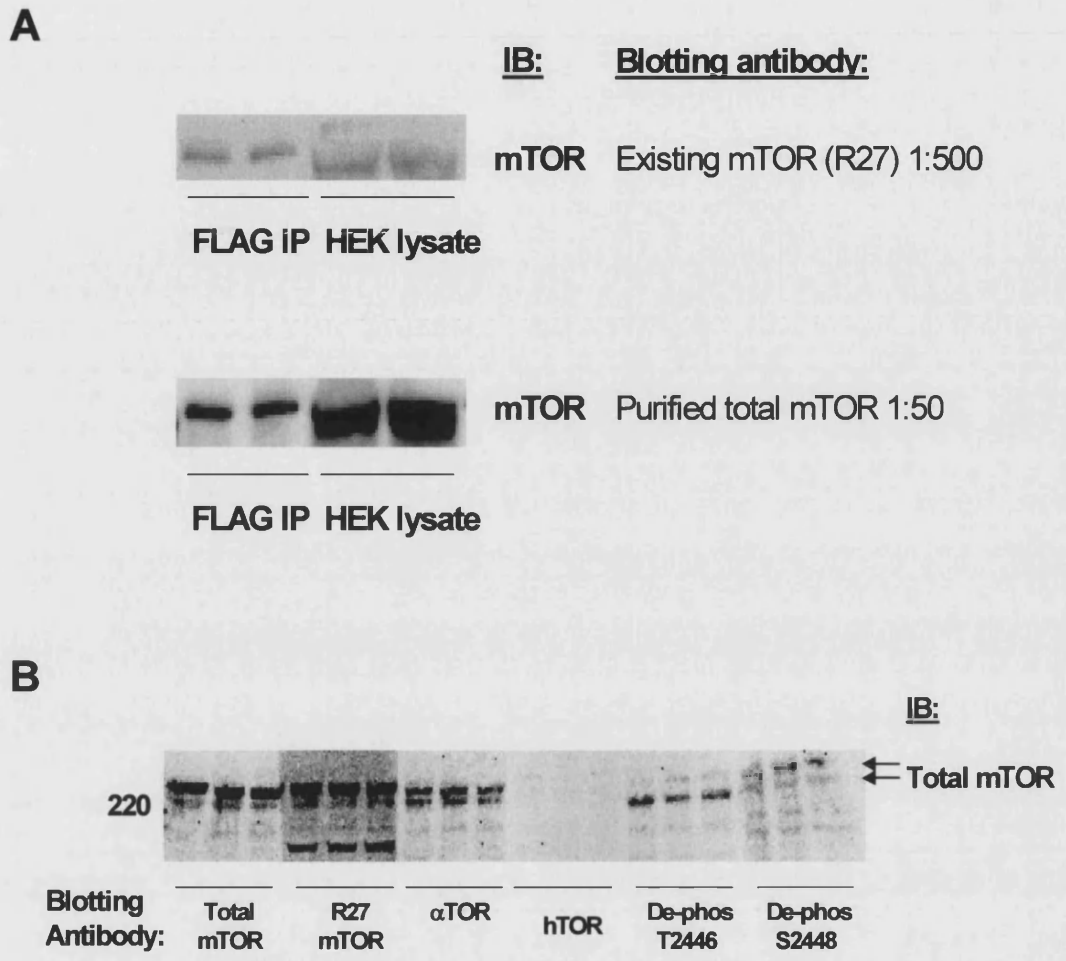

Figure 3.1 Characterisation of mTOR antibody

(A) HEK cells were transfected with wildtype mTOR using the calcium phosphate method. Cells were lysed and immunoprecipitated with the FLAG-tag antibody. Immunoprecipitates and cell lysate were subjected to SDS-PAGE then wet transferred and probed first with the existing mTOR antibody (termed R27), then stripped and re-probed with the newly developed and purified 'total mTOR' antibody. Dilution of the antibody is as indicated.
(B) Min 6 cell whole cell lysates were separated by SDS/PAGE, wet transferred to PVDF then probed with various mTOR antibodies, including total mTOR, R27, αTOR antibody, hTOR (Oncogene), dephospho T2446 and dephospho S2448 antibody. 'De-phos' denotes dephospho antibody and 'IB' denotes immuno-blot.

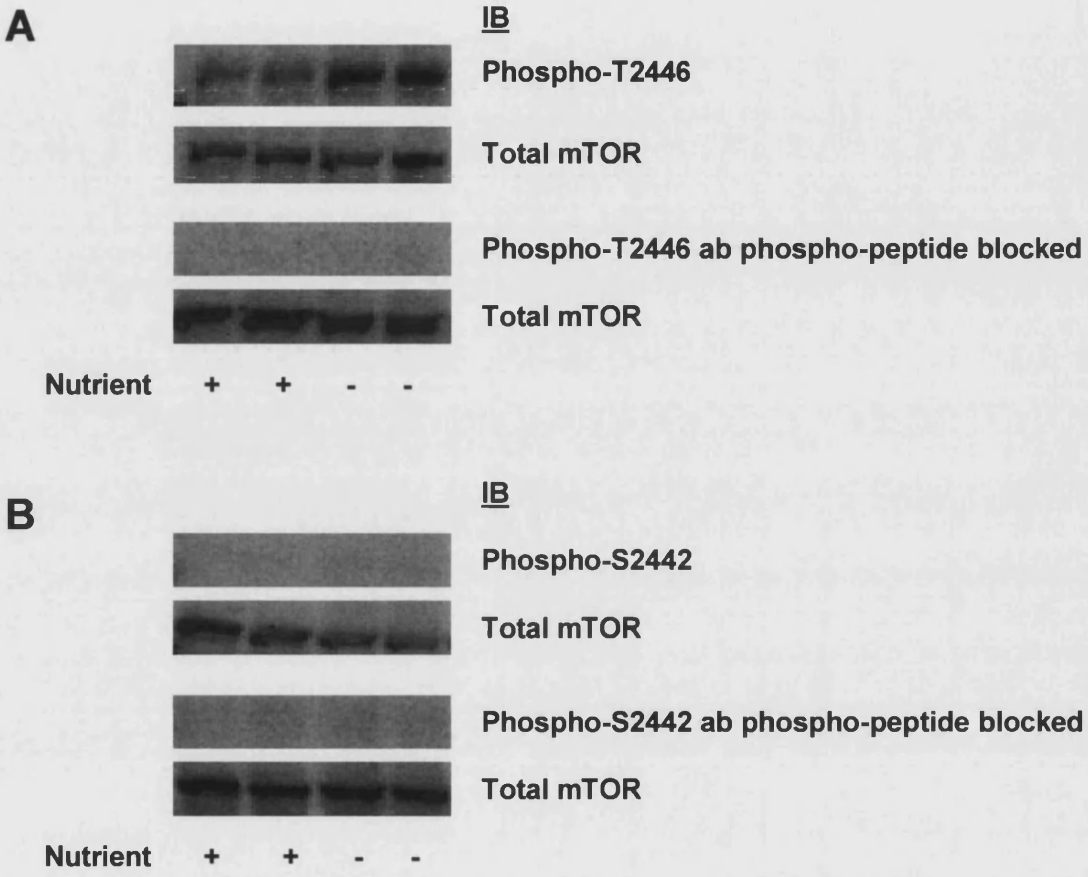

Figure 3.2 Characterisation of phospho-T2446 and phospho-S2442 mTOR antibody

CHO-IR cells were fed with F12 (Ham) supplemented with 10% fetal calf serum or nutrient starved in D-PBS for 1hr then lysed. Lysates were separated by SDS/PAGE, wet transferred to PVDF then probed with: (A) purified phospho-T2446 and; (B) phospho-S2442 mTOR antibody or the equivalent antibody pre-blocked with 1mM of the relevant phospho-peptide, all in 1% BSA in TBS/0.01% Tween20. The same blots were then stripped and re-probed for total mTOR as shown. Lanes are in duplicate.

3.3.3 An inverse relationship exists between phosphorylation of T2446 and S2448 on mTOR

It was previously shown that nutrient deprivation caused a time-dependent decrease in phosphorylation of S2448 in HEK293 cells, as shown by reduced reactivity of mTOR to a phospho-specific S2448 antibody (Navé *et al.*, 1999). This observation was extended here in CHO-IR cells to assess T2446 phosphorylation under the same parameters. Nutrient deprivation caused an increase in reactivity to the phospho-T2446 antibody concomitant with a decrease in reactivity to the phospho-S2448 antibody under the same starvation conditions (Fig. 3.3A). Total levels of mTOR remained unchanged. The reverse was observed when CHO-IR cells were stimulated with insulin: a decrease in reactivity of mTOR with the phospho-T2446 antibody was observed. Prolonged insulin stimulation of CHO-IR cells corresponded to an increase in reactivity to the phospho-S2448 antibody without a change in total mTOR (Fig. 3.3B). S2448 has been established as a nutrient sensitive site (Navé *et al.*, 1999) and here T2446 is also confirmed to be nutrient sensitive. These results demonstrate that conditions supporting T2446 phosphorylation are converse to those required for S2448 phosphorylation suggesting that phosphorylation at these sites may be mutually exclusive.

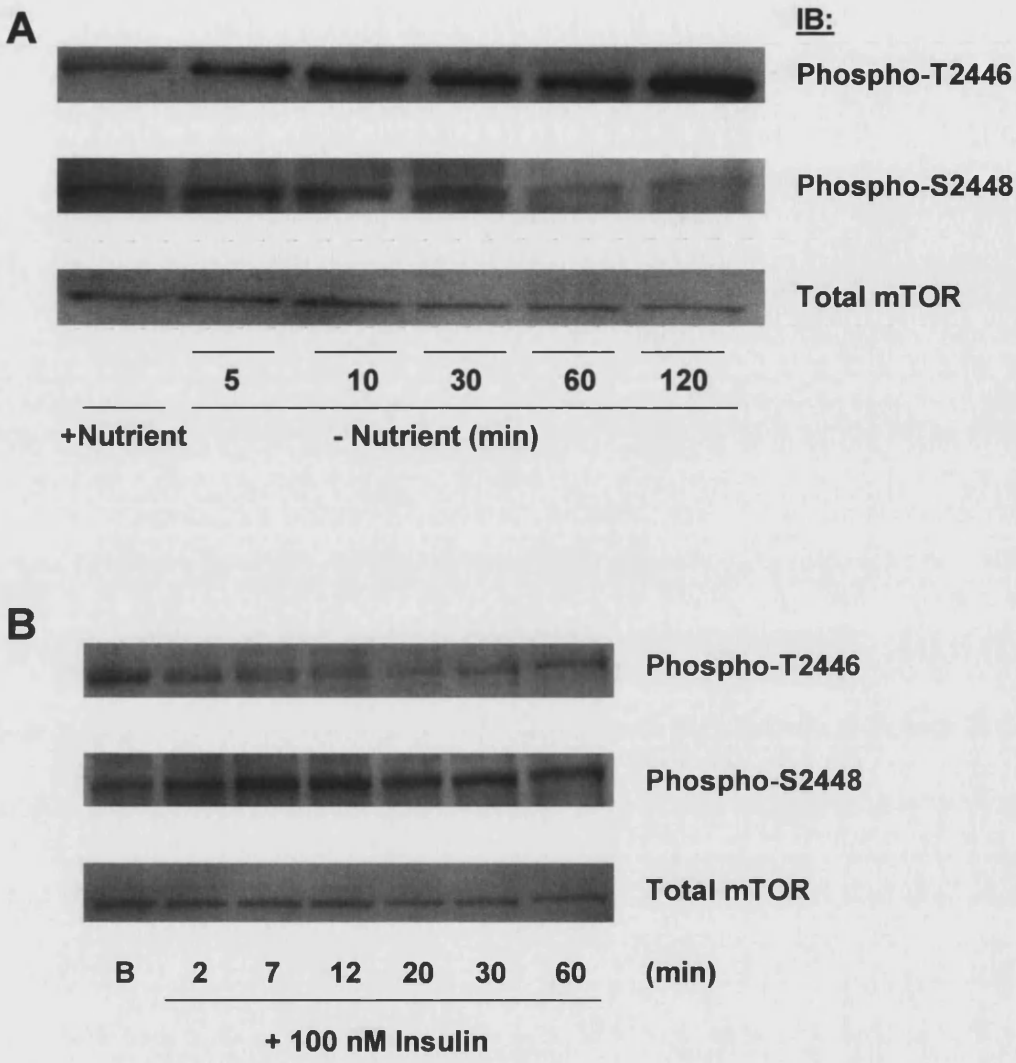

Figure 3.3 An inverse relationship exists between phosphorylation of T2446 and S2448 on mTOR

(A) Effects of nutrient starvation; CHO-IR cells were fed with F12 (Ham) supplemented with 10% fetal calf serum or nutrient starved in D-PBS for the times indicated above. (B) Effects of insulin stimulation; CHO-IR cells were serum-starved overnight in F12 (Ham) media, then stimulated with 100nM insulin for the times indicated. In all cases cells were lysed and lysates were separated by SDS/PAGE, wet transferred to PVDF and probed with the phospho-antibodies as shown. The same blots were stripped and re-probed for total mTOR as shown. Similar results were obtained in three independent experiments.

3.3.4 Effects of various inhibitors on mTOR T2446 phosphorylation compared to S2448 phosphorylation in CHO-IR

To examine the signalling pathways involved in T2446 phosphorylation several, well characterised inhibitors were used to investigate their effects on phosphorylation of T2446 in the presence or absence of nutrients (Fig. 3.4). Wortmannin, a specific inhibitor of PI3K, can cause a block in PKB activation, and has been shown to inhibit PKB dependent S2448 phosphorylation in HEK cells (Navé *et al.*, 1999). As seen in Fig. 3.4B, wortmannin treatment also decreased S2448 phosphorylation under nutrient fed conditions in CHO-IR cells. In contrast wortmannin did not inhibit the phosphorylation of T2446 under nutrient deprivation (Fig. 3.4A). Thus phosphorylation of T2446 is independent of PI3K and PKB activation. Protein phosphatase 2A (PP2A) is a major ser/thr phosphatase involved in the regulation of many signalling pathways and has been shown to be a downstream target of mTOR (Peterson *et al.*, 1999). Here, okadaic acid was used as an inhibitor of PP2A to examine whether it played a part in the regulation of T2446 phosphorylation. Okadaic acid induced no appreciable difference in reactivity to the phospho-T2446 or phospho-S2448 antibody compared to lysates from non-treated cells indicating that an okadaic acid sensitive protein phosphatase may not be involved in regulating phosphorylation at mTOR-T2446. To assess the possible involvement of the MAP kinase cascade in the regulation of T2446 phosphorylation, PD98059 was used. PD98059 is a highly selective inhibitor of *in vivo* MEK1 and MEK5 activation and thus blocks activation of the ERK kinase cascade. PD98059 did not inhibit S2448 phosphorylation in the presence of nutrients nor did it inhibit T2446 phosphorylation under nutrient deprivation thus excluding the MAP kinase pathway in the regulation of T2446 phosphorylation. Finally, treatment with rapamycin also did not affect T2446 phosphorylation in the presence or absence of nutrients (Fig. 3.4A), excluding mTOR itself from regulation of T2446 phosphorylation.

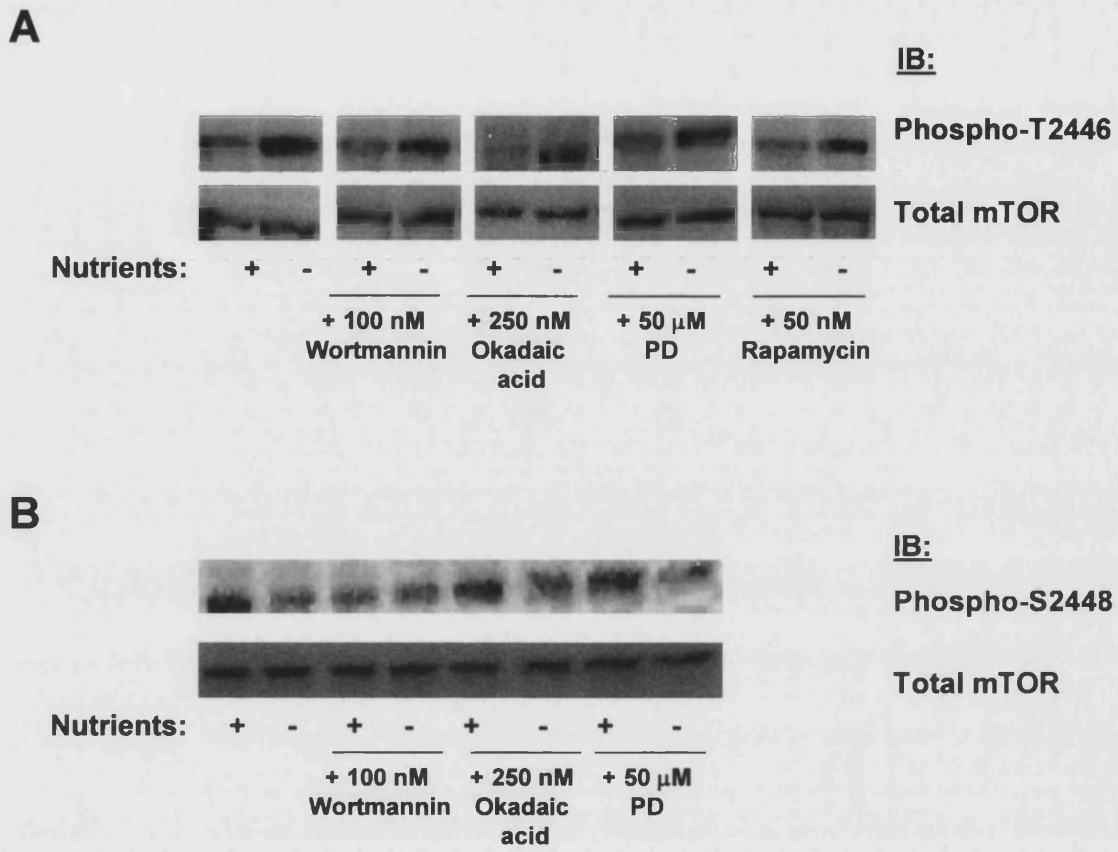

Figure 3.4 Effects of various inhibitors on mTOR T2446 phosphorylation compared to S2448 phosphorylation in CHO-IR

CHO-IR cells were incubated with 100nM wortmannin, 250nM okadaic acid, 50μM PD98059 (denoted as PD) or 50nM rapamycin for 1hour in the presence of nutrients [F12 (Ham) media] or absence of nutrients [D-PBS]. Cells were lysed and lysates were separated by SDS/PAGE, wet transferred to PVDF then probed with (A) phospho-T2446 or (B) phospho-S2448 antibodies as shown then stripped and re-probed for total mTOR. Similar results were obtained in three independent experiments.

3.3.5 PMA stimulates phosphorylation of S2448 on mTOR

The phorbol ester PMA (phorbol-12-myristate-13-acetate) is a potent mouse skin tumour promoter and is used to activate the protein kinase C (PKC) family of ser/thr protein kinases *in vivo* and *in vitro* (Newton, 2001); here it was used to analyse its effects on mTOR phosphorylation. CHO-IR cells were serum starved overnight then stimulated with insulin or PMA for 10 minutes. As observed earlier, T2446 is basally phosphorylated and insulin caused de-phosphorylation of this site (Fig. 3.5). Similarly PMA also caused T2446 dephosphorylation (Fig. 3.5). In contrast, S2448 phosphorylation was stimulated by insulin and also by PMA. The same effect was also observed in mouse myotubes (data not shown). PMA stimulated S2448 phosphorylation has also been demonstrated previously (Iijima *et al.*, 2002). It seems that T2446 and S2448 phosphorylation in response to PMA remains mutually exclusive. Since PMA activates PKC *in vivo*, PKC itself may directly cause the increase in S2448 phosphorylation by phosphorylating mTOR; however scansite analysis of mTOR does not identify S2448 as a potential PKC phosphorylation site. Therefore, the increase in S2448 phosphorylation may actually be indirect because PMA treatment can induce an increase in cellular levels of PtdIns (3, 4, 5)P_3 (Navé *et al.*, 1996). By increasing cellular levels of PtdIns (3, 4, 5)P_3, PDK1 and its downstream effector PKB would be activated and thus *in vivo*, the observed increase in S2448 phosphorylation by PMA treatment could still be via PKB. Insulin and PMA also stimulated T389 phosphorylation of S6K1 (Fig. 3.5), a downstream target of mTOR, indicating that PMA stimulation of mTOR induces normal activation of S6K1.

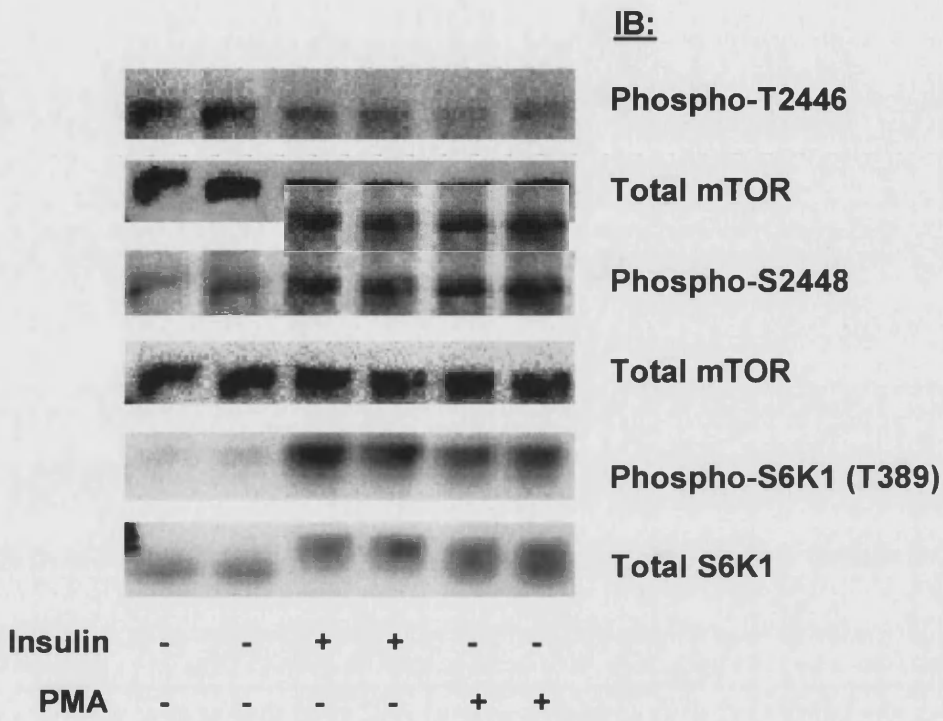

Figure 3.5 PMA stimulates phosphorylation of S2448 on mTOR

CHO-IR cells were serum starved overnight then stimulated for 10min with 100nM insulin or 1μM PMA (phorbol-12-myristate-13-acetate) as indicated. Cells were lysed and lysates were separated by SDS/PAGE, wet transferred to PVDF then probed with the phospho-antibodies as shown, before being stripped and re-probed for total protein. Similar results were obtained in three independent experiments.

3.3.6 cAMP and isoprenaline do not regulate mTOR-T2446 or S2448 phosphorylation

Previous findings indicated that increasing intracellular cAMP attenuated mTOR activity (Scott and Lawrence, 1998). Here, CPT-cAMP (a cell permeable cAMP analogue) and isoprenaline were used to assess the involvement of PKA in T2446 phosphorylation. CHO-IR cells were serum starved overnight before stimulation with 100μM CPT-cAMP or 10μM isoprenaline. Results show that neither CPT-cAMP nor isoprenaline stimulated a change in the phosphorylation state of T2446 or S2448 compared to basal levels, as determined by their respective phospho-antibody (Fig. 3.6). Both the phospho-T2446 and -S2448 antibody identified mTOR as a doublet at the correct molecular weight for mTOR; however this may be an artefact since the total blot only identifies a single band corresponding to mTOR (Fig. 3.6). The evidence here also excludes the involvement of PKA in the regulation of T2446 phosphorylation.

3.3.7 Effect of various serum/nutrients combinations on mTOR T2446 phosphorylation

Having not identified a regulatory pathway for T2446 phosphorylation by use of inhibitors, the effect of nutrients on T2446 phosphorylation was further investigated in CHO-IR cells by incubation with different nutrient combinations (Fig. 3.7A). Presence of 10% serum media complete with all nutrients namely glucose (3mM) and amino acids (at the standard concentration in media) did not induce phosphorylation at T2446. In contrast, cells incubated with any combination of the nutrient elements absent, induced T2446 phosphorylation to similar levels. Cells incubated in the absence of all nutrients (D-PBS) induced maximal stimulation of phosphorylation: approximately 2 fold more reactivity to the phospho-T2446 antibody than any of the other nutrient combinations (Fig. 3.7B). This suggests that T2446 phosphorylation is a general nutrient sensing site since loss of all nutrients results in maximal phosphorylation and addition of any one nutrient reduces phosphorylation to a basal level. Interestingly, the level of mTOR-T2446

phosphorylation was at a similar level whether serum starved for 1 hour or overnight, suggesting that withdrawal of some constituent in foetal calf serum is enough to stimulate basal T2446 phosphorylation. As described in Chapter 1: Introduction (Section 1.7.5.1), AMPK has been implicated in mTOR signalling; therefore the effects of nutrient deprivation on AMPK activation were assessed in parallel with T2446 phosphorylation. AMPK kinase (AMPKK) activates AMPK by phosphorylation at T172 in response to elevated AMP: ATP ratio due to cellular and environmental stress (Hardie *et al.*, 1998). The general trend in AMPK T172 phosphorylation was similar to the profile observed for T2446 phosphorylation (Fig. 3.7A) except where glucose was present in D-PBS which appeared to prevent AMPK phosphorylation. It seems that AMPK is more sensitive to glucose levels and this is unsurprising since glucose starvation elevates the AMP: ATP ratio (Hardie *et al.*, 1998); the presence of glucose would counteract this and thus inactivate AMPK. Nevertheless, loss of all nutrients also induced the highest AMPK activation and the overall similarities between T2446 and AMPK phosphorylation in response to different nutrient conditions suggests they lie in the same or parallel nutrient sensing pathways. ERK phosphorylation was induced by growth stimuli and was inactivated only during initial serum withdrawal and loss of all nutrients or glucose (Fig. 3.7A). As established earlier with the MEK1 inhibitor PD98059 (Section 3.3.4), the MAPK pathway does not seem to be involved in T2446 phosphorylation.

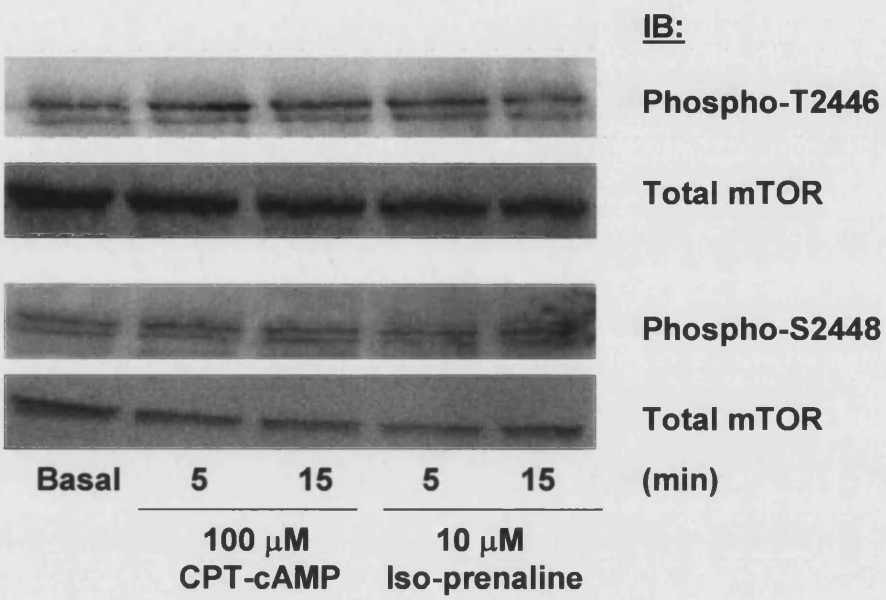

Figure 3.6 cAMP and Isoprenaline do not regulate mTOR-T2446 or S2448 phosphorylation

CHO-IR cells were serum starved overnight in serum free F12 (Ham) media before treatment with 100μM CPT-cAMP or 10μM isoprenaline for the indicated time points. Cells were lysed and lysates were separated by SDS/PAGE, wet transferred to PVDF then blotted with the phospho-antibodies as shown then stripped and re-probed for total mTOR. Similar results were obtained in three independent experiments.

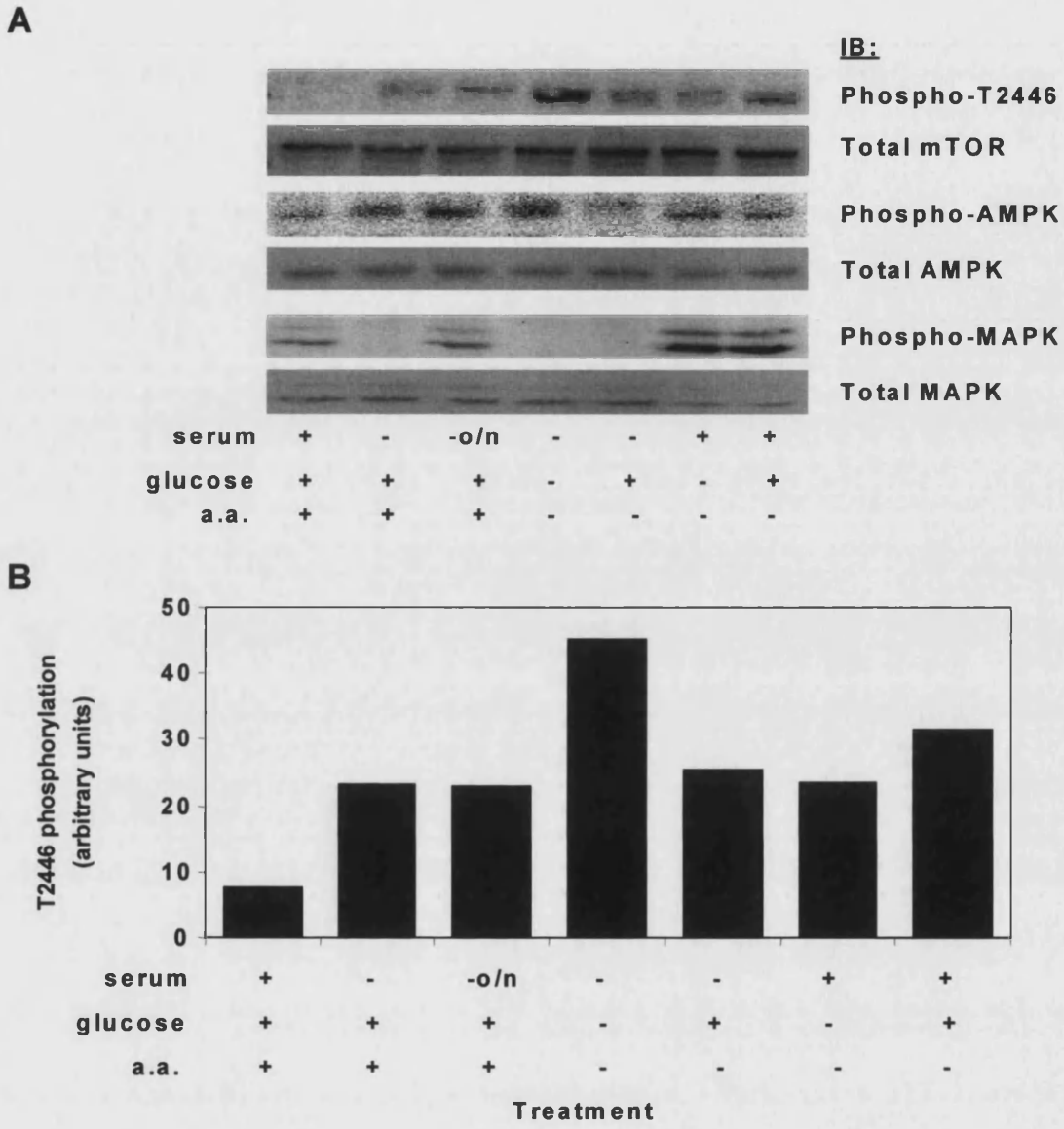

Figure 3.7 Effect of various serum and nutrient combinations on mTOR T2446 phosphorylation

CHO-IR cells with nutrients were incubated in F12 media either with 10% serum, without serum for 1hr (-1hr) or serum starved overnight (-o/n) as indicated. Cells in the absence of nutrients were incubated in D-PBS supplemented with 1.802g/l glucose [3mM], 10% serum or both for 1hr. Cells were lysed and lysates were separated by SDS/PAGE, wet transferred to PVDF and probed with the phospho-antibodies as shown then stripped and re-probed for the total protein (A). Similar results were obtained in three independent experiments. The level of mTOR-T2446 phosphorylation detected from the western blot was quantitated and plotted in (B).

3.3.8 Effect of AMPK activators on T2446 phosphorylation in H2K cells

As established in Section 3.3.7, AMPK phosphorylation and mTOR phosphorylation at T2446 are similarly induced by nutrient deprivation. Therefore to determine the physiological significance of nutrient status in regulating mTOR T2446 phosphorylation in relation to AMPK activation, mouse H2K myotubes were treated with AMPK activators and the effects on T2446 phosphorylation and AMPK activity were assessed. Myotubes were treated with the metabolic inhibitor Dinitrophenol (DNP) which acts to rapidly decrease cellular ATP level or with AICAR riboside, a cell permeable compound that in some cell types (including H2K) is degraded to ZMP, a nucleotide which mimics the effects of 5'AMP and is capable of activating AMPK directly without altering cellular concentrations of ATP, ADP and AMP (Corton *et al.*, 1995). mTOR S2448, ERK and also S6K1 (T389) phosphorylation were increased only in the presence of insulin even when in combination with AICAR (Fig. 3.8A). In contrast, insulin did not stimulate T2446 phosphorylation but DNP did cause an acute increase in T2446 phosphorylation even in the presence of insulin. Treatment with DNP also increased AMPK T172 phosphorylation (Fig. 3.8A) and activity of AMPK (Fig. 3.8B). AICAR had similar effects to DNP treatment on T2446 and AMPK phosphorylation though it was less potent in activation of AMPK. In accordance with results presented earlier where the phosphorylation of AMPK was increased following withdrawal of amino acids in CHO-IR cells (Fig. 3.7A), data here suggest that mTOR T2446 phosphorylation and AMPK activation (as indicated by T172 phosphorylation and activity data) are similarly regulated and possibly within the same pathway.

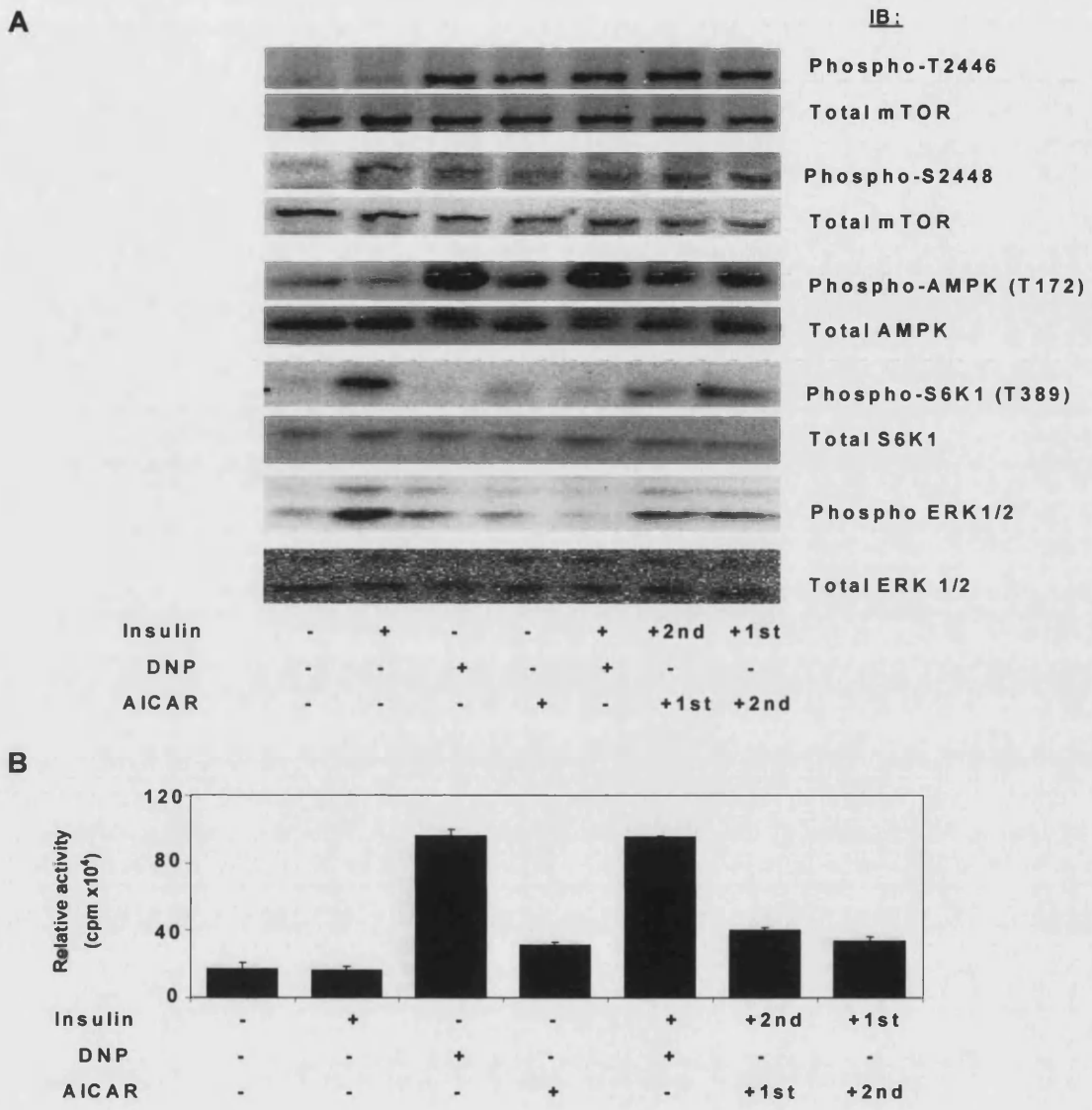

Figure 3.8 Effect of AMPK activators on T2446 phosphorylation in H2K cells

H2K cells were incubated in DMEM [0.5% serum] overnight, which was replaced with HEPES buffered saline [containing 5mM glucose] for 30min treatments with either 100nM insulin, 0.5mM DNP or 0.5mM AICAR as indicated. Where the legend is labelled 1st and 2nd, cells were first stimulated with 100nM insulin for 30min followed by treatment with 0.5M AICAR for 30min or vice versa. Cells were harvested and AMPK was immunoprecipitated using a pan β-subunit AMPK antibody and used to assay AMPK activity against SAMS peptide (B). The remaining lysates were separated by SDS/PAGE, wet transferred to PVDF then probed with the phospho-antibodies as shown in (A), stripped and re-probed for the total protein. Similar results were obtained in three independent experiments. [Experiment performed in collaboration with co-workers in Cheng *et al.*, 2004].

3.3.9 *In vitro* kinase assays with mTOR peptides

Evidence that T2446 phosphorylation and AMPK activation were similarly regulated raised the possibility that AMPK could phosphorylate mTOR at T2446. To investigate this hypothesis and identify potential kinases which may phosphorylate the T2446 region of mTOR, *in vitro* kinase assays were performed using a series of mTOR peptides based on residues 2440-2551. The peptide series included residue changes to alanine and glutamic acid at the S2448 or T2446 positions (Table 3.1). The kinases which were used in the peptide assays included: PKB, AMPK, PKCζ and S6K1.

PKB is well documented as a kinase which can directly phosphorylate mTOR and was used here to assess the effect of T2446 phosphorylation on PKB mediated phosphorylation of S2448. PKB was immunoprecipitated from CHO-IR cells stimulated with insulin. Data presented in Fig. 3.9A shows that PKB mediated maximal phosphorylation of the wildtype peptide from PKB immunoprecipitates obtained after insulin stimulation, demonstrating that the wildtype peptide was a suitable substrate. PKB has been shown to directly phosphorylate mTOR at S2448 (Navé *et al.*, 1999). Evidence presented here shows phosphorylation of the phospho-mimic T2446E peptide by PKB was at a low level compared to phosphorylation of both the wild type peptide and the T2446A peptide (Fig. 3.9B), suggesting that phosphorylation at T2446 prevents S2448 phosphorylation and supporting the hypothesis that phosphorylation of these two sites is mutually exclusive. In addition, PKB phosphorylated the phospho-S2448 and S2448A peptides at approximately 20% and 40% respectively of the phosphorylation observed with the wild type peptide, suggesting that some level of T2446 phosphorylation may be possible by PKB *in vitro*. Since T2446 has a loose consensus to the PKB phosphorylation motif, R-X-R-Y-Z-S/T-hyd (Alessi *et al.*, 1996), it is unsurprising that PKB has some ability to phosphorylate T2446, though this data suggests that T2446 is not a major site of PKB phosphorylation.

To investigate whether AMPK could phosphorylate the mTOR peptides, constitutively active recombinant AMPK was used, comprising His-tagged-

α_1T172D, β_1 and γ_1 subunits - gift from Dr D. Carling (Hammersmith Hospital, London) (Stein *et al.*, 2000). AMPK phosphorylates the motif hyd-(X, bas)-X-X-S/T-X-X-X-hyd where X is any amino acid; hyd is a hydrophobic residue; bas is a basic residue; S/T is the phosphorylatable residue at position zero (P=0). Hence, the P-5 and P+4 positions are occupied by a bulky hydrophobic residue with either the P-4 or P-3 position occupied by a basic residue (Dale *et al.*, 1995). The α_1 isoform of the AMPK catalytic subunit which was used in these experiments is less selective for the phosphorylation motif than other AMPK isoforms; a hydrophobic residue at P+4 is not essential while a basic residue at P-6 is beneficial for phosphorylation (Dale *et al.*, 1995). The wildtype mTOR peptide had a basic residue at P-6 and P-4, so fulfilled requirements for AMPK recognition (see Table 3.1) and AMPK was able to phosphorylate this peptide fully. In contrast, phosphorylation of the T2446E and T2446A peptide was greatly reduced to approximately 20% and 50% respectively of the phosphorylation observed with the wildtype peptide, consistent with the mutated T2446 site being unrecognisable by AMPK (Fig. 3.9C). Changes at the S2448 position also reduced the ability of AMPK to phosphorylate the peptides (Fig. 3.9C). It is possible that the phospho-S2448 and S2448A peptide prevents T2446 phosphorylation by preventing AMPK recognition and phosphorylation.

As shown in Fig. 3.5, PMA stimulates S2448 phosphorylation which may be mediated by a PKC isoform. To determine whether an isoform of PKC could phosphorylate the mTOR peptides, PKCζ, an atypical PKC isofrom was used. PKCζ does not bind phorbol esters (Newton, 2001); here however PMA did stimulate PKCζ activition (Fig. 3.9A). This was most likely via PMA activation of PDK1 which phosphorylates and activates PKCζ *in vitro* (Good *et al.*, 1998). The optimal peptide sequence for PKC phosphorylation is an XRXXS motif where X is any amino acid (Newton, 2001); S2448 is in a XRXXS motif. Here, PKCζ was able to phosphorylate the S6 control peptide and the T2446A and E peptide to the same level of phosphorylation observed with the wildtype peptide. Phosphorylation did not occur when the phospho-S2448 and S2448A peptides were used (Fig. 3.9D), suggesting that PKCζ is capable of phosphorylating the S2448 site *in vitro* or that abrogation of this site prevents phosphorylation of an alternative residue within the peptide sequence. This data does not necessarily imply *in vivo* phosphorylation of

mTOR by PKCζ, since theoretically any peptide containing the XRXXS motif is capable of phosphorylation by PKCζ, as demonstrated by the S6 control peptide. *In vivo* tests would determine the significance of these observations.

The S6K1 phosphorylation motif is K/RXRXX-S/T-hyd, where X is any amino acid and 'hyd' is a hydrophobic residue (Pinna and Ruzzene, 1996). Scansite analysis revealed that T2446 is a potential S6K1 phosphorylation site. Fig. 3.9A presents data showing that S6K1 immunoprecipitated from CHO-IR cells had maximal activity towards the wildtype peptide after insulin stimulation, thus demonstrating that the wildtype peptide was a suitable substrate for S6K1. Assays with S6K1 immunoprecipitates from insulin stimulated CHO-IR cells showed that S6K1 could phosphorylate all the mTOR peptides to between 50% and 200% of the phosphorylation observed with the wildtype peptide (Fig. 3.9E), suggesting general peptide phosphorylation by S6K1 which was not dependent on the status of T2446 or S2448 phosphorylation. Constitutively active recombinant S6K1 was able to phosphorylate the T2446E peptide to approximately 80% of the phosphorylation observed with the wildtype peptide (Fig. 3.9E), suggesting that S6K1 is able to phosphorylate alternative residues to T2446 within the T2446E peptide and supporting the conclusion that S6K1 phosphorylates the mTOR peptides non-specifically. It is possible that the difference between recombinant and immunoprecipitated S6K1 phosphorylation of the mTOR peptides may be from contaminating kinase activity associated with the S6K1 immunoprecipitate.

A truncated PKR construct in pGEX-4T-2 (courtesy of Dr T. Dever, National Institute of Health, USA), which was reported as constitutively active *in vitro* (Ung *et al.*, 2001) was obtained to investigate the possibility that PKR could phosphorylate mTOR. Unfortunately, repeated attempts to express the GST-PKR construct failed and the question of whether PKR could mediate phosphorylation of the mTOR peptides could not be addressed.

Peptide	Sequence
Wild-type	KKRSRTRTDSYSA
T2446E	KKRSRTR<u>E</u>DSYSA
T2446A	KKRSRTR<u>A</u>DSYSA
Phospho-S2448	KKRSRTRTD<u>S</u>*YSA
S2448A	KKRSRTRTD<u>A</u>YSA

Table 3.1 Peptides used as substrates in kinase assays

Peptides used in the peptide assays are shown above. Sequences were based on twelve residues in the mTOR sequence from K2440-A2451 with an additional K residue at the N-terminal to increase the charge for binding to p81 paper. <u>E</u> and <u>A</u> denotes the mutated residue and <u>S</u>* denotes a phosphorylated serine residue.

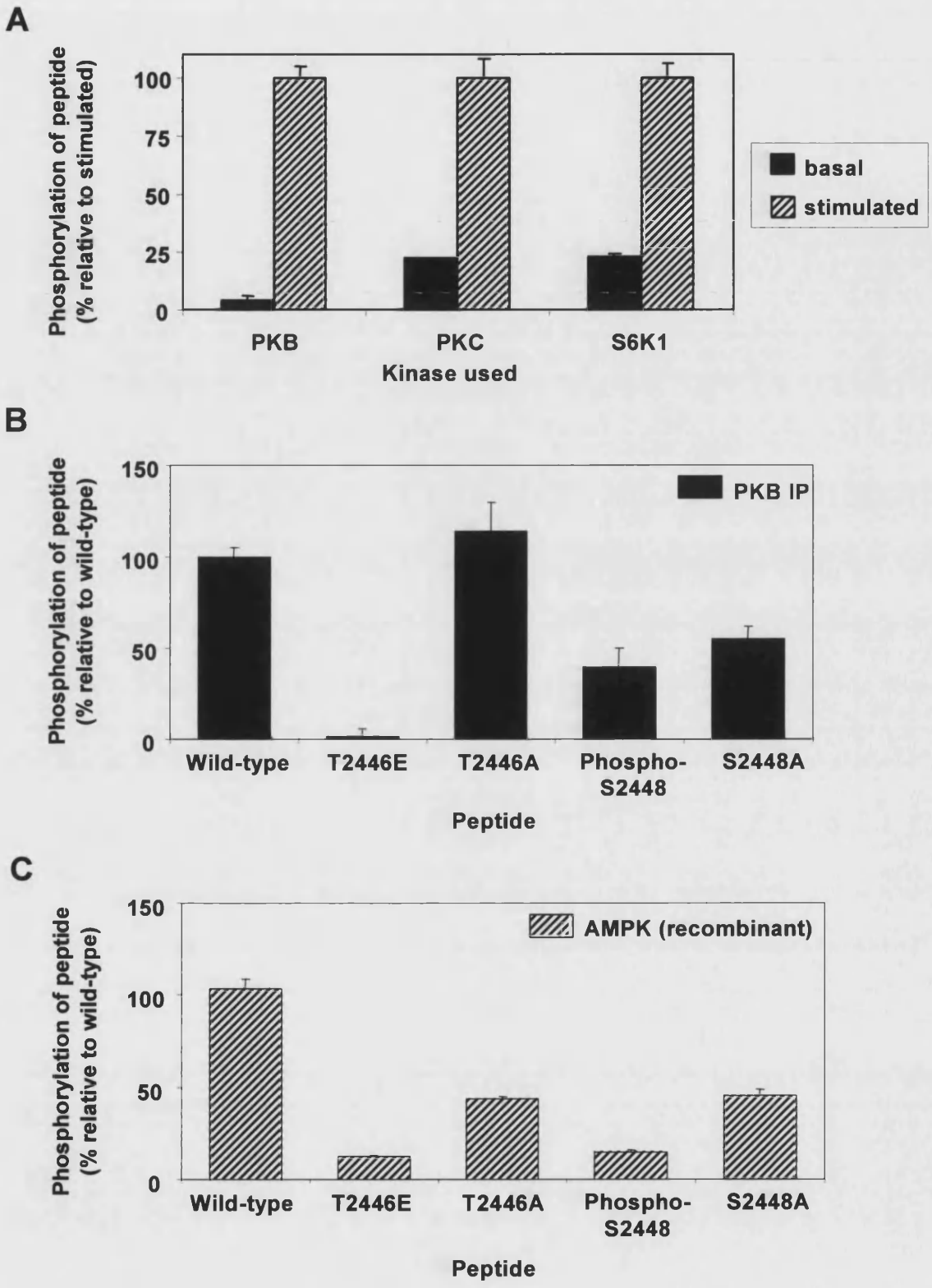

Figure 3.9 *In vitro* kinase assays using mTOR peptides as the substrates

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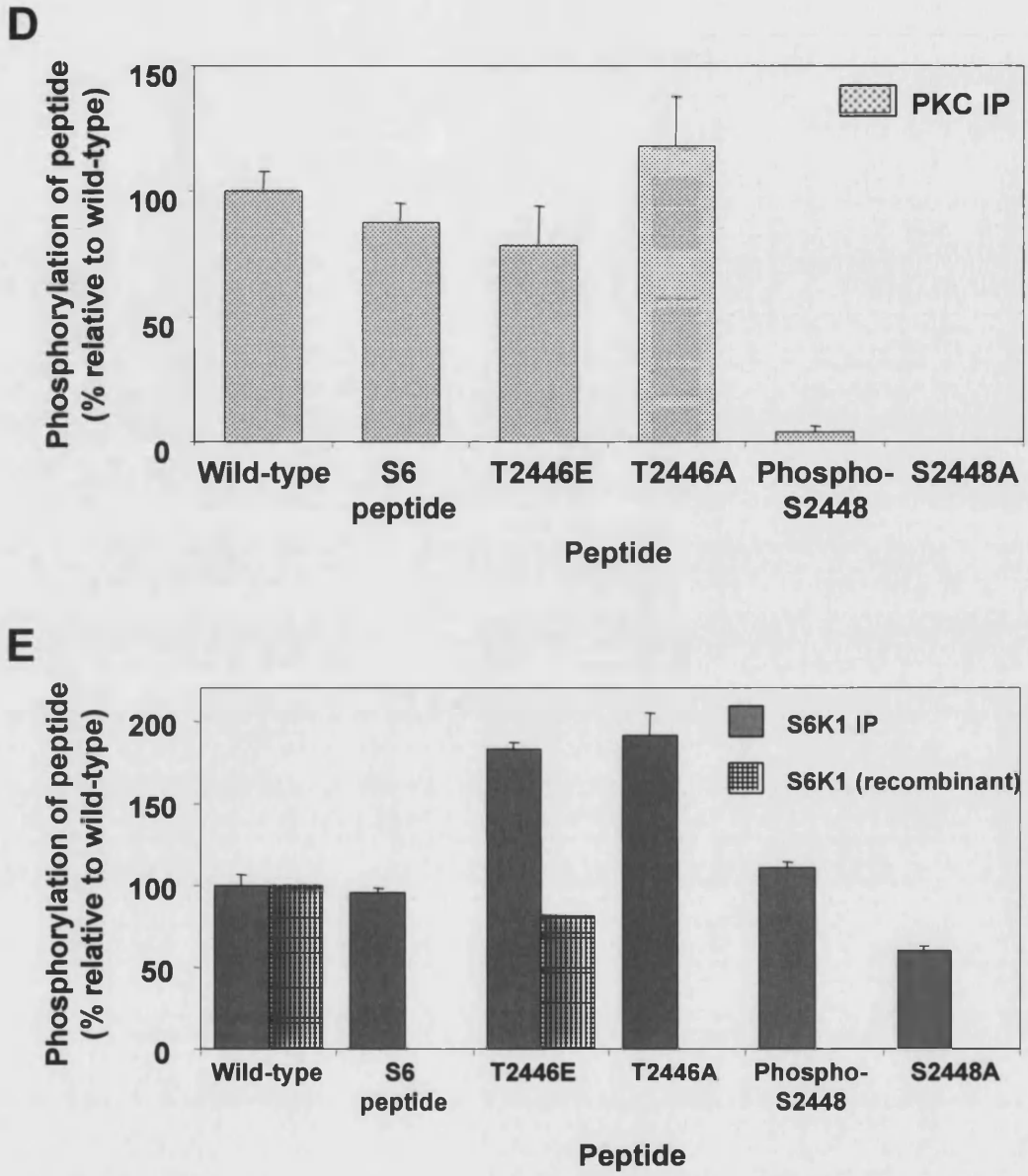

Figure 3.9 *In vitro* kinase assays using mTOR peptides as the substrates

CHO-IR cells were stimulated with insulin or PMA, lysed and immunoprecipitated for PKB and S6K or PKCζ respectively. The immunoprecipitates or recombinant AMPK and S6K1 were subjected to *in vitro* kinase assay against all the peptides shown in Table 3.1. Assays were conducted in the presence of [γ-^{32} P] ATP and Mg^{2+}. % phosphorylation of each peptide was determined relative to phosphorylation of the wildtype peptide. The activity of PKB, PKCζ and S6K immunoprecipitates from basal or stimulated cells against the wildtype peptide is shown in (A). The activities of various kinases towards each peptide in Table 3.1 are presented in (B) PKB, (C) AMPK, (D) PKCζ, (E) S6K1. Results shown are n=3 in triplicate ± SEM

3.3.10 Recombinant AMPK is unable to phosphorylate mTOR *in vitro*

Based on the data obtained from AMPK activators and the peptide assays, recombinant AMPK (α_1T172D, β_1 and γ_1 subunits) was used in an *in vitro* kinase assay to assess its ability to phosphorylate full-length mTOR immunoprecipitated from cells. HEK cells were incubated in the absence and presence of amino acids then lysed and endogenous mTOR was immunoprecipitated with αTOR. The immunoprecipitates were subjected to an *in vitro* kinase assay with recombinant AMPK and no phosphorylation of mTOR was observed (Fig. 3.10A). To increase the quantity of mTOR immunoprecipitated, HEK cells were transfected with wildtype mTOR and the assay was repeated with FLAG immunoprecipitates of mTOR (Fig. 3.10B); there was no noticeable phosphorylation of mTOR by AMPK and a background level of phosphorylation was observed even in the absence of recombinant AMPK. The background phosphorylation observed with FLAG immunoprecipitates may be from a contaminating kinase activity or mTOR autokinase activity (Fig. 3.10B).

The *in vitro* kinase assay was extended to immunoprecipitates of mTOR mutants which substituted T2446 or S2448 with non phosphorylatable alanine or phospho-mimetic glutamic acid (Section 2.2.3.2). The mutants were transfected into HEK cells and isolated by FLAG immunoprecipitation. Again, there was no appreciable difference in AMPK mediated phosphorylation of each mutant and a background level of phosphorylation was observed even in the absence of recombinant AMPK; the background level of phosphorylation was unchanged in the presence of AMPK (Fig. 3.10C). Results here suggest that AMPK does not phosphorylate mTOR *in vitro* though it is possible that immunoprecipitating mTOR with antibody blocks the accessibility to AMPK mediated phosphorylation or that mTOR exists in a complex in which an intermediate kinase may be involved between activation of AMPK and T2446 phosphorylation.

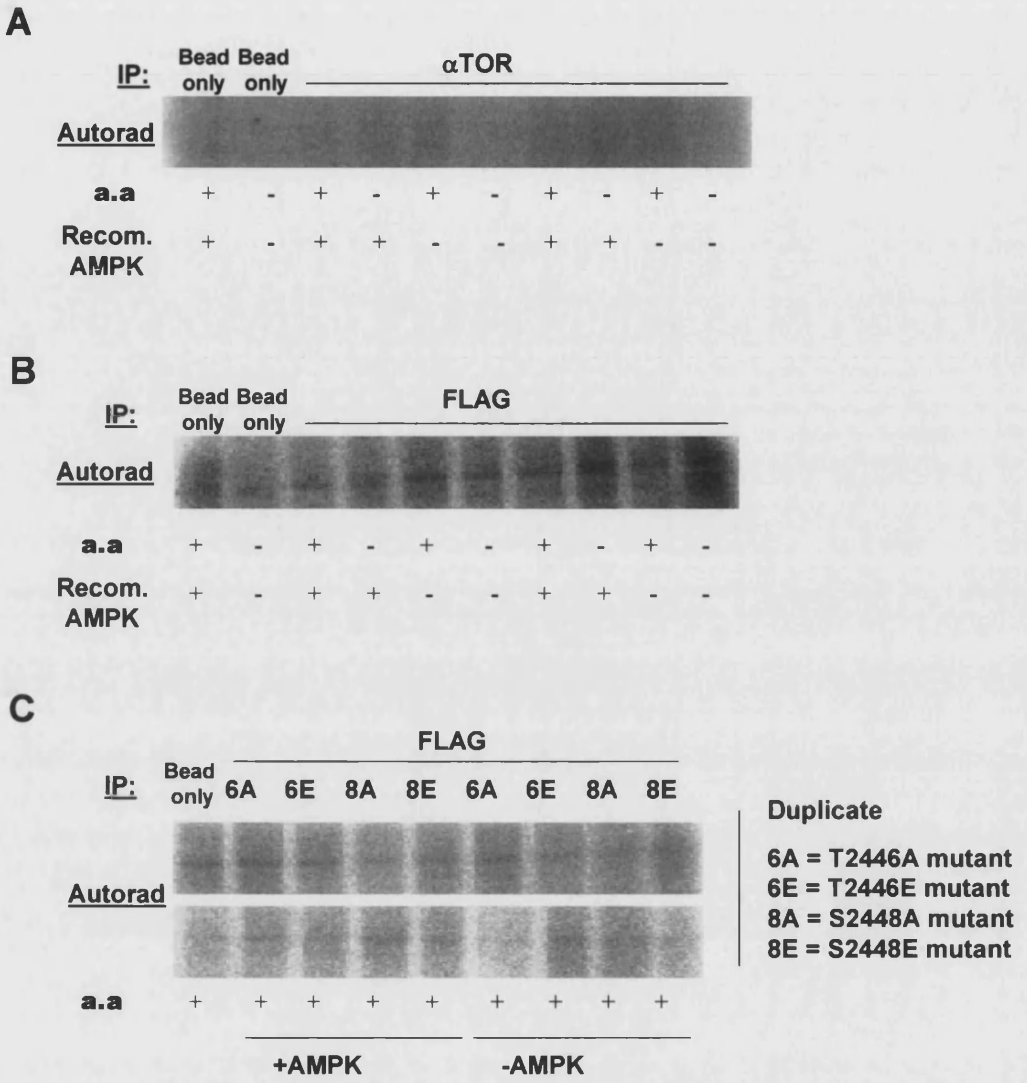

Figure 3.10 Recombinant AMPK is unable to phosphorylate mTOR *in vitro*

HEK293 cells were transfected with wildtype mTOR (B) or mTOR mutant constructs (C) using the calcium phosphate method of transfection. Non-transfected (A) and wild-type transfected (B) cells were incubated with amino acids [DMEM] or amino acid starved in D-PBS supplemented with 5mM glucose for 1hr. Mutant constructs were incubated in DMEM for 1hr. All were lysed then immunoprecipitated with αTOR (A) or FLAG (B and C) as indicated; bead only represents immunoprecipitation with protein A (A) or mouse IgG (B, C) in the absence of antibody. The immunoprecipitates were washed then subjected to an *in vitro* kinase assay with recombinant AMPK in the presence of [γ-^{32}P] ATP and Mg^{2+}. Reaction products were analysed by SDS-PAGE and autoradiography. Results are shown in duplicate and similar results were obtained in another experiment.

3.4 Discussion

The present study supports the notion that the AMPK and mTOR signalling pathways are linked. Previous evidence indicates that the C-terminus region of mTOR is of regulatory importance (Brunn *et al.*, 1997b, Sekulić *et al.*, 2000, Peterson *et al.*, 2000) and results presented here provide evidence that T2446 is a novel mTOR phosphorylation site within this region. Factors which promote phosphorylation at T2446 cause dephosphorylation of the previously identified PKB mTOR phosphorylation site S2448 (Navé *et al.*, 1999). The evidence suggests that T2446 phosphorylation may have negative regulatory effects on mTOR activation since phosphorylation of T2446 is acutely stimulated by nutrient deprivation, a condition associated with reduced signalling through mTOR. Moreover T2446 phosphorylation correlates with an attenuation of insulin-stimulated phosphorylation of S6K1.

Evidence from the peptide assays shows that PKB can phosphorylate at the mTOR-T2446 position since phosphorylation of the S2448A and phospho-S2448 peptides was observed at approximately 50% of the phosphorylation observed with the wildtype peptide. This is not surprising because it weakly fits the PKB consensus sequence. However, T2446 is unlikely to be a major PKB target site. Sekulić *et al.*, (2000), reported that expression of a S2448A mTOR mutant with activated PKB still gave rise to residual T2446 phosphorylation, as shown by increased reactivity to a double phospho-T2446/S2448 antibody termed α-mTORp2. Interestingly the authors also produced a phospho-T2446 antibody though no findings were reported with it. Data presented here suggests that phosphorylation of T2446 and S2448 are mutually exclusive *in vitro* because phosphorylation of the S2448 position is inhibited with use of the mTOR T2446E phospho-mimetic peptide but phosphorylation is normal when T2446 is changed to alanine. Furthermore, *in-vivo* studies in CHO-IR cells show that wortmannin, which blocks activation of PKB and S2448 phosphorylation, does not reduce phosphorylation at T2446, indicating that phosphorylation of this site is independent of PKB activation. The use of selective inhibitors showed that phosphorylation of T2446 is also independent of an okadaic acid-sensitive protein phosphatase activity, the MAP kinase pathway, and even of

mTOR itself. Additionally, the phorbol ester PMA did not stimulate phosphorylation of T2446 and peptide data support the exclusion of PKCs from the regulation of T2446 phosphorylation. However, PMA did stimulate S2448 phosphorylation and also downstream S6K1 activation (indicated by T389 phosphorylation), previously described by Iijima *et al.*, (2002). Iijima *et al.*, (2002) proposed that PMA stimulated phosphorylation of mTOR-S2448 via the PKC mediated c-Raf/MEK/ERK signalling pathway which was not critical during insulin stimulation of cardiomyocytes: this pathway may also operate in CHO-IR cells. The mechanisms of c-Raf/MEK/ERK mediated S2448 phosphorylation have not been demonstrated and the activation of mTOR S2448 phosphorylation observed here could be indirect, as PMA can increase cellular levels of PtdIns (3, 4, 5)P_3 (Navé *et al.*, 1996) and thus activation of PDK1 and PKB. However *in vitro* kinase assays show that the PKCζ isoform had the capability to phosphorylate the wildtype, T2446A and T2446E peptides indiscriminately; it could not phosphorylate the S2448A or phospho-S2448 peptides. This may be because the S2448 site is within a PKC phosphorylation motif and changing the S2448 position with either alanine or phospho- prevents peptide phosphorylation. Phosphorylation of mTOR-S2448 by PKCζ would be a novel observation; however, *in vitro* kinase data may not truly reflect the actual phosphorylation of S2448 by PKC isoforms *in-vivo*. mTOR has been implicated in down-regulation of IRS-1 signalling (Haruta *et al.*, 2000) and in the sub-cellular redistribution of IRS-1 (Takano *et al.*, 2001); PKCζ can also associate and phosphorylate serine residues of IRS-1 in an insulin dependent manner (Ravichandron *et al.*, 2001). It will be of interest to determine whether mTOR and atypical PKCs (which includes PKCζ) are recruited together for down-regulation of IRS-1. Although upstream involvement of PKC in mTOR regulation must be considered, there is evidence that places the novel PKC isoforms (δ and ε) downstream of mTOR (Kumar *et al.*, 2000a; Parekh *et al.*, 1999). Both isoforms were identified as operating via an mTOR dependent pathway as determined by inhibition of PKCδ and PKCε phosphorylation in the presence of rapamycin or with amino acid starvation (Parekh *et al.*, 1999); moreover, PKCδ was found to associate with mTOR and was required for phosphorylation and inactivation of 4E-BP1 (Kumar *et al.*, 2000a). Further *in-vivo* testing is needed to clarify which and how the PKC isoforms are involved in mTOR signalling, whether upstream or downstream.

The data discussed above suggest that phosphorylation of T2446 and S2448 is mutually exclusive since conditions which promote T2446 phosphorylation act to decrease S2448 phosphorylation and vice versa, an event which would require co-ordinate regulation of the kinases involved.

Recent evidence implicates AMPK in the mTOR pathway (Bolster *et al.*, 2002; Kimura *et al.*, 2003) and results here suggest that AMPK is likely to be involved in regulating phosphorylation at T2446. Two lines of evidence support this: nutrient deprivation, DNP and AICAR all activate AMPK and also stimulate phosphorylation of T2446 and; AMPK can directly phosphorylate the wildtype mTOR peptide (residues 2440-2551). Although AMPK is able to phosphorylate the wildtype mTOR peptide, it also phosphorylates both the T2446A and S2448A peptides at approximately 20% and both the T2446E and phospho-S2448 peptides at approximately 60% of that observed with the wildtype peptide. Furthermore, experiments designed to demonstrate AMPK phosphorylation of full length mTOR were inconclusive. From this data it is not possible to confirm the direct phosphorylation of mTOR by AMPK. Although recent data has been published indicating that the γ subunit of AMPK can interact with the C-terminal portion of mTOR, again highlighting the importance of this region, no interaction was observed with full length mTOR (Kimura *et al.*, 2003). It seems more likely that AMPK is indirectly involved in T2446 phosphorylation. A recent study showed that AMPK interacted with TSC2, and AMPK was able to directly phosphorylate TSC2 at T1227 and S1345 to downregulate translation and reduce cell size under energy starved conditions (Inoki *et al.*, 2003b). The findings of Inoki *et al.*, (2003) suggest that TSC integrates signals of energy availability via AMPK leading to inhibition of mTOR signalling to S6K1 and 4E-BP1.

Though AMPK may not regulate mTOR by direct phosphorylation, the mutually exclusive phosphorylation of T2446 and S2448 is likely to be due to phosphorylation of one site which then affects the substrate recognition sequence for subsequent phosphorylation by another kinase. However, the kinase responsible for T2446 phosphorylation remains to be identified.

Additionally, increasing intracellular cAMP has been reported as attenuating mTOR activity (Scott and Lawrence, 1998) but here cAMP treatment did not affect basal T2446 or S2448 phosphorylation. However, conditions did not include CPT-cAMP treatment in conjunction with insulin, which was shown to decrease mTOR phosphorylation by increased reactivity to the mTAb1 antibody (mTOR 2433-2450) which is not specific to T2246 or S2448 phosphorylation (Brunn *et al.*, 1997b). Therefore, it would be speculation to assume the status of mTOR phosphorylation in the presence of cAMP used in conjunction with insulin. Although cAMP inhibited insulin stimulated mTOR activity, this combination of treatment did not attenuate activation of PKB; thus S2448 may still be phosphorylated (Scott and Lawrence, 1998). In addition, the mTAb1 antibody epitope encompasses both mTOR phosphorylation sites and includes other phosphorylatable serine and threonine residues. It was unfortunate that the cAMP + insulin treatment was not included in this investigation, as this would have provided a more conclusive answer as to exactly which mTOR site is phosphorylated.

In summary, T2446 has been identified as a novel phosphorylation site in mTOR which may also be regulated by AMPK signalling. Phosphorylation of T2446 is increased by low nutrient conditions which are normally associated with the negative regulation of mTOR. Phosphorylation at T2446 limits phosphorylation at S2448 and vice versa suggesting that phosphorylation of these sites is mutually exclusive and might act as a switch in controlling the positive and negative signals which regulate protein translation via mTOR.

Chapter 4: Results

4 Investigating the role of phosphorylation in mTOR by mutational analysis

4.1 Summary

The mammalian target of rapamycin (mTOR) integrates growth factor signalling and nutrient availability, culminating with an increase in the rate of translation of specific sub-sets of mRNA. The precise mechanism of mTOR regulation is not clear though identification of nutrient sensitive phosphorylation sites within the mTOR catalytic domain suggested a level of regulation by phosphorylation. Based on this hypothesis, mutants of mTOR were constructed at sites S2442, T2446 and S2448, substituting each site with non-phosphorylatable alanine (A) or phospho-mimetic glutamic acid (E). To allow discrimination between the effects of endogenous mTOR and that of each mTOR site mutant, an additional rapamycin resistance mutation (S2035T) was cloned into each mutant construct. S6K1 activation and 4E-BP1 phosphorylation are regulated via mTOR in response to insulin or nutrient deprivation and were used here as readouts of the effects of the mutant mTOR. The best characterised mTOR phosphorylation site is S2448, which is phosphorylated by PKB when cells are stimulated with insulin and dephosphorylated in response to nutrient deprivation. Results from transfection assays show that the alanine mutation of the S2448 site did not have a dominant negative effect on insulin stimulated S6K1 activity when compared to an mTOR construct with rapamycin resistance but wildtype C-terminal sequence. Likewise, the S2442 and T2446 mutants had no overall effect on insulin stimulated S6K1 activation. Similarly, none of the mutants were able to sustain S6K1 activity in the absence of nutrients. These results suggest that although phosphorylation of T2446 and S2448 is mutually exclusive in response to growth factors and nutrients, their individual phosphorylation may not be enough to have a direct effect on downstream S6K1 activity.

4.2 Introduction

The mTOR catalytic domain is a member of a family of phosphatidylinositol kinase (PIK)-related kinases which share homology to the p110 catalytic subunit of PI-3 kinase (Abraham, 1996). The C-terminal catalytic domain of mTOR exhibits protein kinase (as discussed below) and autokinase activity (Brown *et al.*, 1995; Withers *et al.*, 1997) but lacks lipid kinase activity (Brown *et al.*, 1995). The mechanisms which regulate mTOR protein kinase activity remain undefined. Evidence of the regulatory potential of the mTOR catalytic domain was augmented by identification of the S2448 phosphorylation site. Nutrients and insulin stimulate the phosphorylation at S2448 by PKB (Navé *et al.*, 1999; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000) and the identification of the T2446 phosphorylation site described in the previous chapter suggests that phosphorylation of mTOR is not restricted to a single site. Moreover, the antibody mTAb1 has an epitope directed to residues 2433-2450 and has been used as an mTOR activating antibody (Brunn *et al.*, 1997b). Phosphorylation of S2448 is associated with conditions that also activate mTOR kinase activity (Reynolds *et al.*, 2002; Bolster *et al.*, 2002), though exactly how phosphorylation affects mTOR kinase activity remains unresolved. Thus far, the highly specific mTOR inhibitor, rapamycin, has been extremely useful for identifying pathways which are under the control of mTOR. Two major downstream targets of mTOR were identified both of which are involved in the regulation of translation. One target identified was S6K1 (also known as p70 S6 kinase), which is stimulated by mitogens and phosphorylates the S6 protein of the 40S ribosomal subunit to initiate translation of transcripts containing 5' oligopyrimidine tracts (5'TOP); these mainly include ribosomal mRNAs (Volarević *et al.*, 2000). Rapamycin caused the rapid de-phosphorylation and inactivation of S6K1 (Chung *et al.*, 1992; Kuo *et al.*, 1992) and mTOR was subsequently identified as the rapamycin sensitive upstream regulator of S6K1 (Brown *et al.*, 1995; Hara *et al.*, 1997). Amino acid availability was also a major factor in S6K1 regulation, as nutrient deprivation mirrored the effects of rapamycin treatment (Hara *et al.*, 1998; Shigemitsu *et al.*, 1999; Wang *et al.*, 1998; Xu *et al.*, 1998a). Though S6K1 can be activated via an mTOR independent pathway (Alessi *et al.*, 1997b; Balendran *et al.*, 1999; Pullen *et al.*, 1998), studies showed that mTOR was able to directly phosphorylate S6K1 *in*

vitro in a rapamycin sensitive manner at T389, a site flanked by bulky hydrobobic residues (Burnett *et al.*, 1998; Isotani *et al.*, 1999); phosphorylation at T389 is probably important in S6K1 activation.

The second major target of mTOR identified was the eIF4E binding protein (4E-BP1). Under translationally repressive conditions, such as nutrient deprivation, eIF4E is bound to hypo-phosphorylated 4E-BP1 to inhibit CAP-dependent translation. Upon growth factor stimulation and nutrient availability, 4E-BP1 becomes phosphorylated on at least six sites (T37, T46, S65, T70, S83 and S112) causing the release of eIF4E which is then able to bind eIF4G; binding of eIF4E to eIF4G is the first step in formation of the eIF4F complex which facilitates Cap-dependent translation to progress (Proud, 2002a). Unlike the S6K1 mTOR phosphorylation motif, the 4E-BP1 phosphorylation sites are all proline directed (S/T-P). However, phosphorylation of 4E-BP1 was also shown to be rapamycin sensitive (Beretta *et al.*, 1996; Brunn *et al.*, 1997a, 1997b; Fadden *et al.*, 1997) and mTOR has been shown to directly phosphorylate 4E-BP1 *in vitro* (Brunn *et al.*, 1997a, 1997b; Burnett *et al.*, 1998; Fadden *et al.*, 1997) and by an mTOR-associated kinase (Heesom and Denton, 1999); however the multi-site phosphorylation of 4E-BP1 is considered to occur in an ordered hierarchal manner (Gingras *et al.*, 2001a).

The differences in the S6K1 and 4E-BP1 phosphorylation motifs have led to suggestions that mTOR activity is directed to a protein phosphatase. Studies have shown that mTOR is capable of phosphorylating protein phosphatase 2A (PP2A) with a greater affinity than 4E-BP1 (Peterson *et al.*, 1999). The yeast TOR signalling cascade introduced the nutrient and rapamycin sensitive Sit4/PP2A-catalytic subunit and Tap42 effecter complex (DiComo and Arndt, 1996) (Section 1.6.1). The mammalian homologue of the regulatory Tap42 protein was subsequently identified and termed α4 (Kuwahara *et al.*, 1994) which was able to bind to PP2A; PP2A activity and α4 association were shown to be sensitive to rapamycin treatment (Inui *et al.*, 1998), again implicating mTOR. There is also evidence which suggests that mTOR controls S6K1 activity via downregulation of PP2A/α4 (Parrott and Templeton, 1999).

As described above, a range of conditions regulate mTOR which in turn acts as a crucial intermediate in the regulation of cell growth; however, the mechanisms involved in the regulation of mTOR activity remain an important issue. In this chapter, the functionality of the S2448 PKB phosphorylation site was assessed in parallel to the novel mTOR phosphorylation site at T2446 by site directed mutagenesis. Additionally, S2442 was assessed as a potential PKR phosphorylation site (Section 3.2). Using the Stratagene QuikChange™ site directed mutagenesis kit, wildtype mTOR was mutated at each site to generate mTOR constructs substituted with glutamic acid as a phospho-mimetic mutant or site-substitued with alanine as a non-phosphorylatable mutant. All the mTOR mutants were constructed in a FLAG-tagged vector with a rapamycin resistant backbone which bore the S2035T rapamycin resistance mutation (Chen *et al.*, 1995; Lorenz and Heitman, 1995). The rapamycin resistance mutation was integral in assessing the *in vivo* effects of mutant mTOR. Rapamycin would inhibit endogenous mTOR activity, without blocking the activity of the mutant forms of mTOR which were introduced by transfection. As a proven target of mTOR protein kinase activity, S6K1 activation was used as readout of mTOR activity; additionally the effects on 4E-BP1 phosphorylation were explored.

The present studies suggest that phosphorylation at S2442, T2446 or S2448 is not sufficient to have an affect on S6K1 activation with respect to growth factor stimulation or nutrient availability. The results suggest that phosphorylation at these sites may not directly control mTOR activity *per se*. Additional attempts were made to assess the *in vivo* effects of the mutant mTOR towards 4E-BP1 phosphorylation but *in vitro* mTOR activity could not be consistently attained.

4.3 Results

4.3.1 Testing the integrity of expressed mTOR mutants with phospho-specific antibodies

The mTOR mutant constructs as described in Section 4.2, were made using the Stratagene Quik Change mutagenesis kit as described in Section 2.2.3.2. The accuracy of each mutation was analysed by sequencing; additionally each of the T2446A, T2446E, S2448A and S2448E plasmids were transfected into HEK cells to test the integrity of the mTOR mutants. mTOR expressed from the transgene was immunoprecipitated with FLAG, separated by SDS PAGE, transferred then probed with the phospho-mTOR (S2448) antibody. Under fed conditions, the phospho-S2448 antibody reacted with a band corresponding to the T2446A mutant (Fig. 4.1), suggesting that the T2446A mutant allows phosphorylation at S2448. The phospho-S2448 antibody also reacted faintly with the phospho-mimetic T2446E mutant. Ordinarily conditions promoting phosphorylation of the T2446 site inhibit phosphorylation of the S2448 site and vice versa (Section 3). Partial phosphorylation of S2448 may exist with the phospho-mimetic T2446E mutant, possibly because T2446E does not fully mimic the *in vivo* phosphorylation of endogenous wildtype mTOR. Nevertheless, the phospho-S2448 antibody was unable to recognise the epitope from both of the S2448 mutants, suggesting successful mutation of all the sites.

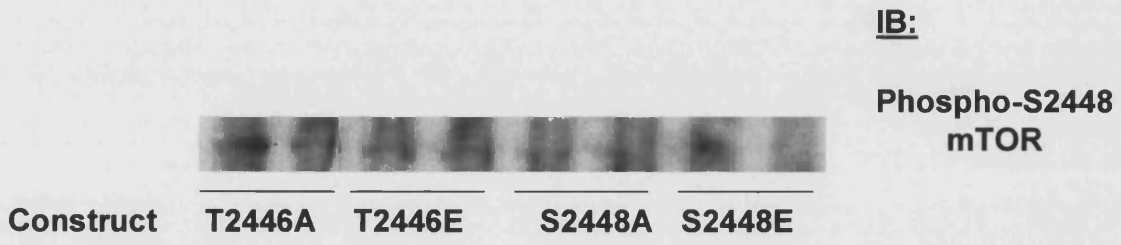

Figure 4.1 Testing the integrity of expressed mTOR mutants with phospho-S2448 antibody

HEK cells were transfected with mutant mTOR constructs as indicated, using the calcium phosphate method of transfection. After 36h, cells were lysed and equal amounts of protein were immunoprecipitated with the FLAG antibody. Immunoprecipitates were subjected to 7.5% SDS-PAGE, wet transferred then probed with the phospho-S2448-antibody. ('IB' denotes immuno-blot).

4.3.2 Variable insulin response from different cell lines

During the development and optimisation of the co-transfection experiments, HEK cells were extensively used since they were relatively easy to transfect with the calcium phosphate transfection method (Section 2.2.4.2.2) and expressed large amounts of protein from the transfected plasmid. However, during the S6K assay optimisation stage it became clear that the HEK cells had a high basal activity, even after 16-28h serum starvation, which depressed the level of insulin stimulation observed (data not shown). Therefore HEK cells were tested against CHO-IR cells which stably expressed the insulin receptor. Three HEK cell lines within the laboratory, but obtained from different sources, were tested using phosphorylation of PKB (S473) as a read-out of insulin stimulation. CHO-IR cells had significantly lower basal PKB (S473) phosphorylation than HEK cells and insulin stimulation was almost double that observed in all three HEK cell lines (Fig. 4.2). Subsequent co-transfections with mutant mTOR and S6K1 were performed in CHO-IR cells.

4.3.3 Insulin stimulated S6K1 activity in the presence of mTOR mutants

As described in Section 1.5.2, the S6 kinases are encoded by two genes designated S6K1 (or S6K1α) and S6K2 (or S6K2β) (Dufner and Thomas, 1999). The isoform used in transfection experiments described here was 70kDa glu-glu tagged S6K1αII (denoted as S6K1 in all subsequent experimental descriptions), a gift from Dr I. Gout (Ludwig Institute for Cancer Research, London). S6K1αII was used because it is cytoplasmic and more sensitive to rapamycin and nutrient deprivation than the S6K2 isoforms (Gout *et al.*, 1998; Minami *et al.*, 2001). To assess the effect of the mTOR mutants on S6K1 activity, the mTOR constructs (excluding the FLAG tagged wildtype), were made in a rapamycin resistant backbone containing the S2035T mutation (Section 2.2.3.2 and 4.2). The influence of each mutant could only be assessed by suppression of endogenous mTOR which was achieved by treatment with rapamycin prior to insulin stimulation.

CHO-IR cells were co-transfected with glu-glu tagged wildtype S6K1 and FLAG tagged mTOR and were treated as described in the Fig. 4.3 legend; cells were lysed and half the lysate was used in a FLAG immunoprecipitation, which was subjected to SDS-PAGE, wet transferred and probed back for mTOR. The other half of the lysate was used in glu-glu immunoprecipitation for S6K1 which was used in an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 80S ribosomes as substrate. Assay products were subjected to SDS-PAGE and the gel cut at the 46kDa marker; the upper portion was wet transferred and probed for S6K1. Activity was determined from the lower portion of the gel which was fixed, dried and exposed to radiographic film. An example of a western blot showing total mTOR and total S6K1 with the 80S autoradiograph from a co-transfection of S6K1 with wildtype or M1 (rapamycin resistant) mTOR, in the absence and presence of rapamycin, is shown in Fig. 4.3A. Within each experiment, control western blots were performed to check mTOR transfection efficiency and mTOR was always equal as demonstrated in Fig. 4.3A. Total S6K1 was quantitated from the western blot to normalise S6K1 activity detected in the autoradiograph of phosphorylated 80S (Fig. 4.3A). The activity data from the same co-transfections is shown in Fig. 4.3B. In these experiments rapamycin completely blocked insulin-stimulated activation of S6K1 in CHO-IR cells transfected with S6K1 and wildtype mTOR (Fig. 4.3B). Cells transfected with S6K1 and the M1 rapamycin resistant mTOR mutant maintained 2 fold activation of S6K1 even after rapamycin treatment (Fig. 4.3B). This demonstrated that the effect of the M1-rapamycin resistant mTOR on S6K1 activity was clearly separable from that of rapamycin sensitive wildtype mTOR.

The S6K1 activity when co-transfected with each mTOR mutant in the presence of rapamycin is presented in Fig. 4.3C. Within each experiment, the M1 mutant was used as the readout of 'normal' S6K1 activity in the presence of rapamycin because although containing the rapamycin resistant S2035T mutation, the rest of the mTOR construct was the same as wildtype mTOR. S2448 is normally phosphorylated with insulin stimulation (Navé *et al.*, 1999) and would be expected to be an activating phosphorylation; however, the non-phosphorylatable S2448A mutant did not significantly decrease S6K1 activity compared to the M1 mutant. Similarly, the S2448E phospho-mimic did not increase basal S6K1 activity compared to both the

M1 and S2448A mutants, suggesting that S2448 phosphorylation is not coupled to activation of mTOR or downstream S6K1 activity in response to insulin.

The T2446 mTOR phosphorylation site identified in Section 3 is not insulin stimulated and was hypothesized to be a negative regulatory site. Here, the T2446E phospho-mimetic mutant was expected to lower S6K1 activity as T2446 phosphorylation was postulated to block insulin responsive S2448 phosphorylation. However, the T2446E mutant displayed similar activation of S6K1 as both the S2448A and S2448E mutants (Fig. 4.3C). Similarly the T2446A mutant was expected to allow more efficient phosphorylation at the S2448 position and increase S6K1 activity; however the T2446A mutant did not show an increase in basal S6K1 activity. Therefore, phosphorylation of T2446 and S2448 may be mutually exclusive but their individual phosphorylation does not seem to considerably affect downstream S6K1 activity in response to insulin.

S2442 was not identified as an mTOR phosphorylation site but the proximity to both the T2446 and S2448 phosphorylation sites may be important for their regulation. However the S6K1 activity profile of both the S2442A and S2442E mutants was comparable to that observed when S6K1 was co-transfected with the M1 mutant (Fig. 4.3C) which also suggests that it is not important in regulation of S6K1 activity.

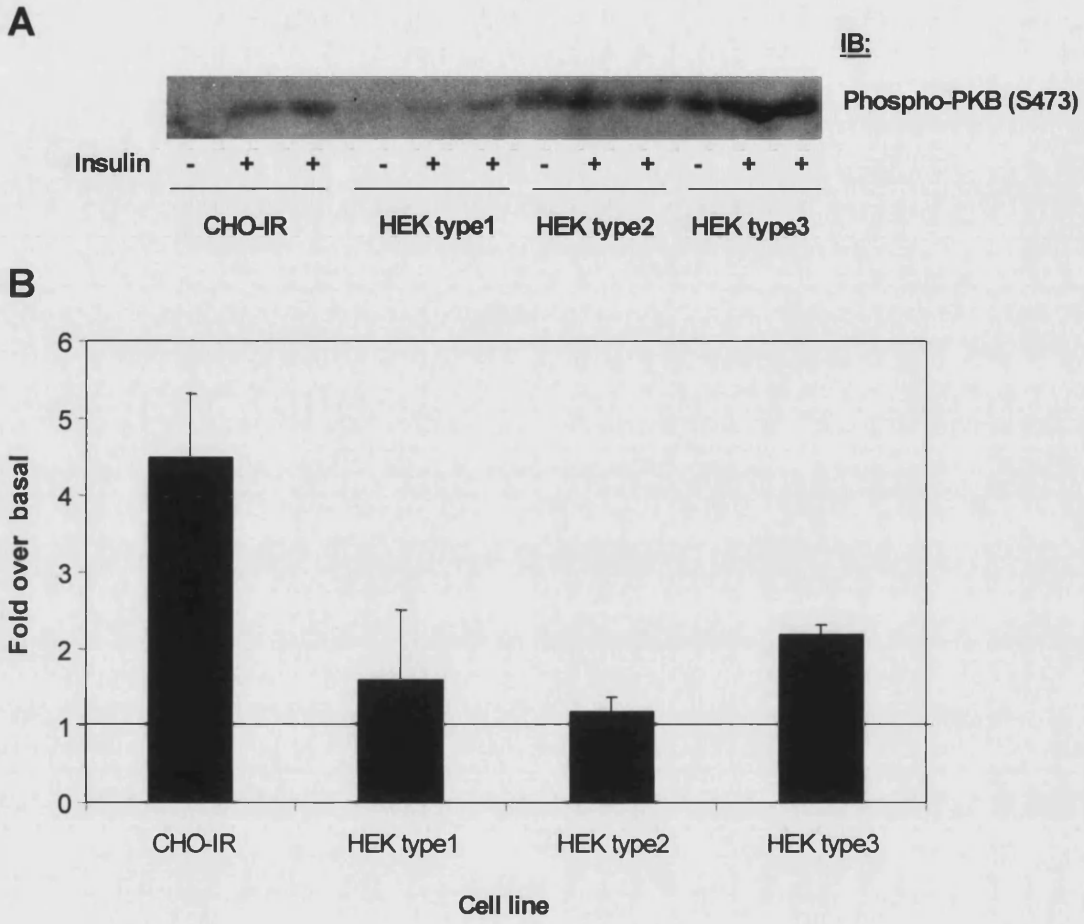

Figure 4.2 Variable insulin response from different cell lines

Three HEK 293 cell-lines from different sources (shown as type1, 2 or 3) and CHO-IR cells were serum starved overnight in DMEM and F12 (Hams) respectively. Cells were stimulated with 100nM insulin for 10min then lysed, the protein concentration was determined and equal amounts of protein were subjected to SDS-PAGE, wet transferred to PVDF then probed with phospho-PKB (S473) as shown (A). Relative fold over basal was determined for each cell type as presented in (B).

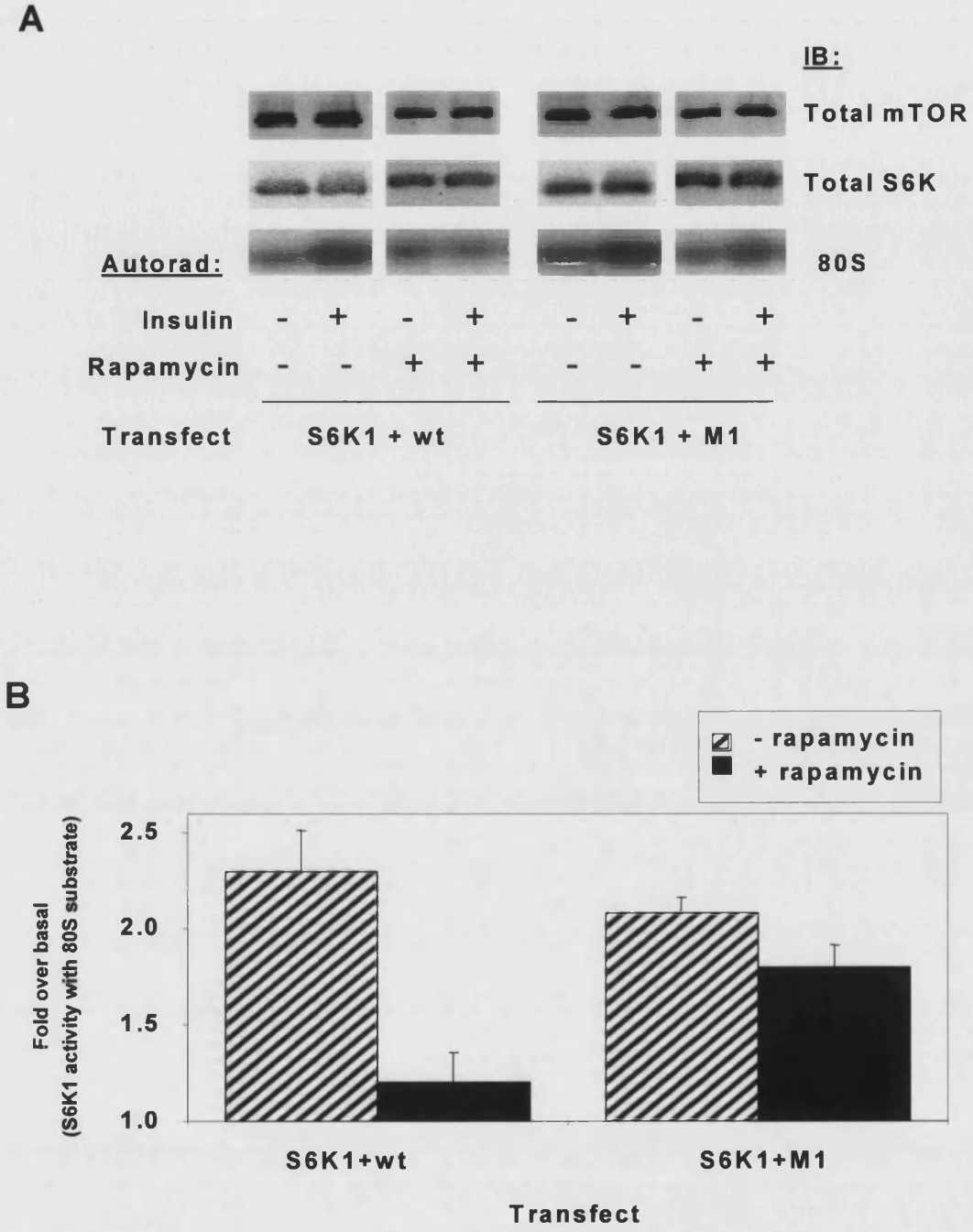

Figure 4.3 Insulin stimulated S6K1 activity in the presence of mTOR mutants

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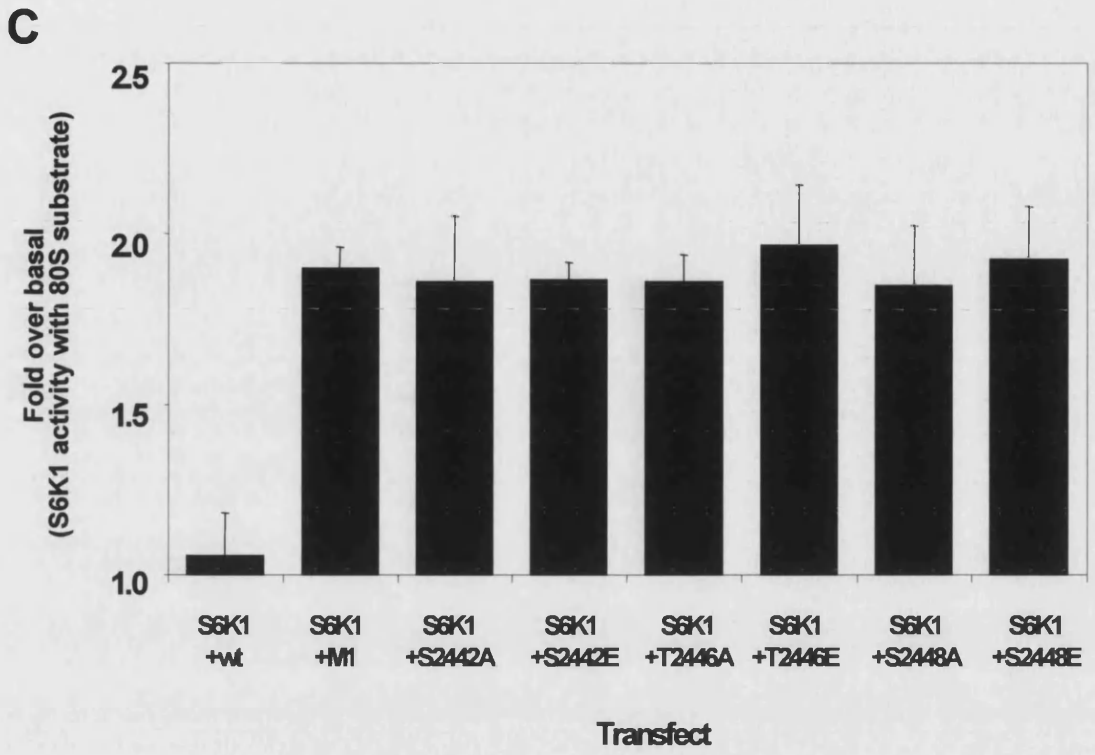

Figure 4.3 Insulin stimulated S6K1 activity in the presence of mTOR mutants

CHO-IR cells were co-transfected with 5µg FLAG-tagged mTOR mutants and 3µg glu-glu tagged S6K1αII (denoted in figures as S6K1) using the lipofectamine method of transfection. Cells were serum starved overnight in F12 medium then pre-incubated with 50nM rapamycin or vehicle for 30min, followed by insulin stimulation for 10min. Cells were lysed and the lysate split: half the lysate was used in FLAG immunoprecipitation, which was subjected to 7.5% SDS-PAGE, wet transferred and probed for mTOR; the other half was used in glu-glu immunoprecipitation for S6K1 which was used in an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 80S ribosomes as substrate. The assay products were subjected to 10% SDS-PAGE and the gel cut at the 46kDa marker; the lower portion was fixed, dried and exposed to radiographic film. The upper portion was wet transferred and probed for S6K1 for normalisation of S6K1 activity.

An example of a western blot showing total mTOR and total S6K with the 80S autoradiograph (autorad) from a co-transfection of S6K1 with wildtype (wt) or M1 (rapamycin resistant) mTOR, in the absence and presence of rapamycin, is shown in (A); the activity data from the same co-transfections is shown in (B). The S6K1 activity when co-transfected with each mTOR mutant in the presence of rapamycin is presented in (C). All data is expressed as fold over basal ± SEM. n=3 in duplicate

4.3.4 Effect of mTOR mutants on the regulation of S6K1 activity by nutrient availability

The effect of the mTOR mutants in response to nutrient availability was assessed with the same principal of co-transfecting CHO-IR cells with glu-glu tagged wildtype S6K1αII (S6K1) and FLAG tagged mTOR, using rapamycin to inhibit endogenous mTOR in the presence or absence of nutrients. Cells were lysed and half the lysate was used in a FLAG immunoprecipitation for SDS-PAGE and western blotting for the presence of mTOR. As described above the other half of the lysate was used in glu-glu immunoprecipitation for S6K1 which was used in an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 80S ribosomes as substrate. Assay products were analysed as described above. An example of a western blot showing total mTOR and total S6K1 with the 80S autoradiograph from a co-transfection of S6K1 with wildtype or M1 (rapamycin resistant) mTOR, in the absence and presence of rapamycin, is shown in Fig. 4.4A.

Within each experiment control western blots were always performed to check mTOR transfection efficiency and total mTOR was always equal as demonstrated in Fig. 4.4A. Total S6K1 was quantitated from the western blot to normalise S6K1 activity detected in the autoradiograph of phosphorylated 80S (Fig. 4.4A). The activity data from the same co-transfections is shown in Fig. 4.4B; S6K1 activity was expressed relative to S6K1 activity when co-transfected with the M1 mutant. This was calculated by standardising S6K1 activity to S6K1 activity when co-transfected with the M1 mutant (in the absence of nutrients), which was used as the readout of 'normal' S6K1 activity. The activity data from the same co-transfections is shown in Fig. 4.4B. In CHO-IR cells transfected with S6K1 and wildtype mTOR, there was a 4 fold (relative to S6K1 co-transfection with M1 -nutrients) activation of S6K1 in the presence of nutrients; this was suppressed to basal in the absence of nutrients. The same profile was observed when S6K1 was co-transfected with the rapamycin resistant M1 mutant. This showed that the M1-rapamycin resistant mTOR construct behaved like wildtype mTOR in the absence of nutrients.

The S6K1 activity with each mTOR mutant in the presence of rapamycin is presented in (Fig. 4.4C). As mentioned previously, the M1 mutant was used as the readout of 'normal' S6K1 activity in the presence of rapamycin because although containing the rapamycin resistant mutation the remainder of the construct was wildtype mTOR. Under nutrient deprivation the S2448 phosphorylation site is dephosphorylated and this was hypothesized to negatively regulate mTOR (Navé *et al.*, 1999); therefore the phospho-mimetic S2448E mutant used here was expected to have an activating influence on downstream S6K1 activity. However, like the M1 mutant it could not sustain S6K1 activity in the absence of all nutrients. The non-phosphorylatable S2448A mutant also activated S6K1 activity in the presence of nutrients, suggesting that phosphorylation of S2448 is not essential for activation of mTOR or downstream S6K1 activity in response to nutrients.

The T2446 mTOR phosphorylation site identified in Chapter 3 is phosphorylated in response to nutrient deprivation so the T2446A mutant would be expected to promote S6K1 activity since it would allow S2448 phosphorylation. However, as established above, S2448 phosphorylation is not enough to enable S6K1 activity, and data from both T2446 mutants suggests likewise. So although phosphorylation of T2446 and S2448 is mutually exclusive in response to nutrients, their individual phosphorylation does not seem to affect downstream S6K1 activity.

The S6K1 activity profiles of S2442A and E were comparable to that observed in the M1 mutant (Fig. 4.4C) which suggests that this site is not important in regulation of S6K1 activity.

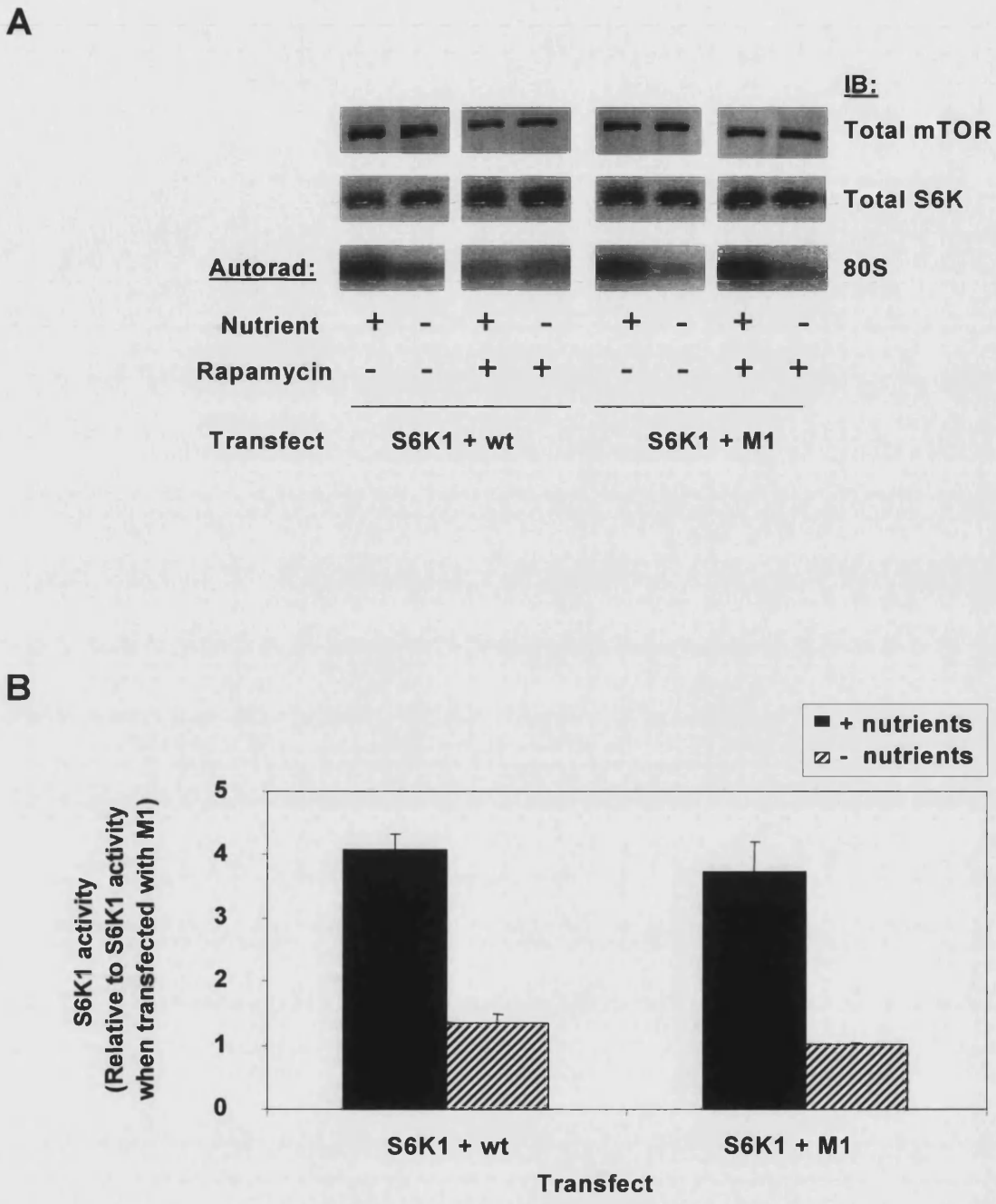

Figure 4.4 Effect of mTOR mutants on the regulation of S6K1 activity by amino acid availability

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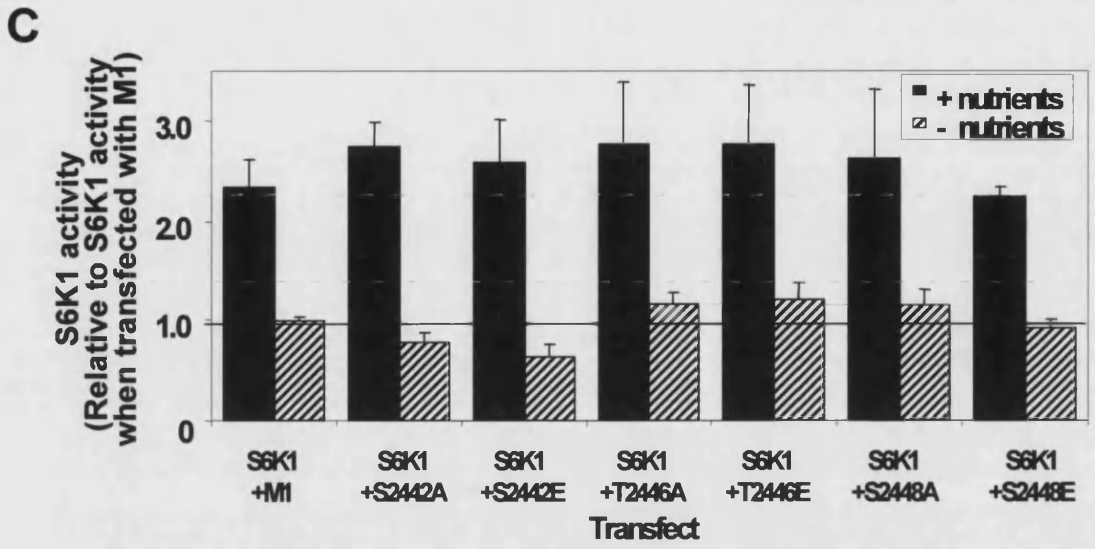

Figure 4.4 Effect of mTOR mutants on the regulation of S6K1 activity by amino acid availability

CHO-IR cells were co-transfected with 5µg FLAG-tagged mTOR mutants and 3µg glu-glu tagged S6K1αII (denoted in figures as S6K1) using the lipofectamine method of transfection. Cells were incubated in F12 medium containing 10% serum for 1 hour or nutrient starved in D-PBS in the presence of vehicle (B) or 50nM rapamycin (C). Cells were lysed and the lysate split: half the lysate was used in FLAG immunoprecipitation, which was subjected to 7.5% SDS-PAGE, wet transferred and probed for mTOR; the other half was used in glu-glu immunoprecipitation for S6K1 which was used in an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 80S ribosomes as substrate. The assay products were subjected to 10% SDS-PAGE and the gel cut at the 46kDa marker; the lower portion was fixed, dried and exposed to radiographic film. The upper portion was wet transferred and probed for S6K1 for normalisation of S6K1 activity.

An example of a western blot showing mTOR and S6K with the 80S autoradiograph (autorad) from a co-transfection of S6K1 with wildtype (wt) or M1 (rapamycin resistant) mTOR, in the absence rapamycin, is shown in (A); the activity data from the same co-transfections is shown in (B). The S6K1 activity when co-transfected with each mTOR mutant in the presence of rapamycin is presented in (C) ± SEM. Data is expressed as S6K1 activity relative to the activity of S6K1 when co-transfected with the M1 mutant (-nutrient sample) which was used as the readout of 'normal' S6K1 activity. n=2 in duplicate.

4.3.5 Blotting phospho-4E-BP1 from co-transfection with T2446 mTOR mutants

As established in Section 1.5.1, 4E-BP1 has been reported as a substrate of mTOR and phosphorylation of 4E-BP1 has been used as readout of mTOR activity. Therefore in parallel to assessing the impact of the mutant mTOR on S6K1 activity, the effects of mutant mTOR on 4E-BP1 phosphorylation were also investigated using phospho-specific 4E-BP1 antibodies (Mothe-Satney *et al.*, 2000a). HA tagged human 4E-BP1 (gift from A.-C. Gingras, McGill University, Canada) was used to transfect HEK cells. Cells were stimulated with insulin then immunoprecipitated with anti-HA antibody; the immunoprecipitates were blotted with phospho-4E-BP1 at positions S65 or T70 (equivalent to rat numbering S64 and T69 respectively) antibodies (gift from J. C. Lawrence Jr., University of Virginia, USA) as shown in Fig. 4.5A. Insulin stimulates the phosphorylation of 4E-BP1 and here phospho-4E-BP1 S65 and T70 seemed able to detect an increase in 4E-BP1 phosphorylation in response to insulin stimulation. Both phospho-antibodies reacted with HA immunoprecipitates from non-transfected cells; the background signal was faintly insulin responsive and it is likely that the HA immunoprecipitation brought down a contaminating protein which was reactive to the phospho-antibody. Preliminary experiments were similar to the S6K1 co-transfections; FLAG-tagged mTOR mutants were co-transfected with HA-tagged wildtype 4E-BP1 in HEK cells. An example of a co-transfection with the M1 and T2446A and T2446E mutants is shown in Fig. 4.5B. Transfected HEK cells were serum starved overnight then treated with 50nM rapamycin prior to insulin stimulation. Cells were lysed and immunoprecipitated with anti-HA; immunoprecipitates were subjected to 15% SDS-PAGE then transferred and sequentially probed with phospho-4E-BP1 at S65 and T70. Cells transfected with wildtype 4E-BP1 were expected to be rapamycin sensitive in the absence of mutant mTOR. The phospho-antibodies detected a non insulin-responsive background, similar to the background from HA immunoprecipitates from non-transfected cells (Fig. 4.5). However, cells co-transfected with 4E-BP1 and the M1 rapamycin resistant mutant were expected to support 4E-BP1 phosphorylation in the presence of rapamycin. However, neither phospho-antibody detected an insulin-stimulated increase in 4E-BP1

phosphorylation. Similarly, no change in 4E-BP1 phosphorylation was detected when co-expressed with either T2446A or T2446E. This interpretation may not be accurate because of the background detected by both phospho-antibodies from HA immunoprecipitates from non transfected cells. The background from the phospho-specific antibodies made it difficult to interpret the effects of the mTOR mutants on 4E-BP1 phosphorylation. This method was rejected in favour of a direct assay of mTOR activity with 4E-BP1 as substrate.

4.3.6 Assessment of 4E-BP1 phosphorylation in mTOR *in vitro* kinase assays at high ATP concentration

Dennis *et al.*, (2001) previously reported that mTOR acted as a sensor of cellular levels of ATP and mTOR activity was dependent on the ATP concentration; their *in vitro* kinase assays were conducted using high ATP concentration, and this method was tested here in both radioactive and non-radioactive assays to assess assay sensitivity. HEK cells were transfected with FLAG-tagged wildtype mTOR and were treated in the presence or absence of nutrients (as described in Fig. 4.6 legend) to assess the effect on 4E-BP1 phosphorylation. Extracts were immunoprecipitated with anti-FLAG then subjected to an *in vitro* kinase assay with 3mM ATP and 4E-BP1 as substrate in the presence (Fig. 4.6 autoradiograph) or absence (Fig. 4.6 western blots) of [γ-^{32} P] ATP. Low level 4E-BP1 phosphorylation was detected in the autoradiograph, though no difference was observed in the presence or absence of nutrients. The low level phosphorylation seems more likely to be background phosphorylation since the 'bead only' (immunoprecipitation with anti-mouse IgG in the absence of FLAG antibody) negative control demonstrates the same level of phosphorylation and the level of phosphorylation detected is significantly lower than that of the positive control. The radioactive kinase assay uses high concentrations of unlabelled ATP and the specific activity of radio-labelled ATP is relatively low, to improve sensitivity of the assay a non radioactive approach, at high ATP concentration, was also used. 4E-BP1 was allowed to phosphorylate in the presence of mTOR immunoprecipitates and the level of phosphorylation was determined using phospho-4E-BP1 antibody as described in Fig. 4.5. Western blot analysis showed that phospho-4E-BP1 (S65) reacted to low level phosphorylation of 4E-BP1 which

was indistinguishable from the background 'bead only' control. Again, the MAPK positive control showed strong phosphorylation of 4E-BP1 (S65) in the western blot which was clearly distinguishable from the background.

4.3.7 mTOR activity towards 4E-BP1 is dependent on the immunoprecipitating antibody

The inability to assay mTOR activity towards exogenous substrates even at high ATP concentration may stem from the method of assay or the immunoprecipitating mTOR antibody, given that mTOR activity has been found to be dependent on the antibody used to immunoprecipitate mTOR (Brunn *et al.*, 1997b; Heesom and Denton, 1999). Therefore, three different *in vitro* kinase methods were used to assay mTOR activity. mTOR was immunoprecipitated with the following mTOR antibodies: αTOR; (Heesom and Denton, 1999) raised against the C-terminal portion of mTOR (a.a. 2433-2450) which has the identical epitope to the activating mTAb1 antibody (Brunn *et al.*, 1997b); anti-FRAP (Calbiochem) a polyclonal antibody raised against a peptide corresponding to residues 2524-2538 in rat mTOR which is conserved in the human sequence; or hTOR (Hosoi *et al.*, 1998) (Oncogene) a mouse monoclonal antibody raised against a peptide corresponding to residues 230-240 of human mTOR, for endogenous mTOR. FLAG antibody was used for transfected wildtype mTOR. Assays were performed in the presence of [γ-^{32} P] ATP and Mg^{2+} using 0.5μg 4E-BP1 as substrate. The first method was previously used successfully in the laboratory to assay mTOR autokinase activity as described in Withers *et al.*, (1997). The other two methods were as described in Brunn *et al.*, (1997a) and Mothe-Satney *et al.*, (2000a). Only the anti-FRAP antibody (Calbiochem) immunoprecipitated mTOR autokinase activity (Fig. 4.7A). In contrast, only the αTOR antibody (Heesom and Denton, 1999) was successful in immunoprecipitating activity towards 4E-BP1 using the Brunn and Mothe-Satney methods. Therefore, isolation of mTOR kinase activity is dependent on the antibody used in the immunoprecipitation. The αTOR epitope is identical to that of the mTAb1 antibody (Brunn et al., 1997b) and encompasses the mTOR T2446 and S2448 phosphorylation sites.

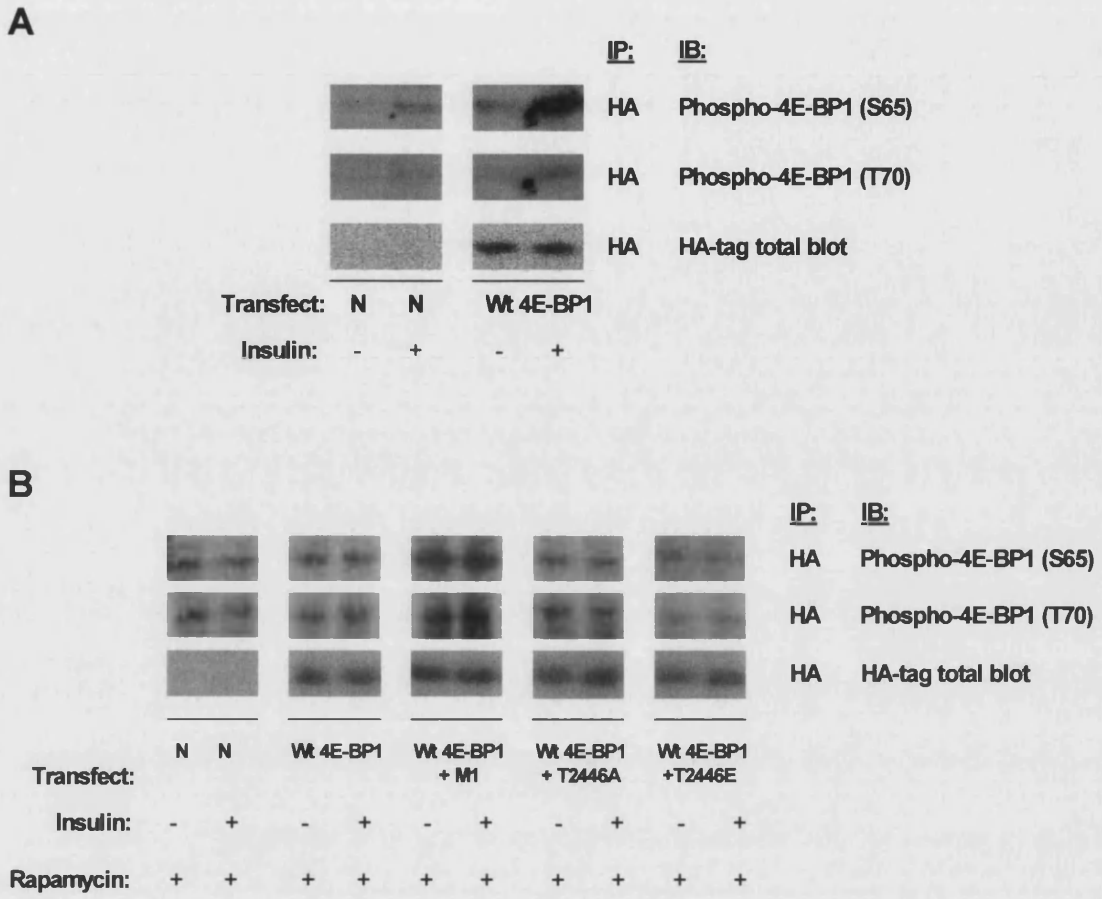

Figure 4.5 Blotting phospho-4E-BP1 from co-transfection with T2446 mTOR mutants

(A) Non-transfected HEK cells (indicated as 'N') and HEK cells transfected with 5µg HA-tagged wildtype 4E-BP1 were serum starved overnight then stimulated with 100nM insulin for 10min. Cells were lysed and equal amounts of protein were immunoprecipitated with HA antibody. The immunoprecipitates were subjected to 15% SDS-PAGE, wet transferred in SDS-free transfer buffer then probed sequentially with phospho-4E-BP1 S65 or T70 and finally with anti-HA to check transfection efficiency.

(B) HEK cells were also co-transfected with 5µg HA-tagged wildtype 4E-BP1 and 5µg FLAG tagged mTOR T2446 mutant as indicated using the calcium phosphate method of transfection. Cells were serum starved overnight then treated with 50nM rapamycin for 30min followed by stimulation with 100nM insulin for 10min. Cells were lysed and the lysates were used in HA immunoprecipitations which were subjected to 15% SDS-PAGE, wet transferred in SDS-free transfer buffer then blotted sequentially with phospho-4E-BP1 S65, T70 and finally with anti-HA to check transfection efficiency.

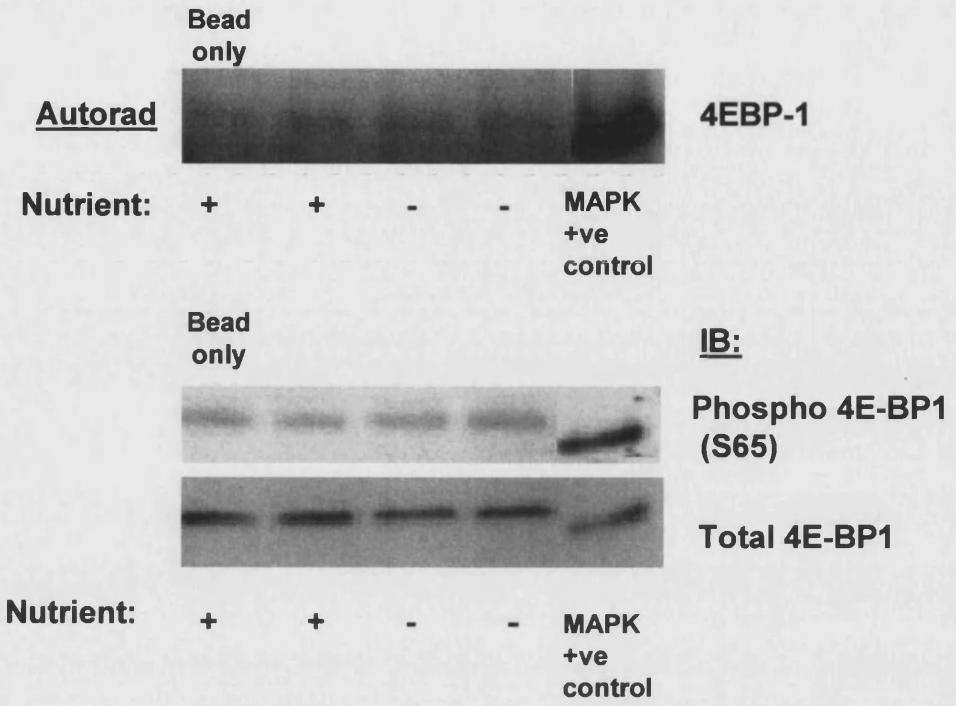

Figure 4.6 Assessment of 4E-BP1 phosphorylation in mTOR *in vitro* kinase assays at high ATP concentration

HEK cells were transfected with FLAG-tagged wildtype mTOR. Cells were treated in the presence or absence of nutrients for 1 hour as indicated. Lysates were immunoprecipitated with FLAG. 'Bead only' represents immunoprecipitation with mouse IgG in the absence of antibody. One set of FLAG immunoprecipitates was subjected to an *in vitro* kinase assay in the presence of 3mM ATP (Dennis *et al.*, 2001), [γ-^{32} P] ATP and Mg^{2+} with 0.5µg 4E-BP1 as substrate. Recombinant MAPK (500ng) was used as positive control. Another set of FLAG immunoprecipitates was subjected to a 'cold' *in vitro* kinase assay in the presence of 3mM ATP and Mg^{2+} with 0.5µg of recombinant 4E-BP1 as substrate. MAPK was again used as positive control. Reactions were stopped with 4× sample buffer then subjected to 12% Tris/tricine SDS-PAGE. Reaction products from the assay with [γ-^{32} P] ATP were analysed by autoradiography as presented in the top panel. Reaction products from the assay without [γ-^{32} P] ATP were wet transferred in SDS-free transfer buffer and probed with phospho-4E-BP1 (S65), then stripped and re-probed for total 4E-BP1. Results are shown in duplicate.

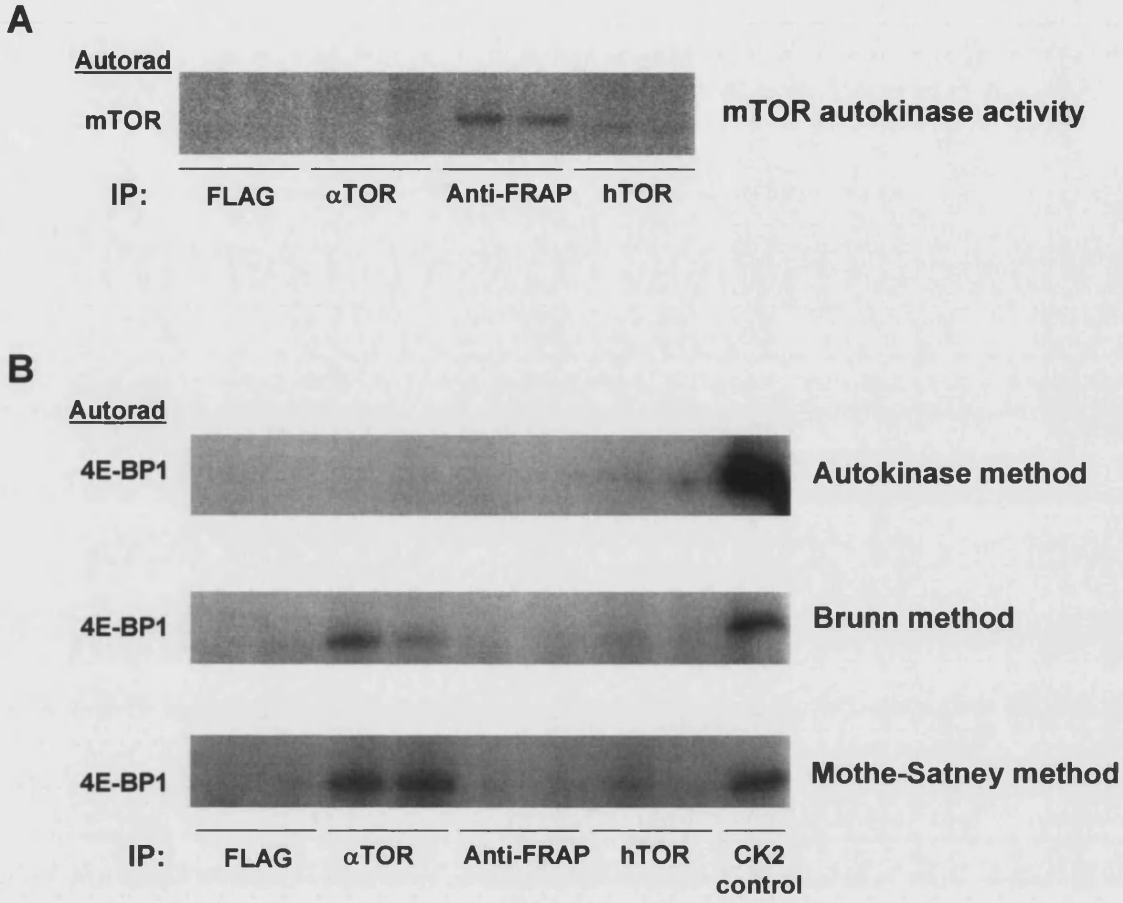

Figure 4.7 mTOR activity towards 4E-BP1 is dependent on the immuno-precipitating antibody

Non-transfected CHO-IR cells or wildtype mTOR transfected (immunoprecipitated with FLAG) CHO-IR cells were lysed and immunoprecipitated with: αTOR antibody (Heesom and Denton, 1999), anti-FRAP (Calbiochem), or hTOR (Oncogene) for endogenous mTOR; or FLAG for transfected mTOR. Immunoprecipitates were processed and subjected to an *in vitro* kinase assay according to 3 published methods as described in Section 2.2.7.3 and 2.2.7.5. Assays were in the presence of [γ-^{32} P] ATP and Mg^{2+} for the autokinase assay as presented in (A) or additionally with 0.5μg 4E-BP1 as substrate (B); recombinant CK2 (500ng) was used as positive control in (B). All reactions were stopped with 4× sample buffer. Reaction products from the mTOR autokinase assay were subjected to 7.5% SDS-PAGE then fixed, dried and analysed by autoradiography as shown in (A). Reaction products from (B) were subjected to 12% Tris/tricine SDS-PAGE, processed as described above then analysed by autoradiography as indicated. Results are shown in duplicate.

4.3.8 FKBP/rapamycin is unable to inhibit endogenous mTOR activity *in vitro*

By identifying the αTOR antibody as the most suitable for immunoprecipitating mTOR activity, it was clear that if mutant mTOR was transfected it would not be possible to isolate mTOR activity with the FLAG antibody. Inhibition of endogenous mTOR would have to be performed *in vitro*, since binding of the FKBP/rapamycin complex is not covalent and would be washed from wildtype mTOR during processing of αTOR immunoprecipitates. Therefore, the mTOR mutants were transfected and immunoprecipitated with αTOR, isolating both endogenous and transgene expressed mTOR. These immunoprecipitates were incubated with FKBP/rapamycin to isolate activity from the rapamycin resistant mutants only. In preliminary trials HEK (Fig. 4.8A) or CHO-IR (Fig. 4.8B) cells were lysed and immunoprecipitated with αTOR or transfected with the M1 mutant then lysed and immunoprecipitated with αTOR. Immunoprecipitates were processed according to the Mothe-Satney method (Mothe-Satney *et al.*, 2000a). A fraction of each immunoprecipitate was extracted prior to the *in vitro* kinase assay to assess the level of mTOR in each sample (top panel Fig. 4.8 A & B). Immunoprecipitates were pre-incubated in assay buffer or with 10μM FKBP and 10μM rapamycin in assay buffer, for 1hr at 25°C, to inhibit endogenous rapamycin sensitive mTOR, then assayed. The amount of mTOR immunoprecipitated was substantial in both cell types (top panel Fig. 4.8 A & B), although HEK cells seemed to express the recombinant gene more efficiently than CHO-IR cells. There only seemed to be a background level of 4E-BP1 phosphorylation detected by autoradiography (lower panel Fig. 4.8 A & B) when compared to the casein kinase II (CK2) positive control. The FKBP/rapamycin additions did not inhibit the αTOR immunoprecipitated from non-transfected cells in either cell type though this is unlikely to be from a defect in FKBP. It is likely that a contaminating kinase was co-immunoprecipitated with mTOR which had low level activity and was insensitive to FKBP/rapamycin, since the 'bead only' immunoprecipitation had the same background. In a similar experiment, CHO-IR cells were transfected with M1 (rapamycin resistant mTOR), the T2446A or the T2446E mutant, then immunoprecipitated and processed as described above, with or without FKBP/rapamycin in the assay. Non-transfected

cells immunoprecipitated with αTOR brought down a background level of activity when compared to the positive control. The background level of phosphorylation was insensitive to FKBP/rapamycin treatment and was present in cells transfected with the M1 mutant. Although there seemed to be a negligible level of phosphorylation from samples with the T2446A or E mutant, this could have been due to transfection efficiency and results here are inconclusive because of the contaminating kinase activity from non-transfected cells. The inconsistency in isolating mTOR activity persisted despite refining conditions.

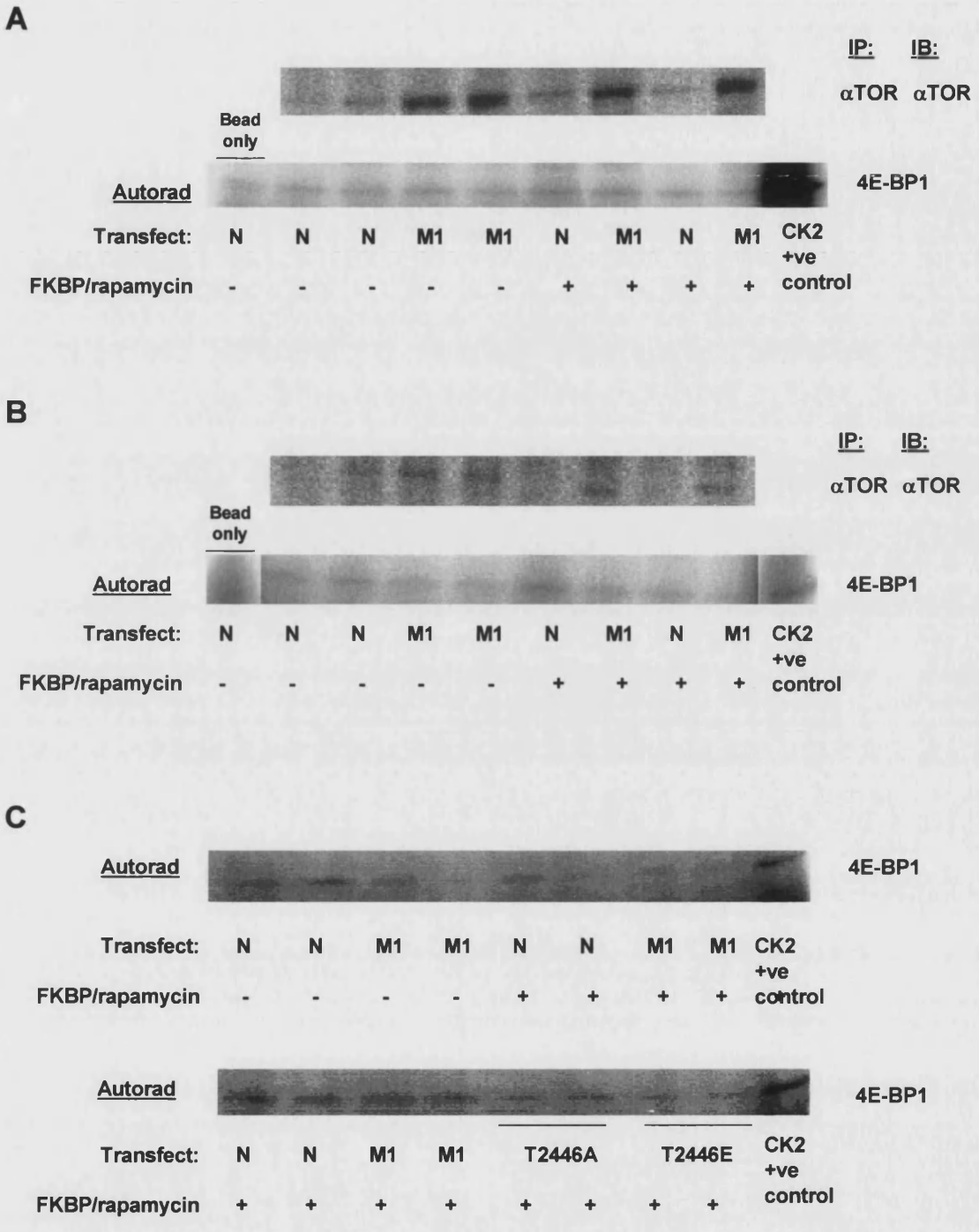

Figure 4.8 FKBP/rapamycin is unable to inhibit endogenous mTOR activity *in vitro*

See legend overleaf.

Figure 4.8 FKBP/rapamycin is unable to inhibit endogenous mTOR activity *in vitro* (legend)

Non-transfected (denoted by N) or M1 (rapamycin-resistant mTOR construct) transfected HEK (A) or CHO-IR (B) cells were lysed and immunoprecipitated with αTOR. 'Bead only' represents immunoprecipitation with protein A in the absence of antibody. A fraction of the mTOR immunoprecipitate was extracted for blotting with αTOR (top panel A & B). The remaining immunoprecipitate was incubated in assay buffer or with 10μM FKBP and 10μM rapamycin in assay buffer for 1hr at 25°C with agitation at 300rpm. Immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 0.5μg 4E-BP1 as substrate. Recombinant CK2 (500ng) was used as a positive control. The assay method was as described by Mothe-Satney *et al.*, (2000). Reactions were stopped with 4× sample buffer then subjected to 12% Tris/tricine SDS-PAGE. Gels were fixed, dried and analysed by autoradiography as shown in the bottom panel of (A) & (B). Results shown are in duplicate and representative of 2 independent experiments.

(C) CHO cells were transfected with M1 or T2446A or E mutant using the lipofectamine method of transfection. Equal amounts of protein were immunoprecipitated with αTOR then processed as described by Mothe-Satney et al., (2000). Immunoprecipitates were incubated in assay buffer or with 10μM FKBP and 10μM rapamycin in assay buffer for 1hr at 25°C with agitation at 300rpm, then subjected to an *in vitro* kinase assay in the presence of [γ-^{32} P] ATP and Mg^{2+} with 0.5μg 4E-BP1 as substrate. Recombinant CK2 (500ng) was used as a positive control. Reactions were stopped with 4× sample buffer then subjected to 12% Tris/tricine SDS-PAGE. Gels were fixed, dried and analysed by autoradiography. Results shown are in duplicate.

4.4 Discussion

In the previous chapter evidence was presented which showed that S2448 and T2446 are sites of reversible phosphorylation in the mTOR catalytic domain. An inverse relationship between these two phosphorylation sites was identified, which was dependent on growth factor and nutrient status, suggesting that phosphorylation at these sites had regulatory effects on mTOR function. The mechanisms by which the S2448 and T2446 phosphorylation affect downstream targets are unclear. The present study used mutational analysis to dissect the effects of phosphorylation at S2442, T2446 and S2448 on S6K1 activity. Evidence shows that none of the individual mutations caused a difference in S6K1 activity when compared with the M1 construct, which contained the rapamycin resistant S2035T mutation but had wildtype S2442, T2446 and S2448. The S2448 phosphorylation site was considered a likely activating site in mTOR since phosphorylation is mediated by mitogenic stimuli which activate the mTOR pathway (Navé *et al.*, 1999; Reynolds *et al.*, 2002; Sekulić *et al.*, 2000). Here, the phospho-mimetic S2448E mutation did not increase basal S6K1 activity and neither did the non phosphorylatable S2448A mutant significantly decrease S6K1 activity compared to the rapamycin resistant M1 construct, or any of the mutant constructs as determined by 80S ribosome phosphorylation. The T2446 phosphorylation site, which is phosphorylated under conditions that normally reduce activity downstream of mTOR, was hypothesized as the mutually exclusive partner site to S2448 and thus considered as a negative regulatory site in response to insulin. Here, the T2446E mutant did not significantly reduce S6K1 activity and the T2446A mutant, which should allow better S2448 phosphorylation, did not increase S6K1 activity. Furthermore, the adjacent S2442 site mutations shared the same S6K1 activity profiles as the other mutants. These data suggest that mTOR phosphorylation does not directly affect S6K1 activity. These findings were confirmed by another group who used a similar approach in which they produced rapamycin resistant T2446A, S2448A and a double T2446A/S2448A mTOR mutant. When these mutants were co-transfected with S6K1, S6K1 activity was not affected (Sekulić *et al.*, 2001).

The effects of mutant mTOR in relation to nutrient availability were not assessed by Sekulić *et al.*, (2000). Data presented here also suggest that each single mutation has no effect on downstream S6K1 activity in response to nutrients. As mentioned earlier the S2448 phosphorylation site is considered an activating phosphorylation. However, the S2448E phospho-mimetic mutant did not support S6K1 activity in the absence of nutrients. In fact none of the mutants could overcome nutrient starvation and S6K1 activity remained at a basal level in response to nutrient deprivation.

The data presented here does not support the hypothesis that phosphorylation of S2448 or T2446 are involved in regulation of mTOR activity towards S6K1 in cells. It is possible that the glutamic acid mutation does not fully mimic the phosphorylated form of mTOR. The effects of mTOR phosphorylation may be more subtle than having an effect on a downstream target and might instead act to regulate the signalling potential of factors associated with mTOR, as reports have suggested that mitogen stimulation does not modulate *in vitro* mTOR kinase activity towards either S6K1 or 4E-BP1 (Dennis *et al.*, 2001; Hara *et al.*, 1998).

As no effects of changes at S2448 or T2446 were detected *in vivo*, we sought to determine whether any effects could be detected using *in vitro* assays under defined conditions. However, it proved extremely difficult to develop a reliable method for consistently assaying mTOR protein kinase activity. mTAb1 has been described and used as an activating mTOR antibody (Brunn *et al.*, 1997b) and its epitope spans a repressor domain in mTOR (Sekulić *et al.*, 2001). Deletion of this region corresponds with an increase in mTOR kinase activity, and relief of repression by mTAb1 is thought to involve substrate presentation (Sekulić *et al.*, 2001). The mTAb1 antibody has been reproduced by several groups using the identical epitope spanning residues 2433-2450 of the mTOR catalytic domain (Sekulić *et al* 2001; Heesom and Denton, 1999). The epitope encompasses all the sites mutated here and it should be noted that the mTAb1 antibody is not a phospho-antibody and recognises mTOR phosphorylation by reduced reactivity in western blot analysis (Scott and Lawrence, 1998). The αTOR antibody used here is identical to the mTAb1 antibody, but we were unable to assay consistent mTOR activity. If actual mTOR kinase activity were to be attained, it is possible that αTOR or mTAb1 may have different

binding affinities for each mTOR mutant which may affect the mTOR activity observed. Heesom and Denton (1999) reported that αTOR (denoted Ab 1 in the reference) retrieved less mTOR by immunoprecipitation from insulin-treated cells because of *in vivo* mTOR phosphorylation within the antibody epitope (presumably S2448). Any 'artificial' activation of mTOR by mTAb1 (or similar antibody) may override and mask the real effects of the mutants themselves. McMahon *et al.*, (2002) also produced a S2448E mutant which did not affect *in vitro* phosphorylation of 4E-BP1 at T37/46 or T70 when activated with the mTAb1 antibody. Sekulić *et al.*, (2001) who also made the T2446 and S2448 point mutations did not test the mutant effect on 4E-BP1 phosphorylation; however, they did produce a deletion mutant (2430-2450) to identify the mTAb1 epitope as a region forming a repressor domain. Taken together, I would expect the mutant mTOR produced here to have no global effect on 4E-BP1 phosphorylation in response to both growth factor stimulation and nutrient deprivation. It is likely that the S2448 phosphorylation (mimicked by the S2448E mutant) cannot relieve inhibition of this repressor domain as observed with the S6K1 data.

The difficulties in obtaining mTOR activity remain problematic as differences in isolation procedures affect the activities brought down in an mTOR immunoprecipitate; these could include mTOR associated kinase activities or other associated proteins which include Raptor. Isolation of mTOR with Raptor is detergent sensitive (Hara *et al.*, 2002; Kim *et al.*, 2002), and Raptor was required for *in vitro* mTOR activity towards 4E-BP1. Therefore, despite the negative data obtained from the mutants they may still prove a useful tool.

One factor which should be considered in the interpretation of these results is the actual principal of assessing the effect of mutant mTOR over endogenously expressed mTOR. The principal was to exploit the rapamycin resistant mutation that was cloned into each construct as S2035T. S2035 has long been identified as the site to mutate for rapamycin resistance (Chen *et al.*, 1995; Lorenz and Heitman, 1995) and was always considered as a silent mutation until recent reports which indicated that mutation at this site affected mTOR substrate selectivity (McMahon *et al.*, 2002). Specifically, S2035I had a much lower mTOR kinase activity for

phosphorylation of 4E-BP1 at T37 and T46 and a moderately lower activity for T70 compared to wildtype mTOR; furthermore several other S2035 substitutions which include S2035T (also E, D and R mutations) affected mTOR kinase activity for 4E-BP1 and S6K1 and only the S2035W mutation was considered truly silent (McMahon *et al.*, 2002). The S2035T mutation was used here and the Sekulić group (2000) used the S2035I mutation. Therefore, it is possible that the rapamycin resistant mutation affected the mutant mTOR activity towards its downstream targets and the effect of phosphorylation at T2446 and S2448 may still prove significant.

Additionally, the inability to achieve mTOR kinase activity sidelined plans to analyse mTOR and PP2A interaction and the effect of mutant mTOR kinase activity towards the PP2A associated phospho-protein α4. Initially, α4 was cloned into a modified pTri-ex vector to incorporate the pTri-ex -HSV and -His tag at the C-terminus; this construct was expressed in bacterial cells to produce recombinant α4 (Section 2.2.3.4). An inducible protein was expressed with molecular weight ~55kDa but could not be isolated sufficiently, possibly because the His-tag had been cleaved or the His-tag was not exposed. The pTri-ex-α4 construct was also used to transfect CHO-IR cells but α4 could not be clearly identified by western blot. Correspondence with Prof. D. Brautigan (Director of Cell Signalling, University of West Virginia, USA) indicated that his collaboration with J. C. Lawrence Jr. (University of Virginia, USA) showed that while mTOR could phosphorylate 4E-BP1, it could not phosphorylate GST-α4; neither had they seen co-immunoprecipitation of α4 with mTOR, or PP2A with mTOR which was also tried unsuccessfully (data not shown). Therefore, the studies with mTOR, PP2A and α4 were abandoned.

Further, in collaboration with Dr I. Gout (Ludwig Institute for Cancer Research, London), a series of experiments were performed to identify whether CoA synthase (CoAsy) was regulated by mTOR. CoAsy is a 63kDa protein found to be weakly associated with S6K (Zhyvoloup *et al.*, 2002). Initial studies showed no association between S6K1 and CoAsy or between mTOR and CoAsy even with less stringent washing of immunoprecipitates. Insulin did stimulate a 2 fold activation of CoAsy

but this was not inhibited by rapamycin treatment, suggesting that mTOR was not involved and this was also not pursued further.

In summary, the established S2448 PKB phosphorylation site does not directly affect S6K1 activity and the mutually exclusive T2246 phosphorylation site does not negatively regulate S6K1 activity in response to growth factors. Moreover, neither alanine nor glutamic acid mutants at S2442, T2446 or S2448, within a rapamycin resistant backbone, were able to overcome the effects of nutrient deprivation on S6K1 activity. Evidence suggests that individual phosphorylation of these sites is insufficient to affect downstream S6K1 activity and the importance of differential phosphorylation at T2446 and S2448 remains to be determined.

Chapter 5: Results

5 The involvement of TSC in upstream signalling of mTOR

5.1 Summary

The tuberous sclerosis complex (TSC) is an inherited human genetic disorder characterised by mutation of either the *TSC1* or *TSC2* gene. TSC1 and TSC2 interact to form a functional complex and epistatic studies in *Drosophila* indicate that TSC functions within the insulin signalling cascade upstream or parallel to the mammalian target of rapamycin (mTOR). PKB is able to directly phosphorylate TSC2 and further studies with TSC revealed that it acted as a negative regulator of two downstream effectors of translation, S6K1 and 4E-BP1, both of which are downstream targets of mTOR. Moreover, targeted disruption of TSC interaction caused constitutive activation of S6K1, suggesting deregulation of upstream signalling elements. To further understand the factors involved in TSC signalling, a mouse embryonic fibroblast cell line derived from mice deficient for TSC2 was used here to analyse defects in the insulin signalling cascade. The loss of TSC2 caused down-regulation of insulin stimulated PKB activation and consequently mTOR S2448 phosphorylation, while simultaneously displaying constitutive activation of S6K1. Moreover, the MAPK kinase pathway was down-regulated and IRS-1 levels were significantly reduced. Low levels of IRS-1 in TSC2 deficient fibroblasts directly impaired IRS-1 associated class 1a phosphoinositide 3-kinase (PI3K) lipid kinase acivity. Similarly, PDGF-stimulated activation of PI3K lipid kinase activity was also impaired. These data suggest that loss of TSC2 impairs insulin signalling by affecting the turnover of the IRS-1 protein which in turn affects associated signalling downstream of PI3K.

5.2 Introduction

The inherited human genetic disorder tuberous sclerosis complex (TSC) occurs via mutation of the *TSC1* and *TSC2* gene which respectively encode the proteins TSC1 (hamartin) and TSC2 (tuberin) (Young and Povey, 1998). TSC functions as a complex and was identified as a negative regulator of cell size and proliferation (Tapon *et al.*, 2001). Epistatic studies in *Drosophila* place TSC parallel to or within the mTOR signalling pathway (Gao *et al.*, 2002; Potter *et al.*, 2001) and PKB is able to phosphorylate TSC2 at S939 and T1462 without affecting association with TSC1 (Dan *et al.*, 2002; Manning *et al.*, 2002). Loss of TSC function results in suppression of PKB activation and the constitutive activation of S6K1 (Kwiatkowski *et al.*, 2002; Gao *et al.*, 2002; Inoki *et al.*, 2002; Jaeschke et al., 2002; Zhang *et al.*, 2003a), a target of mTOR signalling. Over-expression of TSC specifically inhibits phosphorylation of S6K1 at the rapamycin sensitive T389 site (Inoki *et al.*, 2002). Additionally, co-expression of TSC inhibits insulin-stimulated phosphorylation of 4E-BP1 and represses Cap-dependent translation (Tee *et al.*, 2002). Both S6K1 and 4E-BP1 are specific targets of mTOR and these studies suggest TSC has a negative regulatory role specifically within the mTOR signalling pathway. There is no evidence of physical interaction between mTOR and TSC (Gao *et al.*, 2002; Zhang *et al.*, 2003a) but mTOR has been described as a mediator between TSC and S6K1 (Inoki *et al.*, 2002; Tee *et al.*, 2002). However, TSC/p53-null fibroblasts ($TSC2^{-/-}$ fibroblasts) have also been used to establish that TSC inhibits S6K1 via PI3K but independently of mTOR (Jaeschke *et al.*, 2002); this suggests that TSC functions in both an mTOR-dependent and mTOR-independent pathway which might explain the constitutive activation of S6K1 with suppressed PKB activity in the absence of TSC. Such evidence suggests that TSC may be important in regulation of signalling elements upstream of mTOR and PKB.

Insulin stimulation activates the insulin receptor (IR) tyrosine kinase activity which initiates the recruitment and tyrosine-phosphorylation of the insulin receptor substrate (IRS) family of proteins, of which IRS-1 is the most well characterised (White, 1997). The IRS proteins have multiple tyrosine and ser/thr phosphorylation sites (White, 1997) and tyrosine phosphorylation of IRS-1 is the main mechanism of

activating class 1a PI 3-kinases (PI3K) and the ras/MAPK pathway. Multi-site tyrosine phosphorylation of IRS-1 causes the recruitment and activation of PI3K lipid kinase activity and initiation of the insulin signalling cascade (Shepherd *et al.*, 1998). Insulin stimulation also mediates degradation of IRS-1 by a feedback mechanism involving ser/thr phosphorylation of IRS-1, a process which has been implicated in the development of insulin resistance (DeFea and Roth, 1997a, 1997b; Paz *et al.*, 1997; Cengal *et al.*, 1999; Li and DeFea, 1999). However, ser/thr phosphorylation of IRS-1 largely appears to initiate its degradation by the proteasome (Haruta *et al.*, 2000). Evidence suggests that phosphorylation of serine residues targets IRS-1 for degradation via a wortmannin and rapamycin sensitive pathway (Hartley and Cooper, 2002; Hartman *et al.*, 2001; Haruta *et al.*, 2000; Li and DeFea, 1999) and it has also been reported that in response to cytokines, both mTOR and S6K1 are capable of phosphorylating serine residues on IRS-1 *in vitro* (Hartman *et al.*, 2001). Taken together, the absence of TSC2 resulting in constitutively activated S6K1 may bring about deregulation of these feedback mechanisms involving IRS-1.

Since constitutively activated S6K1 is coupled to suppression of PKB activity in the absence of functional TSC (Kwiatkowski *et al.*, 2002; Gao *et al.*, 2002; Inoki *et al.*, 2002; Jaeschke et al., 2002; Zhang *et al.*, 2003a), mouse embryonic fibroblasts (MEFs) derived from mice deficient for TSC2 and the tumour suppressor p53 (as described by Jaeschke *et al.*, 2002) were used to further understand the significance and consequence of constitutively activated S6K1 in the absence of TSC2. The effects of losing TSC2 on feedback mechanisms involving IRS-1 were investigated by assessing IRS-1 associated class 1a phosphoinositide 3-kinase (PI3K) lipid kinase activity.

The present findings show that the loss of TSC2 in TSC2-null/p53-null ($TSC2^{-/-}$) mouse embryonic fibroblasts (MEFs) causes downregulation of factors in the insulin signalling cascade while maintaining constitutive activation of S6K1. Moreover, IRS-1 associated class 1a PI3K lipid kinase acivity was impaired due to significantly reduced IRS-1 levels and lipid kinase activity was not restored by activation of the PDGF receptor.

5.3 Results

5.3.1 Effect of insulin stimulation on $TSC2^{+/+}$ and $TSC2^{-/-}$ cells

To assess the effects of the absence of TSC2 on the mTOR signalling pathway, MEFs derived from mice deficient for TSC2 and the tumour suppressor p53 (denoted throughout as $TSC2^{-/-}$) and control MEFs derived with TSC2 but absent of p53 (denoted throughout as $TSC2^{+/+}$) were used; these were provided by Dr R. Lamb (Institute for Cancer Research, London) (Jaeschke *et al.*, 2002; Zhang *et al.*, 2003a). Cells were serum starved overnight then stimulated with insulin for the times indicated. Whole cell lysates were blotted with various antibodies to determine the protein levels and phosphorylation states of certain proteins in both $TSC2^{+/+}$ and $TSC2^{-/-}$ fibroblasts (Fig. 5.1). Phosphorylation of PKB at S473 indicates activation of PKB. This was only observed in $TSC2^{+/+}$ fibroblasts as described by Jaeschke et al., (2002). In contrast S6K1 was constitutively phosphorylated (T389) in $TSC2^{-/-}$ fibroblasts (Kwiatkowski *et al.*, 2002; Gao *et al.*, 2002; Inoki *et al.*, 2002; Jaeschke et al., 2002; Zhang *et al.*, 2003a); this was also confirmed in the total S6K1 immuno-blot where a slower migrating, hyper-phosphorylated form of S6K1 was observed in $TSC2^{-/-}$ fibroblasts, though total levels of protein were the same. mTOR has been shown to phosphorylate S6K1 at T389 and activate S6K1 activity (Burnett *et al.*, 1998; Isotani *et al.*, 1999). Here, the constitutive activation of S6K1 coupled with downregulation of PKB activity suggests that activation of S6K1 may be independent of PKB in $TSC2^{-/-}$ fibroblasts. Western blot analysis of mTOR phosphorylation showed that insulin stimulated phosphorylation of mTOR at S2448 was only observed in $TSC2^{+/+}$ cells where PKB activation remained intact. mTOR-S2448 was not phosphorylated in the $TSC2^{-/-}$ fibroblasts. Insulin stimulated activation of MAPK was also severely reduced in $TSC2^{-/-}$ fibroblasts, indicating that regulation of the ras/MAPK signalling pathway was impaired. Downregulation of MAPK, mTOR and PKB indicated that the fault in impaired insulin signalling lay further upstream. Indeed, western blot analysis of IRS-1 revealed that total levels of IRS-1 protein were almost absent in $TSC2^{-/-}$ fibroblasts. The general lack of IRS-1

may be from enhanced turnover of IRS-1 which has been targeted for degradation by a feedback mechanism and this was likely to affect IRS-1 associated PI3K activity.

5.3.2 p85 associated lipid kinase activity in TSC2 $^{+/+}$ and TSC2 $^{-/-}$ cells when stimulated with insulin

Taking into account the lack of IRS-1 in TSC2$^{-/-}$ fibroblasts, we wanted to verify that PI3K activity itself was intact. TSC2$^{+/+}$ and TSC2$^{-/-}$ whole cell lysates were probed for levels of p85 and p110 and there were equivalent levels of both PI3K sub-units observed (Fig. 5.2A). In accordance, the p85 associated lipid kinase activities assayed from both sets of fibroblasts were also comparable although p85-associated PI3K activity was slightly lower in TSC2$^{-/-}$ fibroblasts. This data indicated that loss of TSC2 did not affect p85 and p110 protein levels and p85-associated PI3K activity was intact in TSC2$^{-/-}$ fibroblasts; however, the activity was independent of insulin stimulation.

5.3.3 IRS-1 associated lipid kinase activity in TSC2$^{+/+}$ and TSC2$^{-/-}$ cells when stimulated with insulin

Although lipid kinase activity from class 1a PI3K was intact in TSC2$^{-/-}$ fibroblasts, the total protein levels of IRS-1 in these cells suggested that there would be a reduction in IRS-1 associated lipid kinase activity. As expected, immunoprecipitation of IRS-1 recovered trace amounts of tyrosine phosphorylated IRS-1 protein which coincided with a lack of p85 association (Fig. 5.3A). p85 immunoprecipitates also showed lack of IRS-1 association in TSC2$^{-/-}$ fibroblasts (Fig. 5.3A). This was reflected in the lipid kinase activities associated with the IRS-1 immunoprecipitates in which insulin barely stimulated lipid kinase activity above basal from the TSC2$^{-/-}$ fibroblasts. This indicates that loss of TSC2 triggers lack of IRS-1, resulting in the reduced ability of insulin to regulate class 1a PI3K.

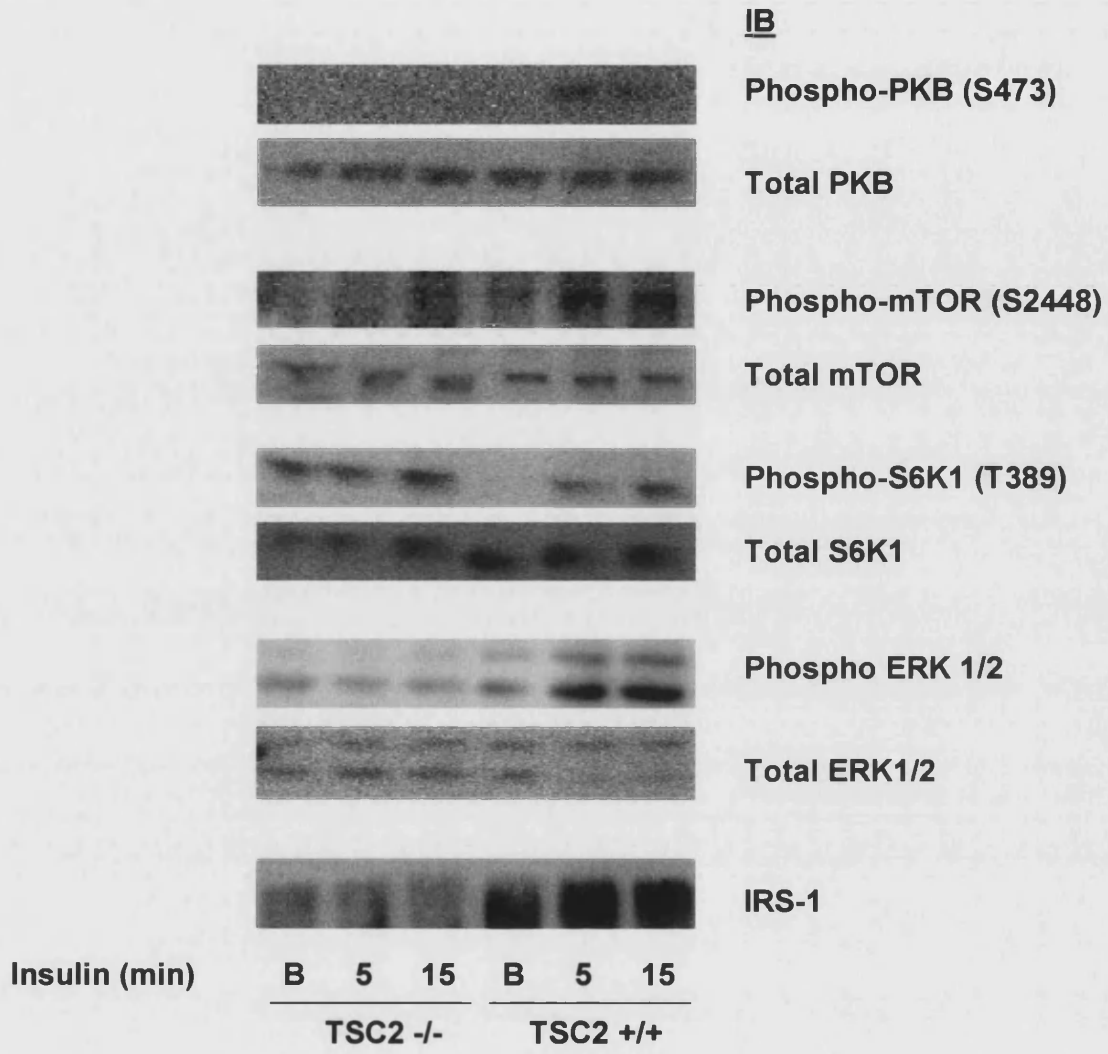

Figure 5.1 Effect of insulin stimulation on $TSC2^{+/+}$ and $TSC2^{-/-}$ cells

$TSC2^{+/+}$ and $TSC2^{-/-}$ fibroblasts were starved overnight in serum free DMEM with 1% antibiotic/antimycotic. Cells were stimulated with 100nM insulin for the times indicated then lysed; equal amounts of protein were loaded and separated by 7.5% SDS/PAGE, wet transferred to PVDF then probed with the phospho-antibodies as indicated then stripped and reprobed for total protein. Similar results were obtained in three independent experiments performed in duplicate. ('B' denotes basal).

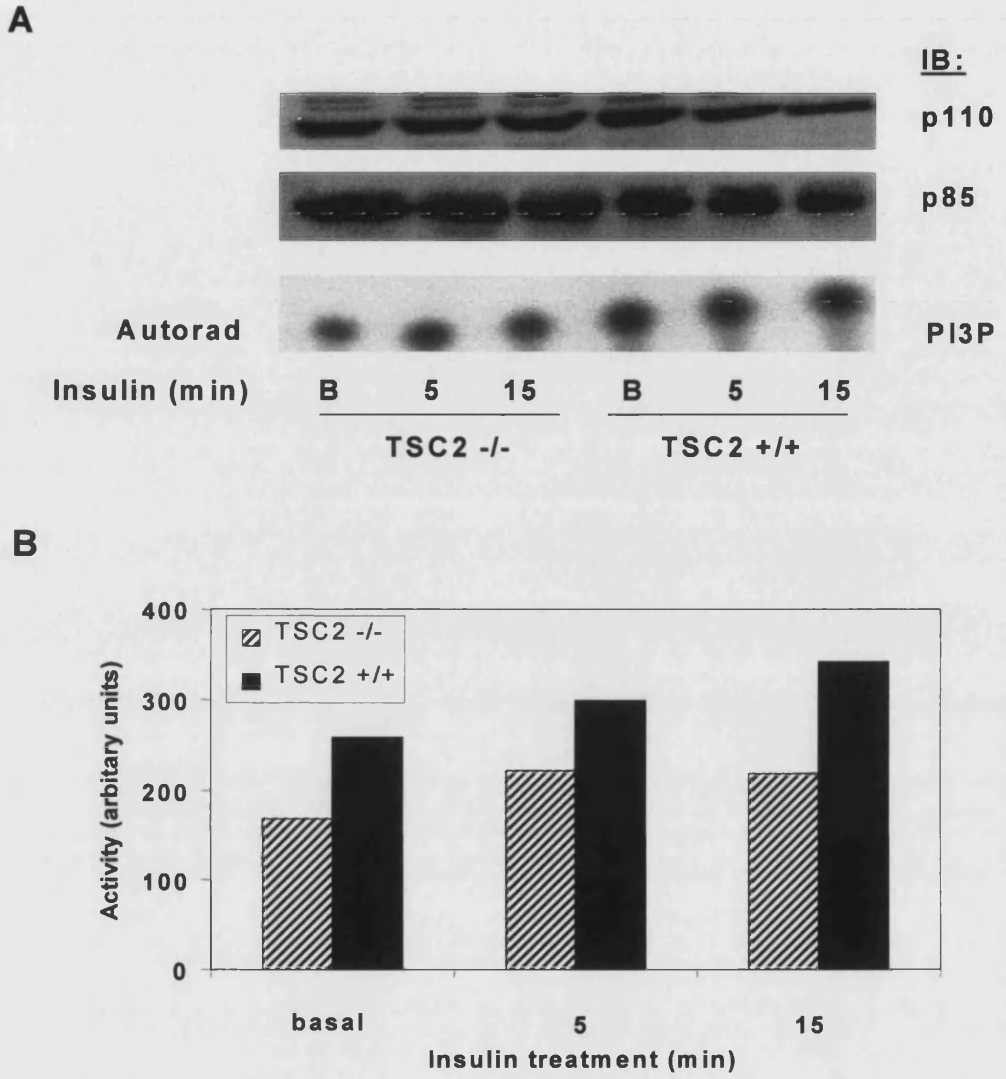

Figure 5.2 p85 associated lipid kinase activity in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells when stimulated with insulin

$TSC2^{+/+}$ and $TSC2^{-/-}$ fibroblasts were starved overnight in serum free DMEM with 1% antibiotic/antimycotic. Cells were stimulated with 100nM insulin for the times indicated then lysed. 100μg of each lysate was separated by SDS/PAGE, wet transferred to PVDF then probed with the antibodies as shown in (A). Similar results were obtained in three independent experiments performed in duplicate. Equal amounts of the remaining lysates were immunoprecipitated with anti-p85 antibody and the immunoprecipitates were assayed for lipid kinase activity using phosphatidylinositol and [γ-^{32}P] ATP as the substrates. Reaction products were analysed by thin layer chromatography and autoradiography. An example of the autoradiograph (autorad) obtained is shown in (A) and relative activity was determined in arbitrary units as shown in (B).

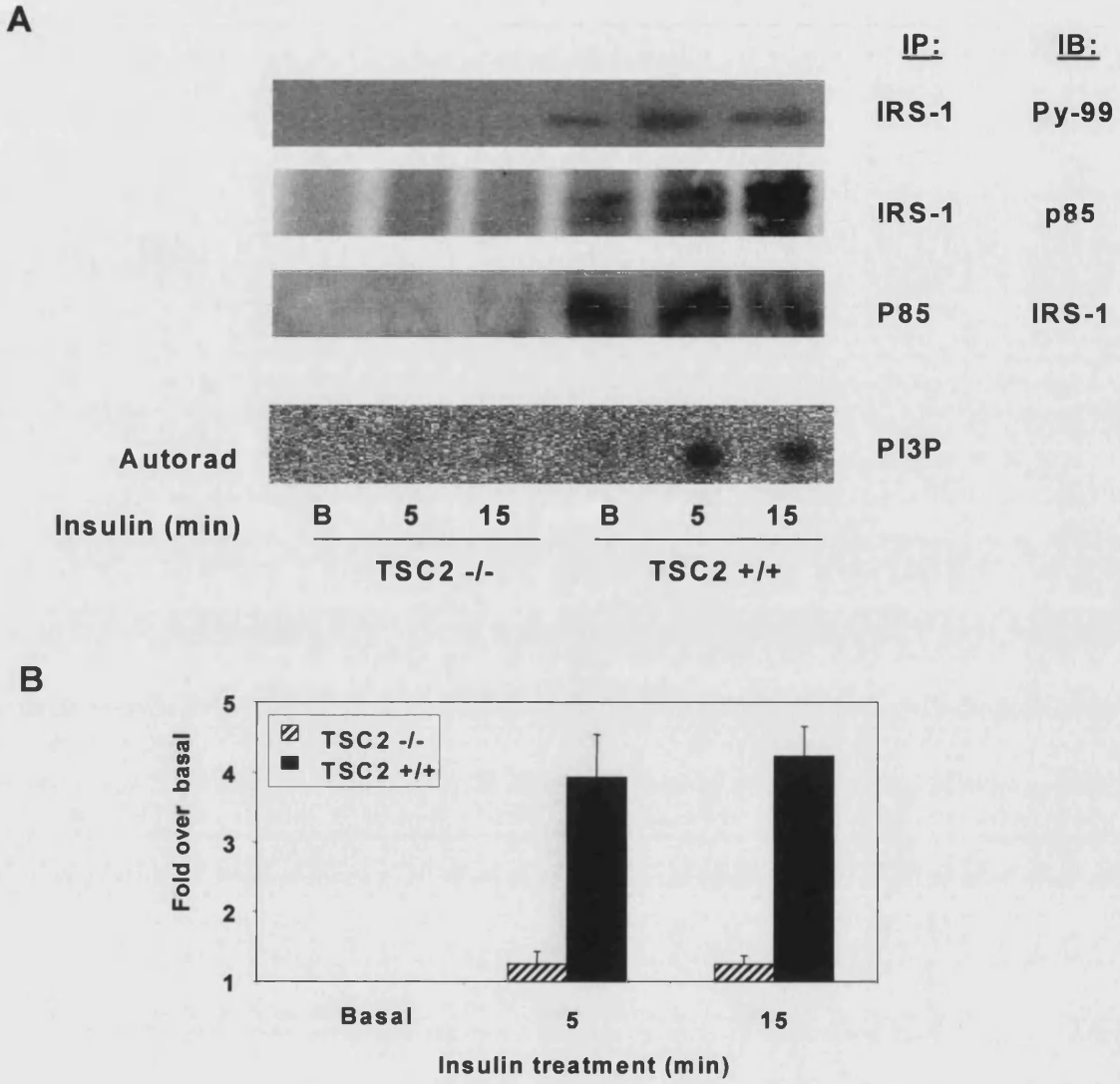

Figure 5.3 IRS-1 associated lipid kinase activity in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells when stimulated with insulin

$TSC2^{+/+}$ and $TSC2^{-/-}$ fibroblasts were starved overnight in serum free DMEM with 1% antibiotic/antimycotic. Cells were stimulated with 100nM insulin for the times indicated then lysed. . Equal amounts of protein were immunoprecipitated and probed with the indicated antibody, as shown above in (A). IRS-1 immunoprecipitates were assayed for lipid kinase activity using phosphatidylinositol and [γ-^{32}P] ATP as the substrates. Reaction products were analysed by thin layer chromatography and autoradiography. An example of the autoradiograph (autorad) obtained is shown in (A) and the lipid kinase activity is expressed as mean fold over basal ± SEM as shown in (B), n=2 in duplicate.

5.3.4 Phospho-tyrosine associated lipid kinase activity in insulin-stimulated $TSC2^{+/+}$ and $TSC2^{-/-}$ cells

The loss of insulin stimulated lipid kinase activity due to lower levels of IRS-1 protein in the $TSC2^{-/-}$ fibroblasts may be compensated for by other IRS proteins e.g. IRS-2. Therefore, phospho-tyrosine (Py-99) immunoprecipitations were performed to assess whether lack of lipid kinase activity was due to an overall reduction in tyrosine phosphorylated proteins available for recruitment of p85. Consistently, no IRS-1 was detected in anti-Py-99 immunoprecipitates from $TSC2^{-/-}$ fibroblasts compared to $TSC2^{+/+}$ (Fig. 5.4A) indicating that any available IRS-1 is poorly tyrosine phosphorylated and so inadequate for p85 recruitment (Fig. 5.4A). The lipid kinase profile obtained from anti-Py-99 immunoprecipitations (Fig. 5.4B) was identical to that observed with IRS-1 immunoprecipitation (Fig. 5.3 B), indicating a general deficiency of tyrosine phosphorylated proteins. The lack of tyrosine phosphorylated proteins may be from impaired insulin-receptor tyrosine kinase activity.

5.3.5 Phospho-tyrosine associated lipid kinase activity in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells when stimulated with PDGF

The defect in insulin mediated lipid kinase activity via IRS-1 and phospho-tyrosine associated proteins raises the question as to whether the PI3K pathway can be activated via another receptor tyrosine kinase. The PDGF receptor was considered since it does not phosphorylate IRS-1 (Shepherd *et al.*, 1998) and therefore any PI3K lipid kinase activity would bypass the requirement of IRS-1 signalling. To test this hypothesis PDGF (BB) was used to stimulate $TSC2^{+/+}$ and $TSC2^{-/-}$ fibroblasts. Anti-Py-99 immunoprecipitates recovered from PDGF stimulated cells did not stimulate PI3K lipid kinase activity; in fact the lipid kinase activity profile observed was identical to that observed with insulin stimulation of $TSC2^{-/-}$ cells. This suggests that PDGF activation of class 1a PI3K lipid kinase activity is also impaired but the defect must occur independently of the lack of IRS-1 protein.

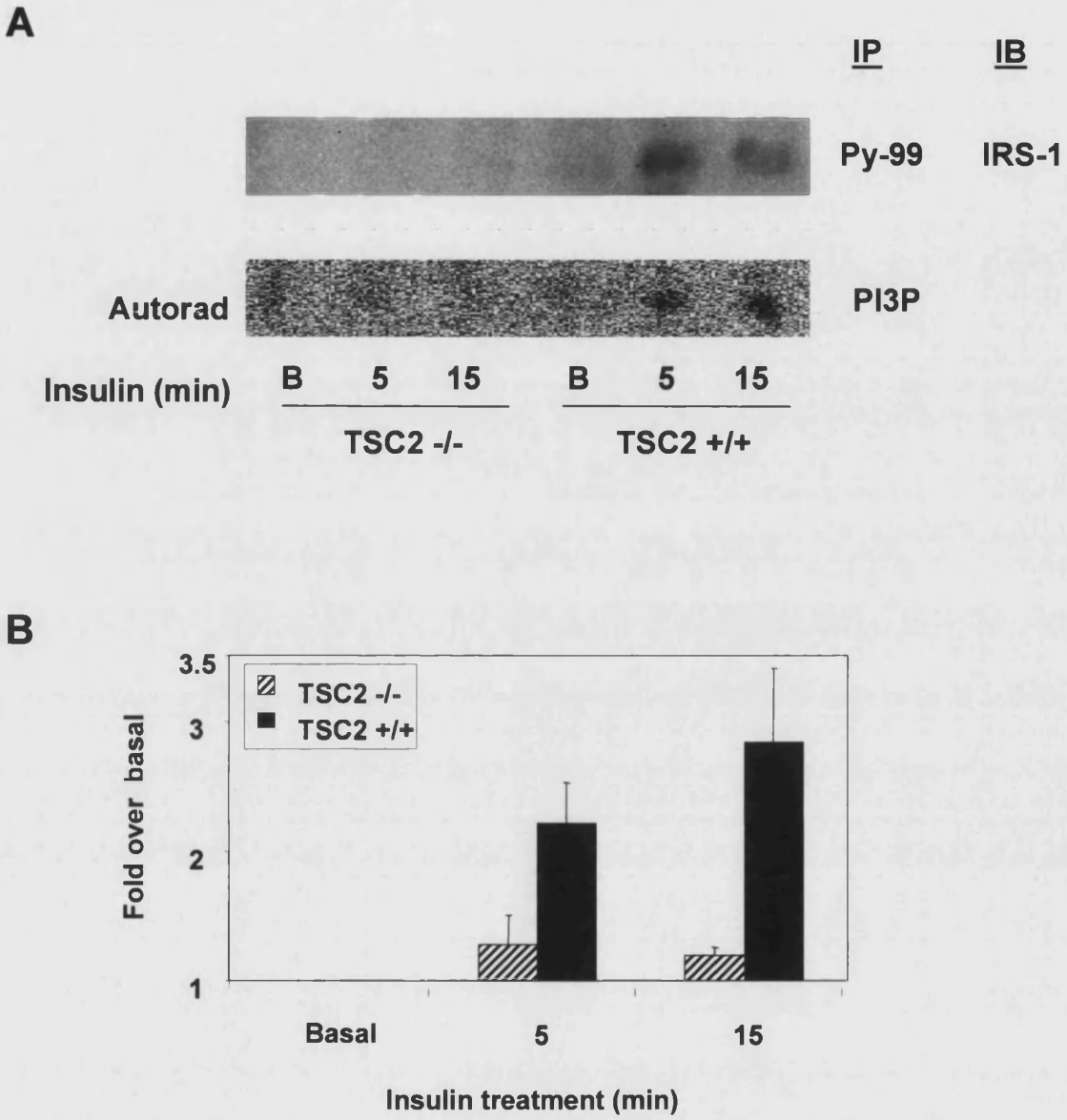

Figure 5.4 Phospho-tyrosine associated lipid kinase activity in insulin-stimulated TSC2$^{+/+}$ and TSC2$^{-/-}$ cells

TSC2$^{+/+}$ and TSC2$^{-/-}$ fibroblasts were starved overnight in serum free DMEM with 1% antibiotic/antimycotic. Cells were stimulated with 100nM insulin for the times indicated then lysed. Equal amounts of protein were immunoprecipitated and probed with the indicated antibody, as shown above in (A). Anti-Py-99 (phospho-tyrosine) immunoprecipitates were assayed for lipid kinase activity using phosphatidylinositol and [γ-^{32}P] ATP as the substrates. Reaction products were analysed by thin layer chromatography and autoradiography. An example of the autoradiograph (autorad) obtained is shown in (A) and the lipid kinase activity is expressed as mean fold over basal ± SEM as shown in (B), n=2 in duplicate.

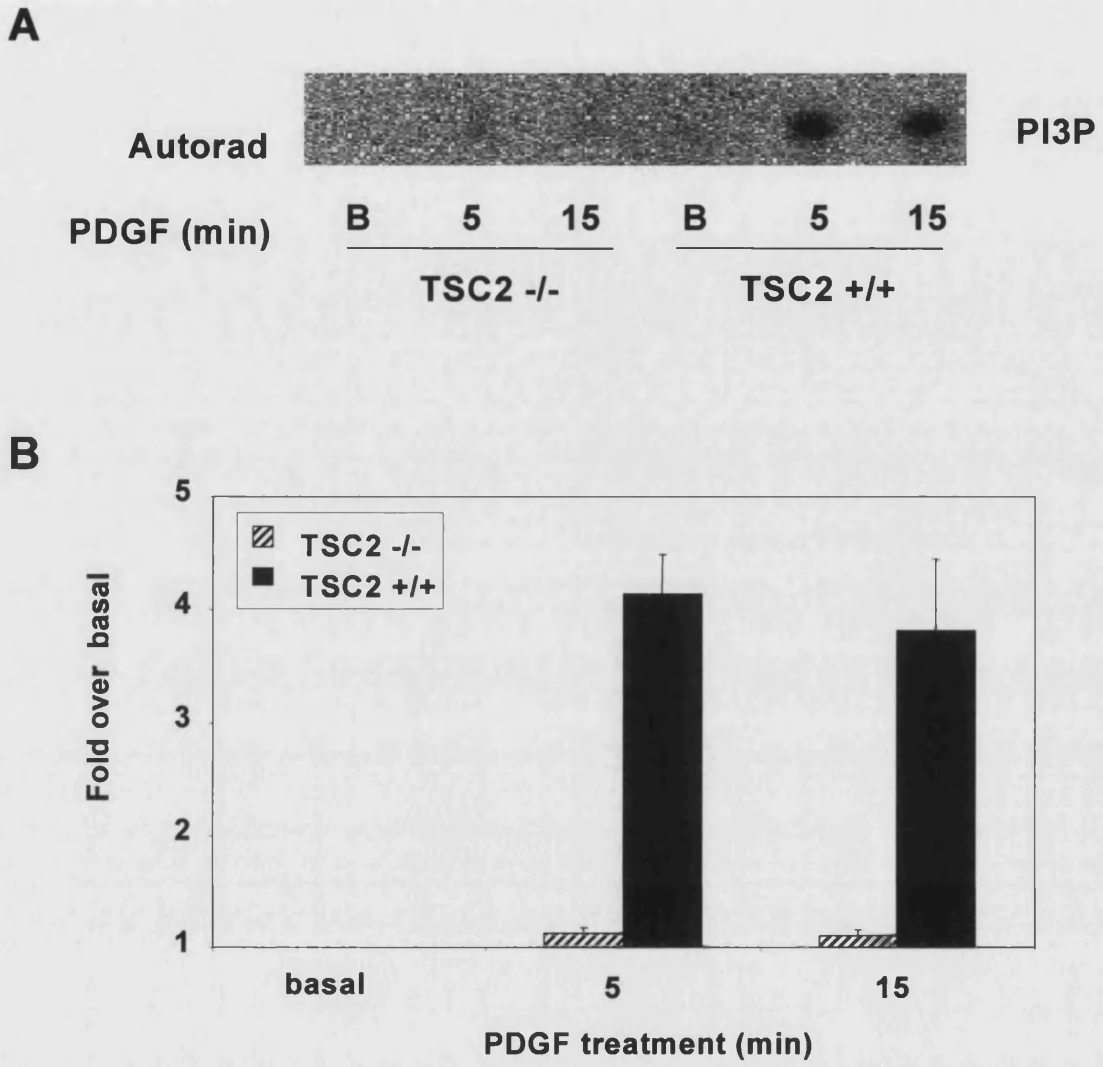

Figure 5.5 Phospho-tyrosine associated lipid kinase activity in TSC2 $^{+/+}$ and TSC2 $^{-/-}$ cells when stimulated with PDGF

TSC2$^{+/+}$ and TSC2$^{-/-}$ fibroblasts were starved overnight in serum free DMEM with 1% antibiotic/antimycotic. Cells were stimulated with 20 ng/ml PDGF (BB) for the times indicated, then lysed. Equal amounts of protein were immunoprecipitated with anti-Py-99 antibody and the immunoprecipitates were assayed for lipid kinase activity using phosphatidylinositol and [γ-^{32}P] ATP as the substrates. Reaction products were analysed by thin layer chromatography and autoradiography. An example of the autorad obtained is shown in (A) and the activity is expressed as mean fold over basal ± SEM as shown in (B), n=2 in duplicate.

5.4 Discussion

Recent studies implicate TSC as a factor in the insulin signalling cascade, acting as a negative regulator of mTOR and its downstream targets S6K1 and 4E-BP1. However, suppression of PKB activity coupled with consititutive activation of S6K1 in $TSC2^{-/-}$ mouse embryonic fibroblasts (MEFs) suggested impaired insulin signalling upstream of mTOR itself. Evidence here suggests that the defect is due, at least in part, to significantly lower levels of IRS-1 which directly affects IRS-1 associated class 1a PI3K lipid kinase acivity. Activation of the PDGF receptor did not restore phospho-tyrosine associated PI3K lipid kinase activity, suggesting additional defects in signalling upstream of TSC and mTOR in $TSC2^{-/-}$ fibroblasts.

As described previously, loss of TSC causes the constitutive activation of S6K1 (shown by phosphorylation at T-389 and activity assays) despite suppression of insulin-stimulated PKB activation (Kwiatkowski *et al.*, 2002; Gao *et al.*, 2002; Inoki *et al.*, 2002; Jaeschke et al., 2002; Zhang *et al.*, 2003a). Constitutively phosphorylated S6K1 (T389) was detected *in vivo* (as described by Jaeschke *et al.*, 2002; Zhang *et al.*, 2003a) in $TSC2^{-/-}$ fibroblasts; however, S2448 phosphorylation was not stimulated by insulin in $TSC2^{-/-}$ fibroblasts and this was likely due to suppressed PKB activation. In contrast, knocking out TSC in HEK cells by RNAi (where PKB activity is unperturbed) increases mTOR-S2448 phosphorylation (Inoki *et al.*, 2002). Whether mTOR phospho-S2448 is significant for mTOR activity is unclear, as discussed in Section 4.4. Constitutively active S6K1, in the absence of TSC, remains rapamycin sensitive (Gao *et al.*, 2002; Kwiatkowski *et al.*, 2002; Zhang *et al.*, 2003a), suggesting that mTOR is the cause of constitutively activated S6K1. If mTOR is the cause of constitutive S6K1 activation in the $TSC2^{-/-}$ fibroblasts it seems to be via a PI3K/PKB independent pathway. TSC is reported to inhibit mTOR *in vitro* kinase activity toward S6K1 (Inoki 2002). In contrast, Zhang *et al.*, (2003a) reported that TSC does not influence mTOR autokinase activity, *in vitro* kinase activity towards 4E-BP1 or cellular phosphatase activity (Zhang *et al.*, 2003a). The *in vitro* kinase activity of mTOR may not reflect *in vivo* mTOR kinase activity (Hara *et al.*, 1998; Dennis *et al.*, 2001) and the effect of TSC on mTOR activity may require intermediate signalling proteins *in vivo*.

What is apparent is that defects in insulin signalling in $TSC2^{-/-}$ fibroblasts are extended to the MAPK pathway which was down-regulated in the presence of insulin. Therefore, despite constitutive activation of S6K1, suppression of PKB activation and the MAPK pathway suggested a defect in upstream insulin signalling; this was confirmed by western blotting for the IRS-1 protein. $TSC2^{-/-}$ fibroblasts had significantly lower levels of IRS-1 compared to $TSC2^{+/+}$ fibroblasts and thus much lower levels of tyrosine-phosphorylated IRS-1 in response to insulin stimulation. Recruitment and phosphorylation of the IRS family of proteins by the insulin receptor is the main mechanism of activating class 1a PI 3-kinases (Shepherd *et al.*, 1998). The lack of IRS-1 caused a decrease in IRS-1 associated PI3K lipid kinase activity despite evidence that p85-associated PI3K activity remained intact in the $TSC2^{-/-}$ fibroblasts. Although, IRS-2 can substitute for the loss of IRS-1 in the activation of PI3K, IRS-1 has a more dominant role in PI3K activation in embryonic fibroblasts (Bruning *et al.*, 1997). The complete lack of lipid kinase activity from phospho-tyrosine immunoprecipitates suggests that there was an overall lack of phospho-tyrosine associated PI3K activity, implying that there was no overall compensation by other IRS isoforms. Undoubtedly, the lack of IRS-1 and phospho-tyrosine associated PI3K lipid kinase activity caused suppression of PKB activation and the Ras/MAPK pathway. PKB activation primarily requires activation of PI3K lipid kinase activity via recruitment by IRS-1 to produce the lipid second messenger phosphatidylinositol (3, 4, 5) triphosphate (PtdIns (3, 4, 5)P_3) (Shepherd *et al.*, 1998). PtdIns (3, 4, 5)P_3 induces the recruitment of proteins containing the pleckstrin-homology (PH) domain which includes phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB); therefore the lack of IRS-1 would thereby inhibit activation of proteins in the insulin signalling cascade through lack of PtdIns (3, 4, 5)P_3 production. The low level of IRS-1 may stem from a general problem with the insulin receptor tyrosine kinase activity and/or from a feedback mechanism due to constitutively activated S6K1. Our collaborators (Harrington *et al.*, 2004) have shown that constitutively active S6K1 in $TSC2^{-/-}$ fibroblasts modulates the expression of IRS-1 by directly phosphorylating serine residues at a region proximal to the IRS-1 phospho-tyrosine binding (PTB) domain (residues 108-516). Suppression of activation by insulin in $TSC2^{-/-}$ fibroblasts is likely due to serine

phosphorylation of IRS-1 by S6K1, which inhibits tyrosine phosphorylation and results in IRS-1 degradation (Harrington *et al.*, 2004). The presence of TSC can therefore prevent IRS-1 degradation. However, it must also be considered that in response to cytokines, both mTOR and S6K1 are able to phosphorylate serine residues on IRS-1 (between residues 511-722) but only mTOR is able to phosphorylate IRS-1 on serine residues which block tyrosine-phosphorylation of IRS-1 (Hartman *et al.*, 2001). This suggests that mTOR may also play a part in regulating the feedback mechanism which controls the levels of IRS-1.

Because of the lack of IRS-1 associated lipid kinase activity we considered whether activation of the PDGF receptor would overcome dysfunction in phospho-tyrosine associated class 1a PI3K lipid kinase activity. The IRS proteins are not phosphorylated by the PDGF receptor and therefore activation of PI3K by the PDGF receptor would bypass the loss of IRS-1. PDGF was also unable to stimulate phospho-tyrosine associated lipid kinase activity in $TSC2^{-/-}$ fibroblasts, suggesting that signalling via the PDGF receptor was also deficient. Zhang *et al.*, (2003a) recently reported impaired insulin, IGF-1 and PDGF signalling in the same TSC2/p53 null MEFs. In accordance with results presented here, there was a significant lack of PI3K lipid kinase activity in $TSC2^{-/-}$ fibroblasts when treated with PDGF (BB). Their extended findings show that reduced PI3K activity from PDGF stimulation is a result of reduced PDGF receptor expression (both α and β isoforms). PI3K and PKB activation was restored by the ectopic expression of the PDGF receptor (Zhang *et al.*, 2003a).

Although defects in PDGF signalling are attributable to reduced expression of the PDGF receptor in $TSC2^{-/-}$ fibroblasts the defect in insulin signalling via downregulation of IRS-1 suggests that another signalling mechanism exists to maintain cell survival and proliferation in $TSC2^{-/-}$ fibroblasts. Additional data from the Shepherd lab (C. Ktori, unpublished 2003) suggests that dysfunction of the 'classical' insulin signalling pathway via class 1a PI3K may be overcome, in part, by up-regulation of the class II PI3-kinases. Class II PI3Ks are large 170kDa proteins predominantly associated with the membrane fraction of cells, though it is not clear which phospholipids they produce *in vivo* (Vanhaesbroeck and Waterfield, 1999).

They are activated by insulin and function in a manner discreet from class 1a PI3K (Brown *et al.*, 1999). Specifically, class II PI3K activity is around 2 fold higher in $TSC2^{-/-}$ fibroblasts than in control $TSC2^{+/+}$ fibroblasts with insulin or PDGF stimulation, suggesting that there may be some compensation of growth factor signalling via class II PI3K.

The $TSC2^{-/-}$ fibroblasts used here were also $p53^{-/-}$. This was necessary to make the cells proliferate in culture and as shown here, loss of both these elements has extensive effects on both insulin and PDGF signalling. Although the authors of Jaeschke *et al.*, (2002) verified that the loss of p53 in MEFs did not disturb the S6K1 signalling pathways; we must consider reports that p53 is negatively regulated by mTOR signalling particularly in malignant cell lines (Castedo *et al.*, 2001, 2002; Huang *et al.*, 2001). Therefore, the additional loss of p53 in the TSC2 null fibroblasts may affect mTOR activity or influence its effects on IRS-1 turnover.

In summary, evidence suggests that loss of TSC2 impairs both insulin and PDGF mediated class 1a PI3K signalling. The loss of TSC2 induces constitutive activation of S6K1 which causes increased turnover of IRS-1 via a feedback mechanism. The mechanism seems to be independent of PKB activation and ultimately leads to down-regulation of IRS-1 associated class 1a PI3K signalling. The PDGF signalling pathway did not restore phospho-tyrosine associated class 1a PI3K signalling because of additional PDGF signalling deficiencies. While reports indicate TSC2 to be a negative regulator of mTOR, data here suggests TSC2 may also be a positive regulator of insulin signalling.

Chapter 6: General Discussion

6 General Discussion

mTOR is a member of the PIK-related kinase family and has demonstrated protein kinase activity to both S6K1 and 4E-BP1, the phosphorylation of which promotes the translation of specific subsets of mRNA to allow cell growth and proliferation, a process dependent on an overall increase in protein synthesis. Cellular control of protein synthesis is closely coupled to mitogen stimulation and nutrient availability, factors which converge at mTOR. While the domains of mTOR are well defined, the precise mechanisms by which mTOR is regulated are not well understood. The catalytic domain of mTOR has been of regulatory interest following the identification of S2448 as a nutrient sensitive and mitogen stimulated phosphorylation site; the significance of S2448 phosphorylation remains to be characterised.

The objectives of the present studies were to identify possible mechanisms by which mTOR is regulated and to determine the functional significance of regulation on downstream effectors.

The C-terminal catalytic domain of mTOR is thought to have regulatory significance, especially in view of identification of the S2448 PKB phosphorylation site (Navé *et al.*, 1999; Sekulić *et al.*, 2000; Reynolds *et al.*, 2002; Bolster *et al.*, 2002). S2448 phosphorylation is stimulated by insulin and negatively regulated by nutrient deprivation which led to the hypothesis that phosphorylation of an adjacent residue or residues may prevent S2448 phosphorylation; this was supported by Scansite analysis (Obenauer *et al.*, 2003) of the region surrounding S2248 which revealed a number of phosphorylatable ser/thr, most notably T2446. By producing a phospho-specific antibody to T2446 we found it to be a novel mTOR phosphorylation site. Moreover, phosphorylation at T2446 was negatively regulated by insulin and positively regulated by nutrient deprivation, conditions converse to those which promote S2448 phosphorylation. The data suggested that these sites were mutually exclusive.

In identifying T2446 as a novel nutrient regulated phosphorylation site we found that conditions supporting T2446 phosphorylation also correlated with activation of AMPK. Further, activation of AMPK by AICAR and DNP also increased phosphorylation at T2446. Though *in vitro* kinase assays with mTOR peptides suggested that AMPK could phosphorylate the region containing the T2446 phosphorylation site, *in vitro* kinase assays with AMPK and full length mTOR proved inconclusive. Although direct phosphorylation of mTOR by AMPK could not be demonstrated, the existence of the mutually exclusive T2446/S2448 phosphorylation sites suggests a level of nutrient regulated control of mTOR. Interestingly there are examples where integration of nutrient signals is regulated by mutually exclusive phosphorylation events, where sites are separated by a single amino acid. Two examples, which co-incidentally involve AMPK, are acetyl CoA carboxylase which is phosphorylated at S77 by AMPK and at S79 by Protein Kinase A (PKA) (Munday, 2002) and hormone sensitive lipase (HSL) which is phosphorylated at S563 by PKA and at S565 by AMPK (Garton *et al.*, 1989; Garton and Yeaman, 1990). The kinase responsible for T2446 phosphorylation remains to be elucidated, though based on studies here we could exclude PKB and S6K1 regulation of the T2446 site and it is likely that additional phosphorylation sites exist, though these sites remain to be identified.

mTOR activity is sensitive to cellular levels of ATP (Dennis *et al.*, 2001) and evidence presented in this dissertation implicates AMPK in the regulation of mTOR in response to ATP levels; however, depletion of cellular ATP levels can lead to inhibition of protein synthesis via an mTOR-independent pathway involving AMPK. Activation of AMPK leads to inhibition of protein synthesis via increased phosphorylation of elongation factor 2 (eEF2) but not by association of eIF4E with 4E-BP1 (Horman *et al.*, 2002; McLeod and Proud, 2002). eEF2 regulates translation elongation and is inactivated by phosphorylation at T56 by eEF2 kinase (Browne and Proud, 2002); insulin stimulates dephosphorylation of eEF2 to allow elongation, a process in which mTOR is peripherally involved because S6K1 can inactivate eEF2 kinase by phosphorylating S366 (Wang *et al.*, 2001). Studies show that eEF2 phosphorylation is rapidly induced and protein synthesis inhibited by AICAR

treatment (Horman *et al.*, 2002; McLeod and Proud, 2002), implicating AMPK in the negative regulation of eEF2. AMPK cannot directly phosphorylate eEF2, as it is a poor AMPK substrate (Horman *et al.*, 2002), but recently AMPK was shown to directly phosphorylate eEF2 kinase at S398 which stimulates eEF2 kinase activity (Browne *et al.*, 2004). Activation of AMPK by depletion of ATP enhanced phosphorylation of eEF2 kinase at S398 and increased phosphorylation of eEF2 (Browne *et al.*, 2004). Under the same conditions, mTOR signalling was not impaired. Furthermore, ATP depletion inhibits S6K1 and 4E-BP1 phosphorylation with slower kinetics than those of eEF2 phosphorylation, suggesting that inhibition of elongation is independent of the mTOR pathway (Horman *et al.*, 2002; McLeod and Proud, 2002), or that the mTOR pathway is involved only after severe depletion of ATP (Dennis *et al.*, 2001; Horman *et al.*, 2002). However, i*n vivo* studies in rats show that AICAR activation of AMPK does inhibit protein synthesis via an mTOR-dependent pathway, primarily through down-regulation of PKB, mTOR (S2448 phosphorylation), S6K1 and 4E-BP1 (Bolster *et al.*, 2002). AICAR treatment activates AMPK (Corton *et al.*, 1995) but may have cellular effects aside from AMPK activation (Kemp *et al.*, 1999). Downregulation of the mTOR pathway with AICAR treatment may involve factors additional to AMPK. However, studies in which AMPK was activated by alternative methods, such as glucose withdrawal or 2-deoxyglucose treatment, also demonstrate inhibition of S6K1 activity by an mTOR-dependent signalling pathway (Inoki *et al.*, 2003b; Kimura *et al.*, 2003).

The above studies do suggest a linkage between the mTOR and AMPK pathways and the fact that AMPK activation and T2446 phosphorylation are stimulated by similar conditions suggest they operate in a common and/or a parallel ATP sensitive pathway. Metformin is an oral anti-diabetic drug used to treat type-2 diabetes which can activate AMPK in skeletal muscle (Zhou *et al.*, 2001). AMPK activation is dependent on the AMP: ATP ratio (Hardie *et al.*, 1998; Carling, 2004); however, a distinct signalling pathway exists whereby metformin activates AMPK independently of the AMP: ATP ratio (Fryer *et al.*, 2002). A preliminary immuno-blot (data not shown) of H2K myotubes treated with metformin showed a concomitant increase in T2446 and AMPK (T172) phosphorylation which suggests that stimulation of mTOR-T2446 phosphorylation is closely coupled to both AMPK

regulatory pathways. These findings need further exploration and the functional significance of T2446 phosphorylation in relation to AMPK activation needs to be determined.

Currently, the involvement of AMPK and TSC in the mTOR signalling pathway is being delineated. Much like AMPK, TSC has been implicated in or parallel to the mTOR pathway; moreover, TSC2 is directly phosphorylated by PKB at S939 and T1462 (Dan *et al.*, 2002; Manning *et al.*, 2002) and more recently TSC2 was also shown to be directly phosphorylated by AMPK at T1227 and S1345 (Inoki *et al.*, 2003b). Unlike mTOR, the TSC -PKB and -AMPK phosphorylation sites are not separated by a single residue. 2-deoxyglucose treatment of HEK293 cells activated AMPK to mediate phosphorylation of TSC2 and decrease signalling through the mTOR pathway. Furthermore, AMPK mediated phosphorylation of TSC2 improved co-immunoprecipitation between TSC2 and AMPK and enhanced its ability to negatively regulate the mTOR pathway (Inoki *et al.*, 2003b); this suggests an important regulatory role for TSC and AMPK in the regulation of the mTOR pathway. Future investigation into the interplay of the AMPK, TSC and mTOR signalling pathways should provide a novel perspective on the regulation of protein synthesis in response to cellular energy levels.

Following identification of the mutually exclusive nature of the phosphorylation at T2446 and S2448, we wanted to determine the functional significance of phosphorylation at each site by mutational analysis. Experiments presented here were pre-empted by those of Sekulić *et al*, (2000); however, data here concurred with their findings. Thus, mutation of S2442, T2446 or S2448 to alanine or glutamic acid does not appear to affect S6K1 activity in response to insulin or nutrient availability when compared to the wildtype rapamycin resistant construct (M1). Though these findings were mainly negative it remains possible that both the T2446 and S2448 phosphorylation sites have some functional effect. Firstly, the aforementioned rapamycin resistance mutations (Section 1.4.1.2 and 4.4), which include S2035T (used here) and S2035I, affect substrate selectivity (McMahon *et al.*, 2002), so the specific effects of T2446 and S2448 site mutations cannot be properly assessed. Secondly, phosphorylation of T2446 and S2448 may not necessarily be coupled to

S6K1 activation. Studies show that binding of mTAb1 or deletion of the mTAb1 epitope (2433-2450) in mTOR, which contains both the T2446 and S2448 phosphorylation sites, has relatively little effect on S6K1 phosphorylation compared to phosphorylation of 4E-BP1 (McMahon *et al.*, 2002). It is therefore possible that phosphorylation of sites in the mTAb1 epitope has no specific effect on S6K1 activity and an alternative substrate may be more suitable to assess the effect of T2446 and S2448 phosphorylation e.g. IRS-1 (Section 1.6.4), PKCδ and ε (Section 1.6.5). However, since these two residues reside within the activating mTAb1 epitope, it suggests there is potential importance in their phosphorylation in the regulation of mTOR activity.

A recurring difficulty is the assessment of mTOR kinase activity under different isolation conditions, leading to inconsistencies in interpreting mTOR kinase activity and function. αTOR antibody was used because it was able to immunoprecipitate mTOR with *in vitro* kinase activity (Section 4.3.7). αTOR has the same epitope as mTAb1, the mTOR-activating antibody (Brunn *et al.*, 1997b) which functions by overcoming repression induced by the region of its epitope (residues 2433-2450) (Sekulić *et al.*, 2000). However, we were not able to consistently obtain mTOR kinase activity and the antibody may act to artificially induce mTOR activity *in vitro*. Brunn *et al.*, (1997b) reported that mTOR phosphorylated 4E-BP1 at residues T37, T46, S65 and T70 (minus 1 for numbering to rat sequence). However, phosphorylation at S65 and T70 was not observed by other groups whether the mTAb1 antibody was used (Gingras *et al.*, 1999a) or not (Burnett *et al.*, 1998). Therefore, mTAb1 provides mTOR with the capability to phosphorylate all the 4E-BP1 sites *in vitro* but this activation may be artificial and other factors may be implicated *in vivo*, particularly mTOR interacting proteins.

mTOR is a large protein and it is not unusual that it should act as a scaffold for associated proteins particularly since it contains a HEAT domain towards its N-terminus; the hydrophobic nature of the HEAT domain makes it a likely target for protein: protein interaction and associated proteins have had reported effects on mTOR kinase activity. Specifically, an mTOR-associated kinase has been co-purified with mTOR immunoprecipitates, though its identity and mechanism of

interaction remain to be identified (Heesom and Denton, 1999). An identified interacting protein is Raptor, a 150kD regulatory protein associated with mTOR that can also interact with S6K1 and 4E-BP1 (Hara *et al.*, 2002). Raptor association seems essential for mTOR activity towards 4E-BP1 (Hara *et al.*, 2002) but not S6K1 (Hara *et al.*, 2002; Kim *et al.*, 2002). Raptor is an external factor affecting mTOR kinase activity, and the Raptor-mTOR complex is highly sensitive to salt, detergent and rapamycin, factors which would similarly affect purification of additional mTOR interacting proteins. The discovery of Raptor will be important for determining other mTOR interacting proteins which may include TSC2 and AMPK. dTSC2 interacts strongly with dTOR but there is no evidence of mammalian TSC2 and mTOR interaction (Gao *et al.*, 2002; Zhang *et al.*, 2003a); similarly, the AMPKγ1 subunit was found to interact with the mTOR kinase domain but not but not with full length mTOR (Kimura *et al.*, 2003). Kim *et al.*, (2002) suggested that mitochondrial metabolism or oxidative stress participates in the regulation of the Raptor-mTOR complex, specifically via intracellular levels of ATP. Raptor may also be important for interaction of mTOR with energy sensing-AMPK. Careful isolation of complexes with Raptor will determine if it scaffolds these proteins together. Raptor interacts primarily with the N-terminus of mTOR via the HEAT domain (residues 728-897) and it has fewer contacts with the C-terminus (Kim *et al.*, 2002); so whether T2446 or S2448 phosphorylation is important for protein: protein interaction is as yet undetermined.

mTOR kinase activity is seemingly dependent on many external factors, thus phosphorylation at T2446 and S2448 may still prove functional under certain conditions. Significantly, S2448 phosphorylation has been demonstrated *in vivo* (Bolster *et al.*, 2002) and *in situ* in rat skeletal muscle, where hypertrophy induced an increase in S2448 phosphorylation and atrophy induced a decrease in S2448 phosphorylation (Reynolds *et al.*, 2002). Hypertrophy induces an increase in mRNA translation, up-regulation of protein synthesis (via an increase in S6K1 activation) and an increase in muscle mass (Baar and Esser, 1999; Bodine *et al.*, 2001; Shah *et al.*, 2000); therefore, an associated increase in S2448 phosphorylation demonstrates it as having a physiologically relevant role in the control of protein synthesis.

Although the mTOR phosphorylation site mutants produced had no apparent effect on the activation of S6K1, the mutants may still prove a useful tool in assessing mTOR localisation, trafficking or interactions with other proteins such as Raptor, PP2A/α4 or TSC.

The final part of this study related to the involvement of TSC within the insulin signalling pathway. Much evidence implicates TSC in the negative regulation of the mTOR pathway (Inoki *et al.*, 2002; Tee *et al.*, 2002) but equally, evidence suggests that TSC functions in an mTOR independent pathway (Jaeschke *et al.*, 2002).

$TSC2^{-/-}$ fibroblasts were used to investigate the role of TSC in upstream insulin signalling. Loss of TSC2 dramatically reduced levels of IRS-1 which had a knock-on reduction in IRS-1-associated class 1a PI3K signalling, confirmed by reduced signalling through PKB, mTOR (S2448 phosphorylation) and MAPK. Downregulation of IRS-1 can be attributable to constitutively active S6K1 in $TSC2^{-/-}$ fibroblasts; S6K1 increased phosphorylation of IRS-1 on ser/thr residues *in vivo* and *in vitro* and thus reduced levels of IRS-1 via a feedback mechanism. Partial recovery of IRS-1 tyrosine phosphorylation was observed with use of rapamycin (Harrington *et al.*, 2004). TSC therefore appears to have an important positive regulatory role in insulin signalling. The dramatic effects of constitutive S6K1 activation on IRS-1 turnover observed here maybe cell-type specific. Over-expressing TSC2 in HEK293 cells caused inhibition of mTOR-S2448 phosphorylation and reduced activity of S6K1; knocking out endogenous TSC2 by RNAi in HEK293 cells promoted mTOR-S2448 phosphorylation (Inoki *et al.*, 2002). The findings of Inoki *et al.*, (2002) were conducted in HEK293 cells in which components of the insulin signalling cascade were intact, albeit with interference in expression of *TSC,* and support the role of TSC in an mTOR-dependent pathway. The $TSC2^{-/-}$ fibroblasts used here suggest an mTOR-independent pathway because of reduced signalling through PKB and mTOR-S2448 phosphorylation. However, mTOR-dependent signalling to S6K1 could also operate if it were independent of PKB activation.

The $TSC2^{-/-}$ fibroblasts interfered not only with insulin signalling but also with reduced signalling through down-regulation of the PDGF receptor (Zhang *et al.*,

2003a). Moreover, additional data from our lab (C. Ktori, unpublished 2003) suggest that impaired activation of class 1a PI3K via IRS-1 can be compensated by class II PI3K. The significance of up-regulated class II PI3K activity in $TSC2^{-/-}$ fibroblasts in response to insulin and PDGF remains uncertain but it may be a cell survival mechanism to overcome the impairment imposed by reduced IRS-1 availability and lack of the PDGF receptor (Zhang *et al.*, 2003a) in the $TSC2^{-/-}$ fibroblasts.

Ultimately, the evidence suggests that TSC is involved in both mTOR-dependent and -independent pathways. As mentioned previously (Section 5.4) the $TSC2^{-/-}$ fibroblasts were also $p53^{-/-}$ which may also affect mTOR function (Castedo *et al.*, 2001; Castedo *et al.*, 2002; Horton *et al.*, 2002; Huang *et al.*, 2001). Though constitutive S6K1 was found to be the cause of IRS-1 down-regulation (Harrington *et al.*, 2004) the possible participation of mTOR cannot be ignored, not only because S6K1 is under the regulation of mTOR but because mTOR itself is implicated in down-regulation of IRS-1 signalling (Hartman *et al.*, 2001; Haruta *et al.*, 2000) and in the sub-cellular redistribution of IRS-1 (Takano *et al.*, 2001). The functional effects of mTOR in the $TSC2^{-/-}$ fibroblasts should prove interesting and the mTOR mutants could be utilized again for further studies. Additionally, we tested the phospho-T2446 mTOR antibody in western blot analysis of $TSC2^{-/-}$ lysates. However, blotting conditions were not optimal and the results were inconclusive so the impact from loss of TSC2 on T2446-mTOR phosphorylation remains to be determined.

Recent evidence has implicated the TSC2 GAP (GTPase-activating protein) activity toward Rheb in the regulation of mTOR signalling (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003). GAPs function by downregulating activated small GTP-bound proteins by accelerating GTP hydrolysis to produce the inactive GDP bound form. The GAP activity of TSC2, when in complex with TSC1, is directed to the small G-protein Rheb (Ras homolog enriched in brain) which is related to the Ras, Rap and Ral super family of small GTP binding proteins (Yamagata *et al.*, 1994). The functions of Rheb are poorly understood though it is predominantly GTP bound. Rheb was identified as a direct target of TSC2 GAP activity *in vivo* and *in vitro* where over-expression of *TSC1* and *TSC2* increased levels of Rheb-GDP (Zhang *et*

al., 2003b); mutations in the GAP domain were associated with tumorigenesis and abolished GAP activity. Further, epistatic studies in *Drosophila* place Rheb in the insulin signalling cascade downstream of dTSC and upstream of dTOR and dS6K (Saucedo *et al.*, 2003; Stocker *et al.*, 2003; Zhang *et al.*, 2003b). Furthermore, activated Rheb has been demonstrated to be a positive regulator of the mTOR signalling pathway as indicated through mTOR S2448 phosphorylation (Inoki *et al.*, 2003a) and the rapamycin-sensitive phosphorylation of S6K1 (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003) and 4E-BP1 (Inoki *et al.*, 2003a). Over-expression of Rheb promoted S6K1 phosphorylation in the absence of amino acids (Inoki *et al.*, 2003a; Saucedo *et al.*, 2003; Tee *et al.*, 2003; Zhang *et al.*, 2003b) and under conditions of energy depletion (Inoki *et al.*, 2003a); this evidence suggests that Rheb can also act in a nutrient and energy sensitive manner. Positive signalling through Rheb and mTOR was negatively regulated by TSC1/TSC2 (Castro *et al.*, 2003; Inoki *et al.*, 2003a; Tee *et al.*, 2003). The additional findings regarding Rheb introduce a further level of regulation to the mTOR signalling pathway via TSC. TSC is still a relatively new addition to the mTOR pathway and time restraints limited the number of studies performed with the $TSC2^{-/-}$ fibroblasts. Further experiments with the mTOR and PI3K reagents described here should prove useful in dissecting further the mTOR-dependent and –independent pathways, particularly in resolving the roles of AMPK and Rheb in -nutrient and -energy signalling to TSC and mTOR. The data presented here and the future experiments suggested should help unravel the intricacies of mTOR signalling and the complex pathways in which it is involved.

In summary, T2446 was identified as a novel mTOR phosphorylation site which is negatively regulated by insulin and positively regulated by nutrient deprivation, conditions which are converse to those which promote S2448 phosphorylation. AMPK may be involved in regulation of T2446 phosphorylation. Phosphorylation at T2446 and S2448 appears to be mutually exclusive and phosphorylation at these two sites may integrate growth factor signalling and nutrient availability. The current data suggest that the individual phosphorylations at T2446 or S2448 are not enough to affect downstream S6K1 activity and the functional significance of phosphorylation at these sites remains to be determined. Additionally, loss of TSC2

caused down-regulation of IRS-1 dependent class 1a PI3K activation as a result of deregulated S6K1 (Harrington *et al.*, 2004).

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