ASSOCIATION OF PROTEIN PHOSPHATASE 2A WITH S6 KINASE IS REGULATED IN AN MTOR-DEPENDENT MANNER

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DEDICATION

To my mother for being my inspiration and motivation, and to my father for his love and encouragement.

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by

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The mammalian target of rapamycin (mTOR) pathway senses nutrient and growth factors to regulate protein synthesis and cell growth. Strict control of the components of this pathway is essential for cells to reach an appropriate size. Aberrant mTOR signaling has been implicated in multiple diseases such as cancer, diabetes, and heart failure. Activation of mTOR by nutrients and growth factors leads to the phosphorylation of its two substrates, S6 kinase 1 (S6K1) and 4E binding protein 1 (4EBP1) to promote translation initiation. Many reports

have demonstrated the rapid dephosphorylation of S6K1 following treatment with the mTOR inhibitor, rapamycin, as well as by the depletion of amino acids. These data suggest a role for a serine/threonine protein phosphatase in mediating this dephosphorylation. This was supported by studies showing the co-immunoprecipitation of the catalytic subunit of protein phosphatase 2A (PP2A) with S6K1. PP2A is a ubiquitously expressed phosphatase that has been implicated in many intracellular signaling pathways. In this dissertation, an association between S6K1 and PP2A was investigated to elucidate the role of this phosphatase in regulating nutrient signaling and cell growth.

Initial studies utilized an inhibitor of PP2A and related phosphatases to block the dephosphorylation of S6K1 under conditions that inactivate mTOR. Immunoprecipitation studies identified an interaction between the PP2A catalytic and scaffolding subunits and S6K1 that was enhanced under conditions that lead to S6K1 dephosphorylation and decreased upon stimulation of the pathway by insulin. An siRNA screen targeting PP2A regulatory subunits was used to identify specific subunits that were mediating this association. Depletion of the B56γ and B56δ regulatory subunits reduced co-immunoprecipitation of PP2A with S6K1. However, functional studies found no effect of knockdown of these subunits on the dephosphorylation of S6K at T389 or on S6K1 kinase activity. There was no effect on cell size in cells depleted of B56γ or B56δ, but cell proliferation was reduced in these cells. It is likely that cell proliferation was affected due to the roles of these subunits in other pathways. Additionally,

knockdown of PP2A-like phosphatases did not definitively identify the phosphatase that dephosphorylates S6K1 at T389.

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LIST OF ABBREVIATIONS

4EBP – eIF4E Binding Protein

AA – Amino Acids

Amp - Ampicillin

α4 – Alpha 4

bp – base pairs

CL-A – Calyculin A

cDNA – Complementary deoxyribonucleic acid

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

eIF2 – Eukaryotic translation initiation factor 2

eIF3 – Eukaryotic translation initiation factor 3

eIF3-PIC – eIF3 Preinitiation Complex

eIF4E – Eukaryotic translation initiation factor 4E

ERK – Extracellular signal-regulated kinase

FACS – Fluorescence Activated Cell Sorting

FBS – Fetal Bovine Serum

FKBP12 – FK506-binding protein 12

FRAP – FKBP12-Rapamycin Associated Protein

FSC-H – Forward Scatter Height

GAP – GTPase Activating Protein

GTP – Guanosine triphosphate

HEAT – Huntington-Elongation Factor 3-protein phosphatase 2A A-TOR

HEK – Human Embryonic Kidney cells

I - Immune

IR – Insulin Receptor

IRS – Insulin Receptor Substrate

kDa – Kilodaltons

K_i – Dissociation constant of inhibitor

Luc - Luciferase

Met-tRNA – methionyl-tRNA

Min – minutes

mRNA – Messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

mTORC – Mammalian Target of Rapamycin Complex

mL – milliliter

mM – millimolar

mg – milligram

NI – Non-immune

nm – nanometer

nM – nanomolar

OA – Okadaic Acid

Oligo – Oligonucleotide

S6K – S6 Kinase

P-S6K – Phospho-S6 Kinase

PAGE – Polyacrylamide Gel Electrophoresis

PARP – Poly ADP-Ribose Polymerase

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDK1 – Phosphoinositide Dependent Kinase 1

PI3K – Phosphatidylinositol 3-kinase

PIKK – Phosphatidylinositol 3-kinase related kinase

PIP_2 – Phosphatidylinositol (4,5)-bisphosphate

PIP_3 – Phosphatidylinositol (3,4,5)-trisphosphate

PKB – Protein Kinase B

PP1 – Protein Phosphatase 1

PP2A – Protein Phosphatase 2A

PP4 – Protein Phosphatase 4

PP5 – Protein Phosphatase 5

PP6 – Protein Phosphatase 6

RAFT – Rapamycin and FKBP target

RAPT – Rapamycin target

Rap – Rapamycin

Raptor – Regulatory Associated Protein of mTOR

Rheb – Ras Homolog Enriched in Brain

RNA – Ribonucleic Acid

RNAi – RNA interference

SDS – Sodium Dodecyl Sulfate

shRNA – Short Hairpin Ribonucleic Acid

siRNA – Short Interfering Ribonucleic Acid

Tap42 – Type 2A-associated protein of 42 kDa

TGF-β – Transforming Growth Factor Beta

TSC – Tuberous Sclerosis Complex

TOR – Target of Rapamycin

tRNA – Transfer Ribonucleic Acid

μL – Microliter

μg – Microgram

μM – Micromolar

Chapter 1

Introduction

A. Regulation of Cell Growth By Nutrients

1. Protein Synthesis

Regulation of protein synthesis is a fundamental process in the modulation of cellular growth and metabolism. Protein synthesis is part of a coordinated process that promotes cell growth to produce a genetically predetermined cell size, organ, and body. This process is also essential for cell proliferation, as cells must reach an appropriate size in order to generate two daughter cells. Cell growth and proliferation are very closely linked, although exactly how cell size and cell division are connected remains unclear (Colon and Raff, 2003). There are a number of signaling pathways that govern cellular growth, and their responses to nutrient availability controls transcription as well as translation of mRNAs important in protein biogenesis. This process is highly demanding in terms of metabolic energy and amino acids as a source of fuels, and must be tightly regulated to ensure that it meets the requirements of the cell.

As precursors for protein synthesis, the availability of amino acids regulates signal transduction pathways that govern mRNA translation (Kimball and Jefferson, 2005). During deprivation of amino acids, protein synthesis decreases due to a decline in the availability of aminoacyl-transfer RNAs (tRNAs). As immediate precursors of protein synthesis, aminoacyl-tRNAs control translation at the point of elongation (Pain, 1994). When amino acids are

depleted, binding of the 40S ribosomal subunit to the initiator form of methionyl-tRNA (met-tRNA) is blocked, preventing formation of the 43S preinitiation complex. This binding is mediated by a complex consisting of the eukaryotic initiation factor (eIF) 2, GTP, and met-tRNA (Kapp and Lorsch, 2004). Low availability of amino acids leads to an accumulation of uncharged tRNAs that can activate the kinase Gcn2p which phosphorylates the α subunit of eIF2 (Hinnebusch, 1990). Phosphorylated eIF2α inhibits the guanine nucleotide exchange factor eIF2B, preventing assembly of the eIF2;GTP;met-tRNA complex and thereby repressing protein synthesis (Scorsone et al., 1987).

Binding of mRNA to the 43S preinitiation complex is the second important regulatory step in translation initiation. This process is mediated by another complex, referred to as eIF4F (Kapp and Lorsch, 2004). This multi-subunit complex controls translation through association of one of its components, eIF4E with its binding proteins, 4EBP1, 4EBP2, and 4EBP3. The best characterized of these is 4EBP1, and its binding blocks the association of eIF4E with the rest of the complex thus inhibiting translation initiation (Ptushkina et al., 1999). The ability of 4EBP1 to bind eIF4E depends on the phosphorylation state of 4EBP1 which is regulated by a growth and nutrient sensing pathways.

The mammalian target of rapamycin protein kinase (mTOR) is activated by amino acids and by growth factors such as insulin via the lipid kinase, phosphatidylinositol 3-kinase (PI3K) (Fruman, Meyer, and Cantley, 1998). Activation of mTOR leads to phosphorylation of 4E-BP1 and prevents it from inhibiting eIF4E association with the preinitiation complex. Additionally, when

mTOR is activated, it can phosphorylate another substrate, p70-S6 kinase (S6K1) (Burnett et al., 1998; Brown et al., 1995). As a result, there is increased phosphorylation of the S6 ribosomal subunit (von Manteuffel et al., 1997). Phosphorylation of S6 was originally thought to regulate translation of a select group of mRNAs containing a 5'-terminal oligopyrimidine tract (TOP) resulting in the upregulation of ribosome biogenesis (Loreni, Thomas, and Amaldi, 2000). However, more recent studies have questioned the role of S6 phosphorylation by S6 kinase in the translation of TOP mRNAs (Pende et al., 2004; Barth-Baus et al., 2002).

In addition to amino acids, protein synthesis is also regulated by growth factors. Extracellular signals such as the hormone insulin bind to their cell surface receptors leading to activation of downstream targets that eventually regulate 4EBP1 and S6K (Gingras, Raught, and Sonenberg, 2001). It is believed that there are both mTOR- and PI3K-dependent signals that together control S6K1 and 4EBP in response to a wide variety of growth stimuli. PI3K is a well-studied component of multiple cellular processes including cell growth, proliferation, survival, and migration (Cantley, 2002). Although signaling by growth factors such as insulin to mTOR occurs through a canonical signal transduction pathway, it is becoming clear that phosphorylation of S6K1 and 4EBP is regulated through the coordination and cross-talk of multiple pathways (Fingar and Blenis, 2004).

2. Mammalian Target of Rapamycin

Also known as FRAP (FKBP12-rapamcyin-associated-protein), RAFT (rapamycin and FKBP target), or RAPT (rapamycin target), mTOR is an evolutionarily conserved serine/threonine kinase that is the central regulator of a pathway that integrates inputs governing cell growth and translation (Wang and Proud, 2006). Activation of mTOR by nutrients such as amino acids and growth factors leads to phosphorylation of its two known downstream targets, 4EBP1 and S6 kinase 1 (Gingras, Raught, and Sonenberg, 2001) (Fig. 1). The mammalian target of rapamycin is a member of the family of phosphoinositide 3-kinase-related kinases (PIKKs). Although mTOR has a kinase domain similar to that of the lipid kinase, PI3K, it has been shown to have protein kinase activity in vitro (Dennis et al., 2001) As depicted in Figure 2A, this 289-kDa protein contains an N-terminal region that consists of 20 tandem HEAT (Huntington, Elongation Factor 3, A subunit of Protein Phosphatase 2A, TOR) repeats which are believed to mediate protein-protein interactions. The C-terminus contains the kinase domain which is next to the FRB (FKBP12-Rapamcyin-Binding) domain which binds the inhibitory rapamycin-FKBP12 complex. In addition, there is a FAT (FRAP, ataxia telangiectasia mutated, transformation/transcription domain-associated protein) domain which is N-terminal to the kinase domain and another FAT domain that is at the very C-terminus of the protein, termed FATC. The FAT and FATC domains are also believed to act as scaffolds for protein-protein interactions (Fingar and Blenis, 2004). Localization of mTOR is predominantly

in the cytoplasm, however cytoplasmic-nuclear shuttling is believed to be important in regulation of its substrates (Kim and Chen, 2000).

Much of the current understanding of the role that mTOR plays in regulating cell growth has come from studies utilizing the bacterial compound, rapamycin. Early studies in yeast identified FKBP12 as a receptor for rapamycin (Harding et al., 1989) and subsequent work in mammalian cells led to the identification and characterization of mTOR (Brown et al., 1994; Sabatini et al., 1995). These studies further demonstrated that the rapamycin-FKBP12 complex can bind mTOR and inhibit its ability to phosphorylate S6K1 and 4EBP1. It was then discovered that rapamycin inhibition of mTOR blocks G_1 cell cycle progression in a variety of mammalian cells (Villela-Bach et al., 1999). More recently, rapamycin was found to cause a reduction in cell size that was associated with G_1 arrest (Fingar et al., 2002). While rapamycin inhibits phosphorylation of S6K1 and 4EBP1, the mechanism remains unclear. It is believed that the rapamycin-FKBP12 complex disrupts association of mTOR with regulatory proteins such as raptor (regulatory associated protein of mTOR), that are important for targeting the enzyme to downstream substrates (Oshiro et al., 2004; Kim et al., 2002). There are two signaling complexes of which mTOR is a component; mTOR complex 1 (mTORC1) is rapamycin sensitive and regulates the metabolic actions of mTOR whereas mTORC2 is not sensitive to rapamycin and has effects on the cytoskeleton (Reiling and Sabatini, 2006; Wullschleger, Loewith, and Hall, 2006).

3. Activation of the mTOR Pathway

Control of protein translation by nutrients and growth factors is a complex process involving multiple signaling pathways that converge at mTOR (Fig. 1). Integration of these signals leads to the phosphorylation and activation of S6K1 and the phosphorylation of 4EBP1. Activation by growth factors such as insulin stimulates mTOR via the PI3K pathway (Nave et al., 1999). Stimulation of this pathway begins at the cell surface upon binding of a ligand such as insulin to its receptor (Cheatham and Kahn, 1998). This leads to tyrosine phosphorylation of a class of adaptor proteins known as the insulin receptor substrates (IRS) (White, 1997). These tyrosine phosphorylated proteins can then recruit signaling molecules containing a Src homology 2 (SH2) domain such as the p85 subunit of PI3K (Cheatham and Kahn, 1998). PI3K phosphorylates phosphoinositide-bisphosphate (PIP_2), generating phosphoinositide-trisphosphate (PIP_3) leading to the membrane recruitment of downstream effector kinases including Akt and PDK1 (3-phosphoinositide-dependent kinase 1) (Alessi et al., 1996). Akt is a positive regulator of mTOR signaling through its ability to phosphorylate and inactivate the tuberous sclerosis complex (TSC), which is a negative regulator of cell growth (Tee, Anjum, and Blenis, 2003; Manning et al., 2002). This complex is comprised of two proteins, hamartin (TSC1) and tuberin (TSC2), the latter of which has GTPase-activating protein activity towards the small GTPase Rheb (Ras homolog enriched in brain) (Li et al., 2004) The mechanism for how Rheb activates mTOR is unknown, but binding to mTOR is dependent on Rheb being in the GTP-bound state (Long et al., 2005). The TSC heterodimer acts a tumor

suppressor through its negative role in cell growth and thus mutations in these genes can result in the formation of benign tumors called hamartomas (Green, Smith, and Yates, 1994).

The mechanism through which mTOR senses amino acids is not as well-defined as its stimulation by growth factors. Most of the current knowledge on amino acid requirements for mTOR signaling has come from studies showing dephosphorylation of S6K1 and 4EBP1 by amino acid depletion (Fox et al., 1998; Hara et al., 1998). In particular, the branched-chain amino acid leucine was able to rescue S6K and 4EBP1 dephosphorylation in a rapamycin-dependent manner (Xu et al., 1998). These findings triggered a search for a leucine receptor that can signal the presence of leucine to cellular pathways. Although a receptor was not discovered, these studies led to the development of leucine derivatives that have been useful in further dissecting the regulation of mTOR by nutrients (Hidayat et al., 2003). Recently, evidence has emerged that implies a role for the lipid kinase, hVPS34 in amino acid-induced stimulation of mTOR and the subsequent phosphorylation of S6K1 (Byfield, Murray, and Backer, 2005; Nobukuni et al., 2005). This study showed that in the absence of PI3K, insulin stimulation does not lead to phosphorylation of S6K1 or Akt, but amino acid stimulation still induced phosphorylation of S6K1. However, there is much yet to be learned about nutrient stimulation of mTOR signaling and how it is regulated.

4. Regulation of S6K1

Ribosomal S6 kinase 1 is a ubiquitously expressed enzyme that is activated by a wide variety of signals, primarily via the mTOR and PI3K pathways. Mammalian cells possess two S6 kinase genes, S6K1 and S6K2 (Martin and Blenis, 2002). Most studies have focused on the 70 kDa isoform of S6K1 (p70-S6K1) which is predominantly cytoplasmic, but there is also an 85 kDa isoform (p85-S6K1) that localizes to the nucleus due to a nuclear localization sequence (NLS) at the N-terminus (Martin and Blenis, 2002). However, the two isoforms of S6K2 are both nuclear and only recently have investigators begun to study its role in cellular processes (Martin et al., 2001).

Activation of S6K1 by mTOR is characterized by a number of phosphorylation events that ultimately lead to the phosphorylation of its substrate, the 40S ribosomal protein S6 (Jeno et al., 1988). It is through coordination of these multiple phosphorylations that this molecule is able to regulate translation and cellular growth. Near the C-terminus of S6K1 lies a linker region that connects the C-terminus terminal end of the protein to the catalytic domain (Fig. 2B). Within this linker region lie two phosphorylation sites, threonine 389 (T389) and serine 371 (S371), both of which are essential for S6K1 activity (Pearson et al., 1995; Moser et al., 1997) . T389 is phosphorylated *in vitro* by mTOR and is rapamycin-sensitive, but the kinase for Ser371 is unknown (Burnett et al., 1998). Furthermore, biochemical studies have suggested a role for S6K1 autophosphorylation in the regulation of T389 phosphorylation (Romanelli, Dreisbach, and Blenis, 2002). The C-terminal region of S6K1 contains an

autoinhibitory pseudosubstrate domain that allows S6K1 to remain in an inactive conformation in which the pseudosubstrate domain interacts with the catalytic domain (Martin and Blenis, 2002). Additionally, there are four proline-directed phosphorylation sites in the C-terminus that when phosphorylated, are thought to "relax" the inhibitory effect of the pseudosubstrate domain, thus allowing phosphorylation at T389 by mTOR. Phosphorylation at T389 destabilizes the interaction between the catalytic and pseudosubstrate domains, creating a docking site for PDK1, which then phosphorylates threonine 229 (T229) in the activation loop resulting in full activation of S6K1 (Biondi et al., 2001). The kinases responsible for phosphorylation of the C-terminal residues have yet to be identified.

Activation of S6K1 by phosphorylation is an intricate process that has multiple steps, each of which plays a critical role in ribosome biogenesis. It is therefore essential that this process is tightly regulated otherwise it can lead to lower or even incomplete activation of the kinase. Many studies on S6K1 activity have demonstrated rapid dephosphorylation of T389 following rapamycin treatment or amino acid withdrawal (Pearson et al., 1995; Weng et al., 1998). While this process involves a protein phosphatase, the enzymes responsible for dephosphorylation of mTOR *in vivo* have not been identified.

B. Protein Phosphatases

1. Serine/Threonine Phosphatases

Protein phosphorylation is one of the most important regulatory events in eukaryotes and can dramatically alter the function of a cell. This reversible process is controlled by the opposing actions of protein kinases and protein phosphatases. Although protein kinases have received a tremendous amount of attention, protein phosphatases are also important players in cellular regulation. Through their ability to alter the properties of key regulatory proteins, serine/threonine phosphatases are an integral part of many signaling pathways. The most striking feature of serine/threonine phosphatases is the diversity that they can achieve through the formation of heteromultimeric holoenzymes (Virshup, 2000).

Eight classes of serine/threonine phosphatases have been identified in vertebrates. Classified based on a conserved phosphatase domain, protein phosphatases 1, 2A, 2B/calcineurin, 2C, 4, 5, 6, and 7 are members of the PPP gene family and can be grouped into two broad categories, the type-1 or PP1, and the type-2 phosphatases (Shenolikar, 1994). Much of the current knowledge regarding serine/threonine phosphatase function has been attained through the use of pharmacological inhibitors. The most widely used is okadaic acid (OA) which inhibits protein phosphatase 2A (PP2A) with a K_i of 0.2 nM and protein phosphatase 1 (PP1) with a K_i of 2 nM (Cohen, Holmes, and Tsukitani, 1990). Other serine/threonine phosphatases are insensitive to OA treatment, although protein phosphatases 4 (PP4) and 5 (PP5) were shown to be inhibited at higher K_i

values than PP2A (Chen et al., 1994; Hastie and Cohen, 1998). Calyculin A is another inhibitor of serine/threonine phosphatases. PP2A is most potently inhibited by calyculin A with a K_i in the low nanomolar range. However, other PP2A-like phosphatases such as PP4, PP5, and PP6 and to some degree PP1, are also calyculin-A sensitive (Cohen, Philps, and Vazquez-Martin, 2005). These phosphatases are able to carry out their cellular functions through numerous protein-protein interactions. For example, protein phosphatase 1 (PP1) has been reported to interact with over 70 proteins (den Hertog, 2003). PP4 has two known regulatory subunits in mammals that interact with the catalytic subunit to control activity (Cohen, Philps, and Vazquez-Martin, 2005). The less abundant PP5 is a single polypeptide that interacts with substrates through a tetratricopeptide repeat (TPR) (Chinkers, 2001).

2. Protein Phosphatase 2A

PP2A refers to a family of enzymes with a shared core structure that are involved in numerous cellular signaling processes (Janssens and Goris, 2001). Although previously thought of as a single enzyme, it is now widely accepted that PP2A exists in multiple forms and can therefore be described as a family or group of enzymes. PP2A is a heterotrimeric complex that consists of a core dimer that includes a scaffolding A subunit (PR65), and the catalytic C subunit (Fig. 3). The third component of the PP2A trimeric holoenzyme is the regulatory subunit, which is also referred to as the B subunit (Fig 3). The core dimer is present in most but not all forms of PP2A and its association with a vast array of regulatory

and interacting proteins generates considerable diversity. By interacting with the core dimer through both the scaffolding and catalytic subunits, the regulatory subunits are essential in targeting PP2A to intracellular locations and specific substrates (Janssens and Goris, 2001).

Phosphatase activity is conferred by the C subunit, which exists in two isoforms, α and β, that share 97% sequence identity (Stone, Hofsteenge, and Hemmings, 1987; Green, Yang, and Mumby, 1987). Although expression of both isoforms is ubiquitous, the α isoform is 10 times more abundant than the β isoform (Khew-Goodall and Hemmings, 1988). Studies attempting to over-express the catalytic subunit in mammalian cells have been difficult, suggesting the expression levels of this subunit are tightly regulated. The catalytic subunit is also critical for cellular survival as deletion of the C subunit gene resulted in embryonic lethality in mice (Gotz et al., 1998). Similar to the catalytic subunit, the scaffolding subunit has two isoforms. The α and β isoforms are ubiquitously expressed and share 86% sequence identity, but the α isoform is far more abundant (Hemmings et al., 1990). Structurally, the A subunit is comprised entirely of 15 HEAT repeats allowing it to participate in protein-protein interactions with the catalytic subunit and the many regulatory subunits. Additionally, mutations in the β isoform of the scaffolding subunit have been identified in 15% of primary lung and colon cancer-derived cell lines suggesting a role as a putative tumor suppressor (Wang et al., 1998). A more recent study demonstrated the inability of mutant Aβ to form a complex with and regulate the

phosphorylation status of RalA, thereby keeping Ral A active so that it can drive cells to a tumorigenic state (Sablina et al., 2007).

Targeting of the A/C core dimer to cellular substrates and to specific subcellular localizations is mediated by association with the regulatory or B subunits (Fig. 3). Expression of these regulatory proteins is cell- and tissue-specific as well as developmentally regulated (Janssens and Goris, 2001). Regulatory subunits have been grouped mainly into three families based on sequence similarity. The R2 regulatory subunit family which is also commonly known as the B or PR55 family consists of four isoforms that encode a 55 kDa subunit. The isoforms are expressed in a tissue-specific manner with the α and δ isoforms having wide expression and the β and γ isoforms being highly expressed in the brain and testes (Mayer et al., 1991; Zolnierowicz et al., 1994). Studies in *Drosophila* have led to a better understanding of the functions of many regulatory subunits. In *Drosophila*, low levels of the R2 subunit leads to death of larvae or abnormalities in larval brain cells in early adult flies (Mayer-Jaekel et al., 1993). Additionally, depletion of the R2 subunit in drosophila S2 cells by RNA interference delays activation of the MAP kinase pathway (Silverstein et al., 2002).

The R3 or B" subunit family members PR72 and PR130 were identified from human heart muscle and brain cDNA libraries, respectively (Hendrix et al., 1993). While they are believed be alternatively spliced products from the same R3α subunit gene, PR72 expression is primarily in the heart and skeletal muscle, whereas PR130 is widely expressed (Hendrix et al., 1993). Two other family

members were identified more recently using a yeast two-hybrid screen. PR59 expression was detected in a variety of tissues including testis, kidney, heart, liver and brain, but it was not found in skeletal muscle. It was identified through its interaction with the retinoblastoma-related p107 protein (Voorhoeve, Hijmans, and Bernards, 1999). The R3 isoform, PR48, localizes to the nucleus and interacts with the Cdc6 protein that is important in DNA replication (Yan et al., 2000). Interestingly, overexpression of either PR59 or PR48 causes cells to arrest in the G_1 phase of cell cycle (Voorhoeve, Hijmans, and Bernards, 1999; Yan et al., 2000).

The third major family of PP2A regulatory subunits is the R5 group. Also commonly known as the B' or B56 subunits, these isoforms have been associated with the regulation of a multitude of cellular pathways. Five isoforms for the R5 regulatory subunits have been identified. Expression of the α and γ isoforms is primarily in muscle, β and δ are highly expressed in brain, and ε in brain and testis (McCright, Brothman, and Virshup, 1996). Additionally, there are multiple splice variants of each isoform, adding another level of diversity to the number of PP2A holoenzymes that can be generated. Almost all isoforms can also be phosphorylated, suggesting that they are subject to post-translational regulation (McCright et al., 1996). The five isoforms and their variants have been implicated in many cellular signaling pathways and play an important role in regulating components of these pathways. The B56α subunit was shown to behave as a tumor suppressor through its association with the oncogene c-Myc, thus enhancing c-Myc degradation (Arnold and Sears, 2006). B56β was also

found to regulate c-Myc by interacting with the Pim-1 protein kinase (Ma et al., 2007). Depletion of B56β by short hairpin RNA (shRNA) caused stabilization of Pim-1 protein levels, allowing it to induce lymphomas via c-Myc. Very few functional studies involving the B56ε subunit have been reported. However, one group has demonstrated a positive role for B56ε in *Xenopus* embryogenesis by regulation of Wnt/β-catenin signaling (Yang et al., 2003).

B56γ is one of the most widely studied PP2A regulatory subunits. Dephosphorylation of p53 by B56γ-containing PP2A heterotrimers inhibits cell proliferation and transformation (Li et al., 2006) These data support previous reports suggesting the role of B56γ as a tumor suppressor by its ability to partially reverse tumorigenicity of human lung cancer cells lines (Chen et al. 2004). This was further supported in studies using a mouse melanoma cell line containing an alteration of the gene encoding B56γ that results in a truncated form of the protein that was associated with an aberrant cell cycle checkpoint leading to less apoptosis and increased metastasis (Ito et al., 2003). B56γ is also regulated through phosphorylation by ERK (Letourneux, Rocher, and Porteu, 2006). Phosphorylation by ERK caused dissociation of the A/C core dimer from a complex containing ERK and the early response gene IEX-1, resulting in sustained ERK activation.

The role of B56δ in cellular processes is not as well-characterized. However two recent studies have shown that it plays an important role in mitosis and in neuronal signaling (Margolis et al., 2006; Ahn et al., 2007). Margolis et al. used a *Xenopus* model to show that DNA damage-responsive checkpoints regulate

B56δ-mediated dephosphorylation of the mitotic activator Cdc25 and subsequent mitotic entry. In the other study, activation of dopamine D1 receptors causes activation of B56δ-containing PP2A resulting in the dephosphorylation of DARP-32, a protein important in modulating dopamine levels in the brain (Ahn et al., 2007). In both cases, B56δ activity towards a substrate was controlled by phosphorylation suggesting that similar to B56γ, this subunit is subject to regulation by this post-translational modification.

A potential fourth regulatory subunit family was identified based on a conserved region, shared with the R5 family. Striatin (P110) and S/G2 nuclear autoantigen (SG2NA; PR93) are members of this putative B''' family. These proteins bind the PP2A core dimer and also interact with calmodulin in a calcium-dependent manner (Moreno et al., 2000). Both proteins were identified in large multi-protein complexes, suggesting that they may function as scaffolds (Moreno et al., 2000).

3. Protein Phosphatases and TOR Signaling

One of the earliest studies that showed a connection between TOR signaling and a protein phosphatase was performed in yeast. A genetic screen identified an interaction between the phosphatase 2A associated protein of 42 kDa (Tap42) with the yeast PP2A catalytic subunits Pph21 and Pph22 and Sit4, the yeast homolog of PP6 (DiComo and Arndt, 1996). Tap42 did not associate with the type 1 phosphatase Glc7. Overexpression of Tap42 alone did not affect growth rate whereas overexpression of Pph21/22 reduced growth. However,

when both were overexpressed, there was an even greater reduction in growth than by PP2A-C alone (DiComo and Arndt, 1996). This suggests that Tap42 was somehow regulating PP2A-C activity, possibly by targeting it to a specific substrate. Furthermore, cells that contained a temperature sensitive allele of *TAP42* displayed rapamycin resistance and treatment with rapamycin induced dissociation of Tap42 with these type 2 phosphatases (DiComo and Arndt, 1996) suggesting that the association between Tap42 and PP2A-C (and Sit4) is TOR-dependent. A more recent study has shown that interaction with Tap42 inhibits the activity of PP2A-like phosphatases by sequestering them to TOR complexes on the membrane. Upon rapamycin treatment, the association of the Tap42-phosphatase complex with TORC is abrogated and phosphatases are released to the cytosol (Yan, Shen, and Jiang, 2006).

The mammalian homolog of Tap42 is the α4 protein with which it shares 24% sequence identity. Despite reports showing association of α4 with the catalytic subunits of PP2A, PP4, and PP6 (Prickett and Brautigan, 2006), there is no evidence suggesting its role in mTOR signaling is conserved. Experiments in *Drosophila* showed that dTap42 does not contribute to dTOR signaling and its depletion by RNAi did not affect S6K phosphorylation (Cygnar et al., 2005; Bielinski and Mumby, 2007).

The role of protein phosphatases in mediating dephosphorylation of S6 kinase 1 in mammals has remained largely unresolved. Use of pharmacological inhibitors has demonstrated a role for a PP2A-like phosphatase in T389 dephosphorylation as use of these drugs blocks dephosphorylation of T389 by

starvation, rapamycin treatment or cellular stress (Grove et al., 1991; Parrott and Templeton, 1999). However, the specific phosphatase that is being inhibited remains unclear since the concentrations of inhibitors used would inhibit even the less sensitive enzymes. A biochemical study has shown association of S6K1 with the catalytic subunit of PP2A and rapamycin treatment in Jurkat cells increased the activity of PP2A toward 4EBP1 (Peterson et al., 1999). This report also showed the inability of a rapamycin-resistant mutant of S6K1 to interact with PP2A-C and demonstrated phosphorylation of PP2A by mTOR *in vitro*. This was supported by another study in which the catalytic subunit of PP2A was isolated in complex with S6K following cross-linking of soluble brain extracts (Westphal et al., 1999). The only reported association of a PP2A holoenzyme with S6K came from work on TGF-β mediated cell cycle arrest (Petritsch et al., 2000). Activation of the TGF-β receptor (TβRI) by ligand induced its binding to the Bα (PR55α) regulatory subunit of PP2A which was then able to recruit the A/C core dimer to bind and dephosphorylate S6K (Petritsch et al., 2000). However, complexes containing TβRI, PP2A, and S6K were not identified suggesting that activated phosphatase is released from the receptor to target the substrate. It is interesting to note that rapamycin treatment did not induce association of PP2A with S6K in the polarized mammary epithelial cells used in the study; association was only seen upon TGFβ stimulation.

Even with the evidence provided in the reports discussed in the previous section, there still does not appear to be a definitive answer regarding phosphatase regulation of S6K dephosphorylation. What is clear is that this enzyme is

sensitive to the inhibitors, calyculin A and okadaic acid. The bulk of published reports support the idea that this phosphatase is PP2A. However, the data are not fully convincing and also conflict with each other with regard to how association of PP2A with S6K is regulated. The project described in this dissertation utilized immunoprecipitation studies under conditions that alter mTOR activity to determine if there is a correlation between S6K phosphorylation and association of PP2A with S6K. The molecular basis of this interaction was then investigated to identify the PP2A regulatory subunit associated with S6K and determine if it is modulating dephosphorylation at T389. The application of short interfering RNA (siRNA) technology was used to screen regulatory subunit interactions with S6K and regulation of T389 dephosphorylation by PP2A-like phosphatases.

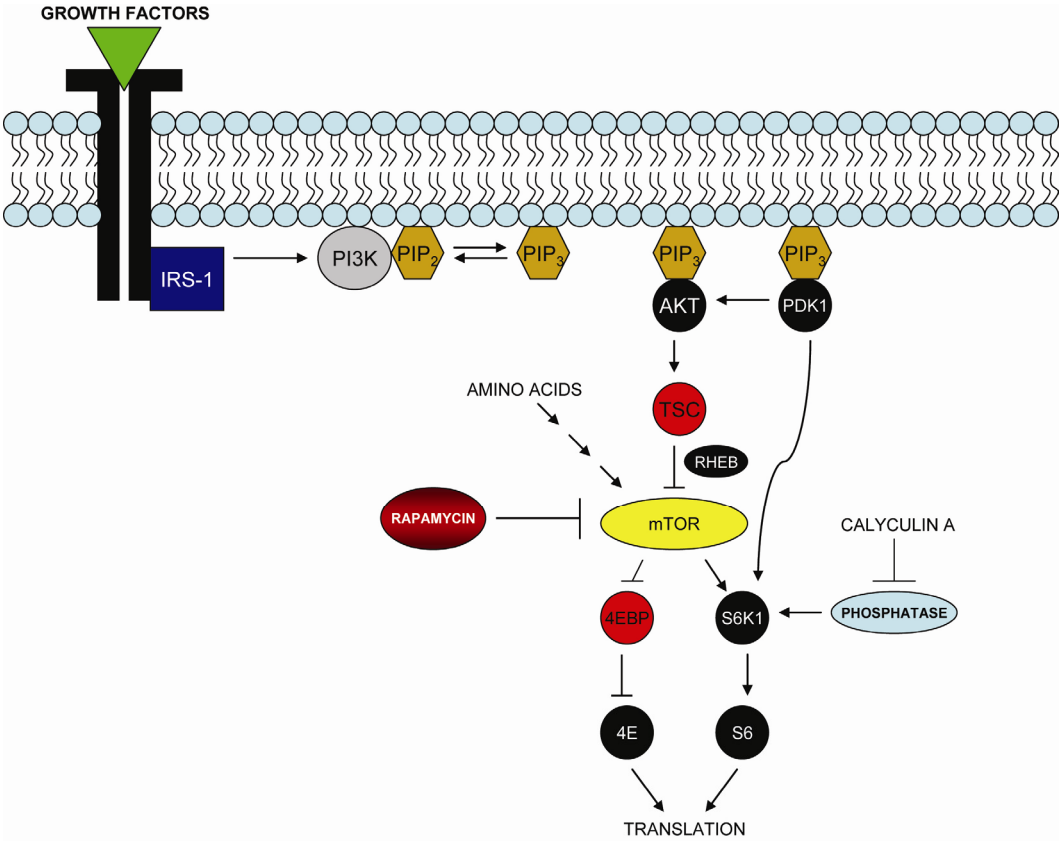

Figure 1. The mTOR signaling cascade. Stimulation by insulin or growth factors activates PI3 kinase which converts PIP_2 to PIP_3. This recruits Akt and PDK1 to the membrane where they are able to phosphorylate substrates. Phosphorylation of TSC2 by Akt prevents it from inhibiting Rheb, thus activating mTOR. mTOR phosphorylates its two known substrates, 4EBP1 and S6K1. Phosphorylation of 4EBP1 causes its dissociation from eIF4E (4E) and allows formation of the preinitiation complex. Phosphorylation of S6K1 by mTOR relieves its autoinhibition allowing PDK1 to phosphorylate it in the catalytic domain, leading to full activation. S6K1 can then phosphorylate the ribosomal protein S6 and initiate translation. Rapamycin is a bacterial agent that can potently inhibit mTOR activity. As a result, S6K1 is rapidly dephosphorylated by a calyculin A-sensitive phosphatase. Activation of mTOR is also regulated by amino acid levels although the pathways involved in this process are not clear.

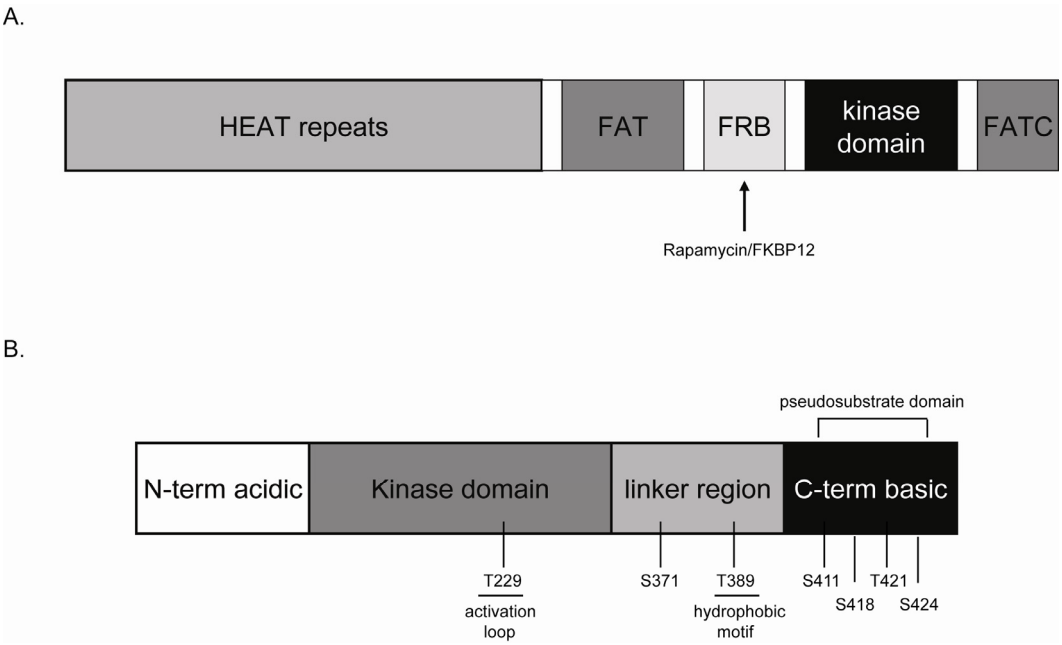

Figure 2. Domain structure of mTOR and S6K1. A) The domain architecture of mTOR is shown. The HEAT repeats are believed to mediate protein-protein interactions between mTOR and its binding partners. The FRB domain is where the Rapamycin/FKBP12 complex binds. FAT and FATC domains are found in all PIKKs. Adapted from Fingar and Blenis, 2004. B) The many important phosphorylation sites on S6K1 are shown in this schematic. Phosphorylation at T389 in the hydrophobic motif by mTOR relieves autoinhibition by the C-terminal pseudosubstrate domain allowing phosphorylation of T229 in the catalytic loop by PDK1. This leads to full activation of the kinase. The kinases that phosphorylate the C-terminal residues are unknown. Adapted from Romanelli and Blenis, 2002.

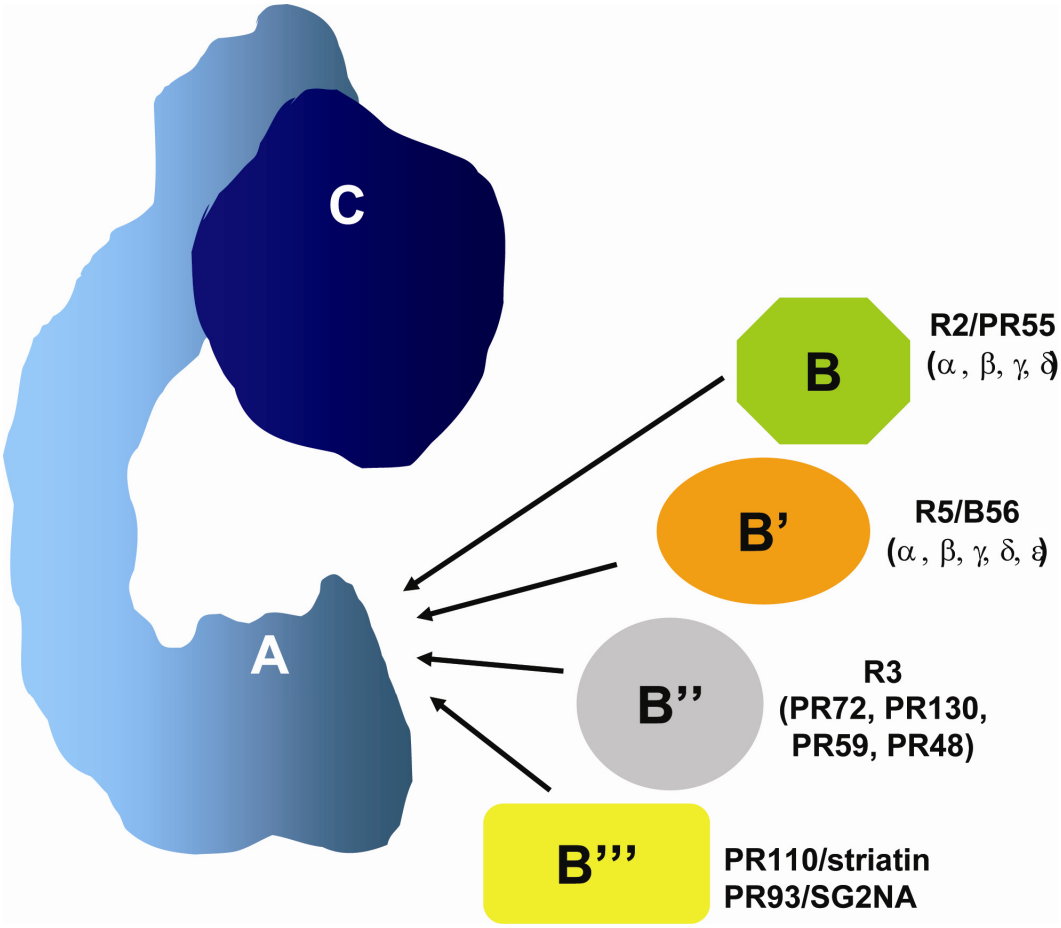

Figure 3. The PP2A holoenzyme. The catalytic (C) subunit is bound to the scaffolding (A) subunit to form the core dimer. The three major regulatory subunit families (B, B', and B") are shown as well as a potential fourth group (B'''). The A/C core dimer binds a regulatory subunit to form a heterotrimeric complex termed the PP2A holoenzyme. Adapted from Janssens and Goris, 2001.

Chapter 2

Association of PP2A with S6 Kinase 1

A. Introduction

Regulation of mTOR activity is an important process in nutrient signaling and in the response to cellular stress. Activation of mTOR by mitogens and nutrients leads to the phosphorylation of its two known downstream targets, S6 kinase and 4EBP1 (Gingras, Raught, and Sonenberg, 2001). Cells are then able to progress through the G_1 phase of the cell cycle, resulting in cellular growth (Chou and Blenis, 1995). Upon treatment with an osmotic stress agent such as sorbitol, nutrient starvation, or energy depletion, mTOR activity is down-regulated leading to dephosphorylation of S6K and 4EBP1 (Fox et al., 1998; Hara et al., 1998; Parrott and Templeton, 1999). In particular, S6K has been shown to be dephosphorylated by a serine/threonine phosphatase that is sensitive to the inhibitors, calyculin A and okadaic acid (Parrott and Templeton, 1999; Grove et al., 1991). These findings support previous reports that S6K associates with the catalytic subunit of protein phosphatase 2A (PP2A) (Peterson et al., 1999; Westphal et al., 1999; Petritsch et al., 2000). One of these studies demonstrates an increase in the associated catalytic subunit upon rapamycin treatment (Peterson et al., 1999).

Although PP2A has been suggested to be the phosphatase that dephosphorylates S6K upon mTOR inactivation, it is unclear how this process is regulated. Studies in yeast have suggested a model where the yeast homolog of the mammalian protein α4, Tap42, inhibits PP2A function through association

with the catalytic subunit of PP2A upon TOR activation (Jiang and Broach, 1999; Zabrocki et al., 2002). As a result, the enzymatic activity of the PP2A catalytic subunit is sequestered away from the regulatory and scaffolding subunits, preventing dephosphorylation of the substrate. However in mammalian cells, phosphatase regulation of S6K activity has been controversial and remains largely unresolved.

The goal of this chapter is to 1) verify that a calyculin A-sensitive phosphatase can block dephosphorylation of S6K by rapamycin and various cellular stresses by monitoring the phosphorylation sate of T389; 2) determine whether PP2A A and C subunits associate with S6K, and see if the association is regulated in an mTOR-dependent manner; 3) identify the regulatory subunit(s) mediating association of PP2A with S6K.

B. Experimental Procedures

1. Materials

Rapamycin, DMSO, sorbitol, insulin, poly-L-lysine hydrobromide, and protein A sepharose were obtained from Sigma (St. Louis, MO). Calyculin A was purchased from Cell Signaling Technology (Danvers, MA). Pfu Turbo was obtained from Stratagene (La Jolla, CA). The B56δ variant 1 and variant 2 clones were purchased from Open Biosystems (Huntsville, AL). The B56γ clone was previously described (Tehrani, Mumby, and Kamibayashi, 1996). The pCS2-MT vector was a generous gift from Hongtao Yu (UTSW, Dallas, TX).

Lipofectamine 2000 and Opti-MEM Reduced Serum Medium were purchased from Invitrogen (Carlsbad, CA).

2. Antibodies

Anti-phospho-p70 S6K (T389) (1A5) and anti-GAPDH were purchased from Cell Signaling Technology (Danvers, MA). Polyclonal anti-p70 S6K, anti-PP2A B56α, and anti-PP2A A subunit were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-p70 S6K was obtained from Upstate (Billerica, MA) and anti-Myc was obtained from Roche (Indianapolis, IN). Anti-B56δ was purchased from Novus Biologicals (Littleton, CO). Anti-PP2A C subunit, B56γ, and Bα have been previously described (Mumby et al., 1987; Mumby, Green and Russell, 1985).

3. Cell Culture

HEK 293T cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37^0C and 5% CO_2. For amino acid starvation media, DMEM was prepared according to the manufacturer's directions but all amino acids were left out.

4. siRNA

For short interfering RNA (siRNA) studies, 21 nucleotide RNA directed against target DNA sequences were obtained from Dharmacon (Lafayette, CO).

The DNA targeting sequences for PP2A-B56α were 5'-TGAATGAACTGGTTGAGTA-3' and 5'-CAATACAAGTGCCGAATAA-3'. The target sequences for PP2A-B56γ were 5'-GGAGAGAGATTTTCTTAAA-3' and 5'-TGATGGCACTTCTCAAATA-3'. PP2A-B56δ target sequences were 5'-TCCATGGACTGATCTATAA-3' and 5'-GCTTATATCCGTAGGCAGA-3'. The DNA target sequence for PP2A-Bα and Luciferase were 5'-CTTCTCCTCTGCTATGAGA-3' and 5'-TCGAAGTATTCCGCGTACG-3', respectively.

5. Cloning of B56γ and B56δ into pCS2-MT vector

PCR primers were designed to produce full-length human B56δ variant 1, B56δ variant 2, and B56γ variant 3 cDNA with *Eco*RI and *Xba*I restriction site 5' and 3' of each sequence, respectively. The resulting PCR products and the vector were digested with *Eco*RI and *Xba*I and separated by electrophoresis on a 1% agarose gel. Vector and insert were cloned into the pCS2-MT vector with 6-Myc tags using T4 ligase overnight at 16^0C. TOP10 *E. coli* cells were transformed using ligated vector and insert by incubating at 42^0C for 45 seconds. Cells were then put on ice for 2 minutes and allowed to recover in 100 μL of LB media for 1 hour prior to plating on LB-AMP plates overnight at 37^0C. After overnight incubation, colonies were picked, overnight cultures were grownm and mini-preps performed (SV Miniprep Kit) (Promega, Madison, WI). Restriction digests were performed to verify that the Myc-tag was in-frame with the cDNA sequence.

6. Calyculin A Treatments

$5x10^5$ HEK 293T cells were plated in 2mL of media in 35mm dishes coated with poly-L-lysine. Cells were incubated overnight at 37^0C in a 5% CO_2 atmosphere. The following day, media was replaced with new growth medium containing 50 nM calyculin A for 45 minutes. Media was aspirated and replaced with new media containing either 10 nM rapamycin or 200 mM sorbitol, or with media without amino acids in the presence of 50 nM calyculin A for indicated time-points. Control cells were incubated with DMSO alone in place of calyculin A. Cells were harvested by washing with 5 mL 1x ice-cold PBS and then lysed directly on the plate by addition of 5x SDS buffer (5% SDS, 0.5% 2-mercaptoethanol, 50% glycerol, 312.5 mM Tris- HCl pH 6.7, 0.5% bromophenol blue). Cells were scraped, transferred to Eppendorf tubes, briefly vortexed, and then boiled for 3 minutes. Samples were allowed to return to room temperature, followed by further vortexing for 30 seconds. Lysates were then centrifuged at 14,000 rpm at room temperature for 10 minutes. Protein concentration of lysates was determined using the amido black assay (modified from Schaffner and Weissman, 1973). Samples were run on an 8% SDS-PAGE and proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA) followed by Western blotting.

7. Transfection of HEK 293T Cells

Cells were plated at a density of $2.4x10^6$ cells per 100 mm dish. 16-18 hours after plating, transfections were carried out using Lipofectamine 2000

according to the manufacturer's directions. 600 picomoles of each siRNA were transfected per plate. For DNA transfections, 5 μg of Myc-B56δ variant 1 or Myc-B56δ variant 2 was transfected per plate, while 15 μg of Myc-B56γ or HA-Bα was used.

8. Cell Lysis and Immunoprecipitations

48 hours after transfection, cells were washed once with cold 1x PBS and then 300 μL of cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO) was added directly to the plate. Cells were scraped, transferred to an Eppendorf tube and incubated on ice for 30 minutes. Lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4^0C and protein concentration was determined using the BCA method (Pierce, Rockford, IL). For immunoprecipitations, 2.5 mg of lysate was incubated with 40 μL of protein A sepharose beads and 2 μg of anti-S6K polyclonal antibody for 2.5 hours at 4^0C with gentle rotation. Beads were washed 5 times with 500 μL of wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1% SDS) containing a protease inhibitor cocktail and phosphatase inhibitor cocktails 1 and 2. After the last wash, a 27-guage needle was used to vacuum out any residual buffer. Samples were then solubilized by adding 2x SDS buffer and boiled for 3 minutes. The entire eluate was run on an 8% SDS-PAGE, transferred to a nitrocellulose membrane and Western blotted.

C. Results

1. S6K1 is Dephosphorylated at T389 by a Calyculin A-Sensitive Phosphatase.

S6K has been previously shown to be dephosphorylated at T389 in response to growth factor and nutrient deprivation (Grove et al., 1991) as well osmotic stress (Parrott and Templeton, 1999). However, this effect can be blocked by treatment with the phosphatase inhibitor, calyculin A (Parrott and Templeton, 1999; Peterson et al., 1999). To optimize conditions under which S6K dephosphorylation can be blocked most effectively by calyculin A in HEK 293T cells, the phosphorylation level of T389 was monitored by Western blot analysis. Time course experiments to observe S6K dephosphorylation following rapamycin treatment, amino acid starvation, and sorbitol treatment were carried out (Fig. 4). Control cells that were not treated with calyculin A showed a decrease in phosphorylation at T389 over time following treatment with 10 nM rapamycin (Fig. 4A; left). Complete dephosphorylation at this site was seen on both p70- and p85-S6K by 30 minutes. Pre-treatment of cells with 50 nM calyculin A blocked rapamycin-induced dephosphorylation and maintained high levels of phospho-T389 (Fig. 4A; right). Basal levels of T389 phosphorylation were also elevated upon calyculin A treatment prior to addition of rapamycin (time=0).

Similar to rapamycin treatment, incubation of HEK 293T cells in medium lacking amino acids resulted in a time dependent dephosphorylation of S6K at T389 (Fig. 4b; left). When cells were pre-treated with 50 nM calyculin A,

dephosphorylation was blocked and high levels of phospho-T389 were observed (Fig. 4b; right). This effect was seen for both p70- and p85-S6K. Treatment with the hyper-osmotic stress agent, sorbitol, also caused dephosphorylation of T389 (Fig. 4c; left). The kinetics of dephosphorylation by sorbitol appeared to be faster than those observed for rapamycin and amino acid starvation as almost all phoshpho-T389 signal was gone by 10 minutes. Pre-treatment of HEK 293T cells with 50 nM calyculin A blocked sorbitol-induced dephosphorylation of T389 and maintained elevated levels of phospho-T389 throughout the time course. Blotting for total S6K levels showed a shift to less phosphorylated species over time in control samples without inhibitor. S6K from calyculin A treated samples had a slower mobility and are not as well resolved due to phosphorylation. A band shift in these samples was also observed as a result of hyper-phosphorylation at this site. Additionally, the intensity of S6K bands in calyculin A-treated samples is lower than DMSO-treated samples due cell death caused by this drug that results in reduced protein synthesis. These results are consistent with previous reports that inhibition of mTOR activity by rapamycin, amino acid starvation, and sorbitol treatment cause dephosphorylation of S6K T389 buy a calyculin A-sensitive phosphatase.

2. The PP2A Core Dimer Associates with S6 Kinase in an mTOR-Dependent Manner.

Previous reports have suggested that the catalytic subunit of PP2A is associated with S6K (Peterson et al., 1999; Petritsch et al., 2000; Westphal et al.,

1999). In order to confirm these observations and characterize the interaction in more detail, S6K was immunoprecipitated and associated PP2A was detected by immunoblotting. S6K1 was immunoprecipitated from naïve HEK 293T cells and co-immunoprecipitation of both the A and C subunits was observed suggesting that there is association even under normal growth conditions in complete medium (Fig. 5A). S6K was also immunoprecipitated using two additional antibodies that recognize distinct epitopes on S6K and under these conditions, the PP2A core dimer was also co-immunoprecipitated (data not shown). As previously shown, rapamycin treatment, amino acid starvation, and sorbitol treatment all result in dephosphorylation of S6K at T389. To determine if stress treatments affected the amount of PP2A core dimer associated with S6K1, cells were treated with either rapamycin or sorbitol, or starved of amino acids. As seen in Figure 5B, this resulted in dephosphorylation of S6K at T389 (lower panel). Under these conditions there was a marked increase in the amount of PP2A core dimer that co-immunoprecipitated with S6K1 when compared to untreated control cells (Fig. 5B; upper panel). An increase in both A and C subunits was observed under each stress treatment. As in Figure 5A, a low level of basal association was observed in untreated cells.

Insulin is an activator of the mTOR kinase resulting in phosphorylation of S6K1 at T389. To test the effect of insulin treatment on association of PP2A with S6K1, immunoprecipitations were performed from cells starved of serum overnight, and then treated with 100 nM insulin for 30 minutes. Compared to untreated and serum starved samples, immunoprecipitations from insulin-treated

cells had a higher level of associated PP2A core dimer (Fig. 5C). Untreated and serum starved samples had comparable amounts of co-precipitated A and C subunits.

3. Identification of PP2A Regulatory Subunits Mediating Association with S6 Kinase.

To determine if there was a specific PP2A regulatory subunit that was regulating the association of the A and C subunits with S6K, an siRNA screen targeting many of these subunits was utilized. Members of the B56 regulatory subunit family were knocked down by siRNA in HEK 293T cells followed by immunoprecipitation to observe any changes in associated A and C. Interestingly, two members of this family, B56δ and B56γ both appeared to have an effect (Fig. 6A; upper panel). Two distinct siRNA oligos targeting B56γ and B56δ were used. One of the B56γ siRNA treatments showed a slight decrease in the amount of PP2A A and C subunits that co-precipitated compared to Luciferase control siRNA, while both B56δ siRNAs yielded a more dramatic decrease.

These immunoprecipitates were then probed with an antibody against B56δ and endogenous protein was found to be associated with S6K. As expected, no B56δ was detected in samples from B56δ siRNA-treated lysates. Endogenous B56γ was not detected (data not shown). The efficiency of knockdown was determined in lysates from immunoprecipitated samples by Western blotting. B56δ siRNA treatment resulted in highly efficient knockdown whereas B56γ

siRNA was not as efficacious but still caused greater than 50% reduction in protein levels (Fig. 6A; lower panel).

The previous experiment was repeated using siRNA against B56δ and B56γ individually, as well as siRNA against both in combination (Fig. 6B; upper panel). As before, knockdown of B56δ had the most dramatic effect, reducing levels of co-immunoprecipitated core dimer to levels that were barely detectable by Western blotting compared to control samples. Treatment of cells with siRNA against B56γ also slightly reduced the amounts of A and C subunit that were pulled-down. However, when knockdown of B56γ and B56δ were combined, there was an additive effect that resulted in greater reduction in associated A and C than either siRNA treatment alone. It is interesting to note that of the five members of the B56 family, γ and δ are the most closely related with respect to sequence similarity (McCright et al., 1996). Western blotting of lysates showed that knockdown efficiency was high in samples treated with both siRNAs (Fig. 6B; lower panel)

To investigate the specificity of B56γ and B56δ in mediating the association of the core dimer with S6K1, a related family member, B56α was knocked down by siRNA treatment. Once again, highly efficient knockdown was achieved as observed by immunoblotting protein levels in lysates from which immunoprecipitations were performed (Fig. 7A; lower panel) using different siRNA duplexes corresponding to distinct B56α mRNA target sequences. Immunoprecipitations carried out in B56α knockdown lysates showed no difference in associated A and C subunits compared to those from control

Luciferase siRNA-treated samples (Fig. 7A; upper panel). This suggests that B56α does not mediated association of PP2A with S6K. Additionally, endogenous B56δ co-precipitated with S6K1 in both Luciferase and B56α siRNA treated samples.

A specific role for B56γ and B56δ was further investigated by looking at a regulatory subunit from a distinct family. Bα (PR55α) belongs to the B family of regulatory subunits and knockdown by siRNA caused a significant reduction in protein levels (Fig 7B; lower panel, lower band). A single siRNA construct was used to knockdown Bα in duplicate and then S6K was immunoprecipitated as before. In comparison to control Luciferase siRNA treated samples, there was no effect of Bα knockdown on the amount of co-precipitating A and C subunits suggesting that it does not play a role in S6K association with PP2A (Fig. 7B; upper panel).

4. Association of Exogenously Expressed B56γ and B56δ with S6K.

The knockdown experiments suggested the involvement of both B56γ and B56δ in mediating association of PP2A with S6K. This was investigated further by over-expression of Myc-tagged constructs of each of these subunits in HEK 293T cells followed by immunoprecipitation of S6K1 (Fig. 8). Both variants 1 and 2 of Myc-tagged B56δ co-immunoprecipitated with S6K. Myc-B56γ variant 3 also co-immunoprecipitated with S6K at levels that were lower than the two Myc-B56δ variants. However, Western blots of immunoprecipitation lysates show that Myc-B56γ was expressed at lower levels than Myc-B56δ which may

explain why there appeared to be less in the immunoprecipitated sample. These data further support the observation that both B56δ and B56γ associate with S6K.

Reciprocal immunoprecipitations were carried by using an antibody against Myc to pull-down the B56 subunits and to look for association of S6 kinase (data not shown). In these experiments, co-immunoprecipitating endogenous S6K1 could not be detected from cells expressing Myc-B56γ or Myc-B56δ. However, these variants were over-expressed at such high levels that the fraction associated with S6K1 may have been too low to detect by Western blotting.

S6K1 was also immunoprecipitated from lysates over-expressing an HA-tagged construct of the regulatory subunit, Bα (HA- Bα). In both HA-Bα over-expressing cells and empty vector transfected cells, both PP2A A and C subunits were co-immunoprecipitated with S6K at similar levels (Fig. 9). However, no associated HA- Bα was detected even though the levels expressed in the lysates were very high.

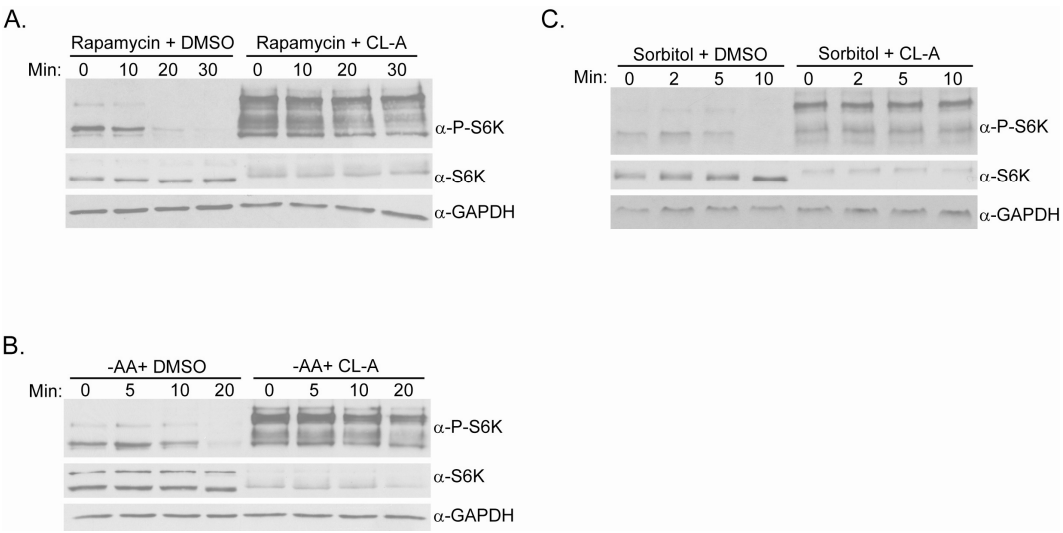

Figure 4. Dephosphorylation of S6K at T389 is blocked by calyculin A. HEK 293T cells were pre-treated with 50 nM calyculin A (CL-A) or DMSO in complete media for 45 minutes. New media containing (A) 20 nM rapamycin or (C) 400 mM sorbitol was then added in the presence of 50 nM calyculin A or DMSO for indicated time points. (B) For amino acid starvation treatments (-AA), media lacking all amino acids was added with calyculin A. Samples were harvested by washing once in cold PBS and then 5% SDS sample buffer was added directly to the plate. Samples were boiled, vortexed, and centrifuged prior to loading on 8% SDS-PAGE. Western blot analysis was carried out using antibodies against phospho-S6K (T389), total-S6K, and GAPDH as a loading control.

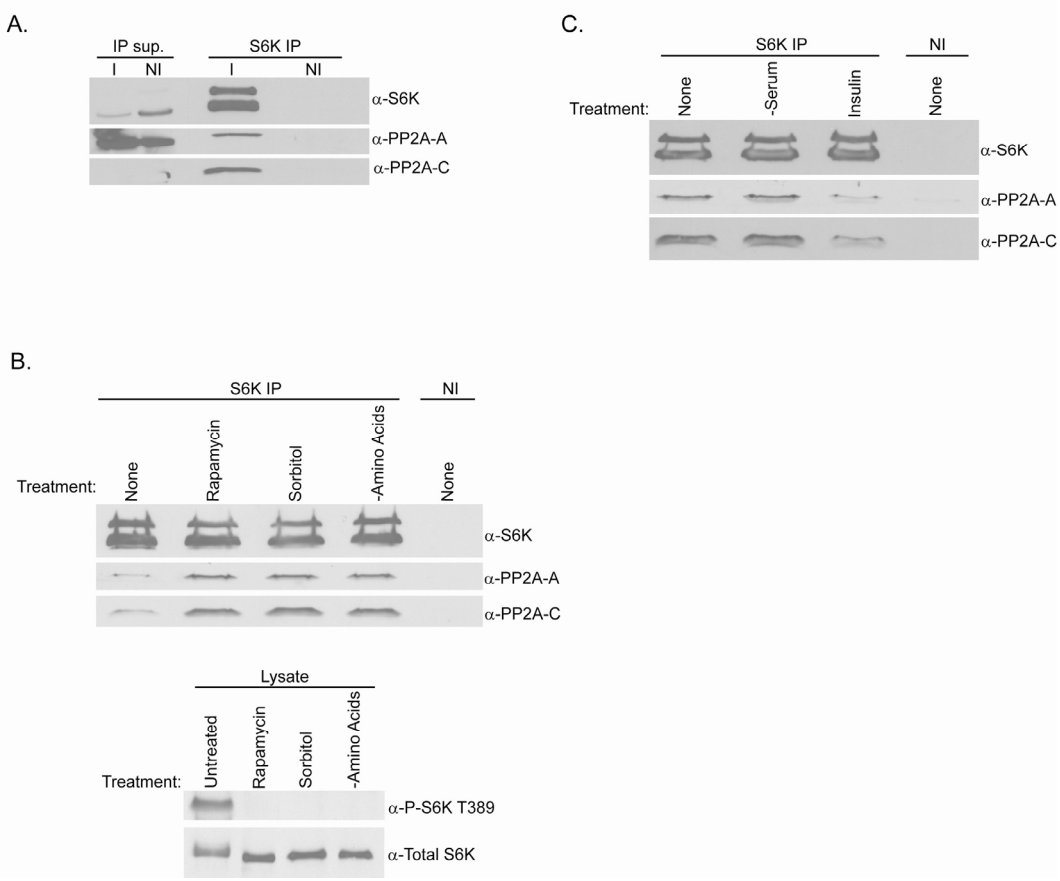

Figure 5. Association of the PP2A core dimer with S6K1 is regulated by mTOR. (A) Co-immunoprecipitation of PP2A A and C subunits with S6K1 in untreated cells. (B) For inactivation of mTOR, cells were treated with either 20 nM rapamycin, 400 mM sorbitol, or starved of amino acids for 45 minutes. (C) For insulin treatment, cells were serum-starved overnight and then placed in media with serum and 100 nM insulin for 30 minutes. Cells were lysed and immunoprecipitations were carried out by incubating lysates with protein A beads and antibody for 2.5 hours at 4^0C. Non-immune (NI) samples were incubated with rabbit IgG. Pellets were washed 5x, solubilized in SDS sample buffer and then analyzed by 8% SDS-PAGE and immunoblotting.

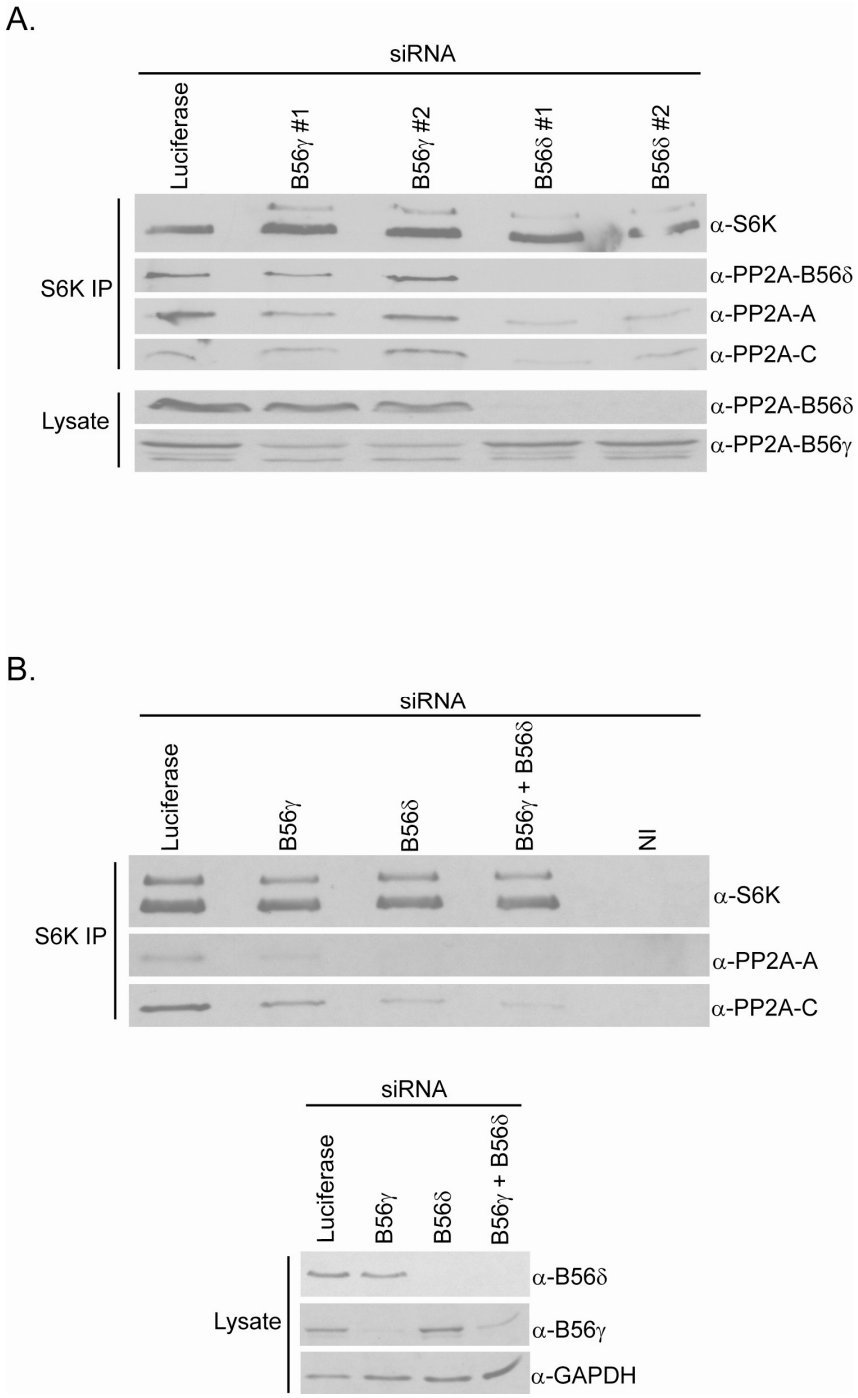

Fig 6. Knockdown of B56γ and B56δ reduces association of PP2A with S6K1. (A) Cells were plated on 100 mm dishes and transfected the following day with 600 picomoles of siRNA. (B) For double knockdowns, cells were transfected with 600 picomoles of each oligo. Cells were harvested 48 hours after transfection and S6K1 was immunoprecipitated. Co-immunoprecipitation of A and C was detected by Western blotting.

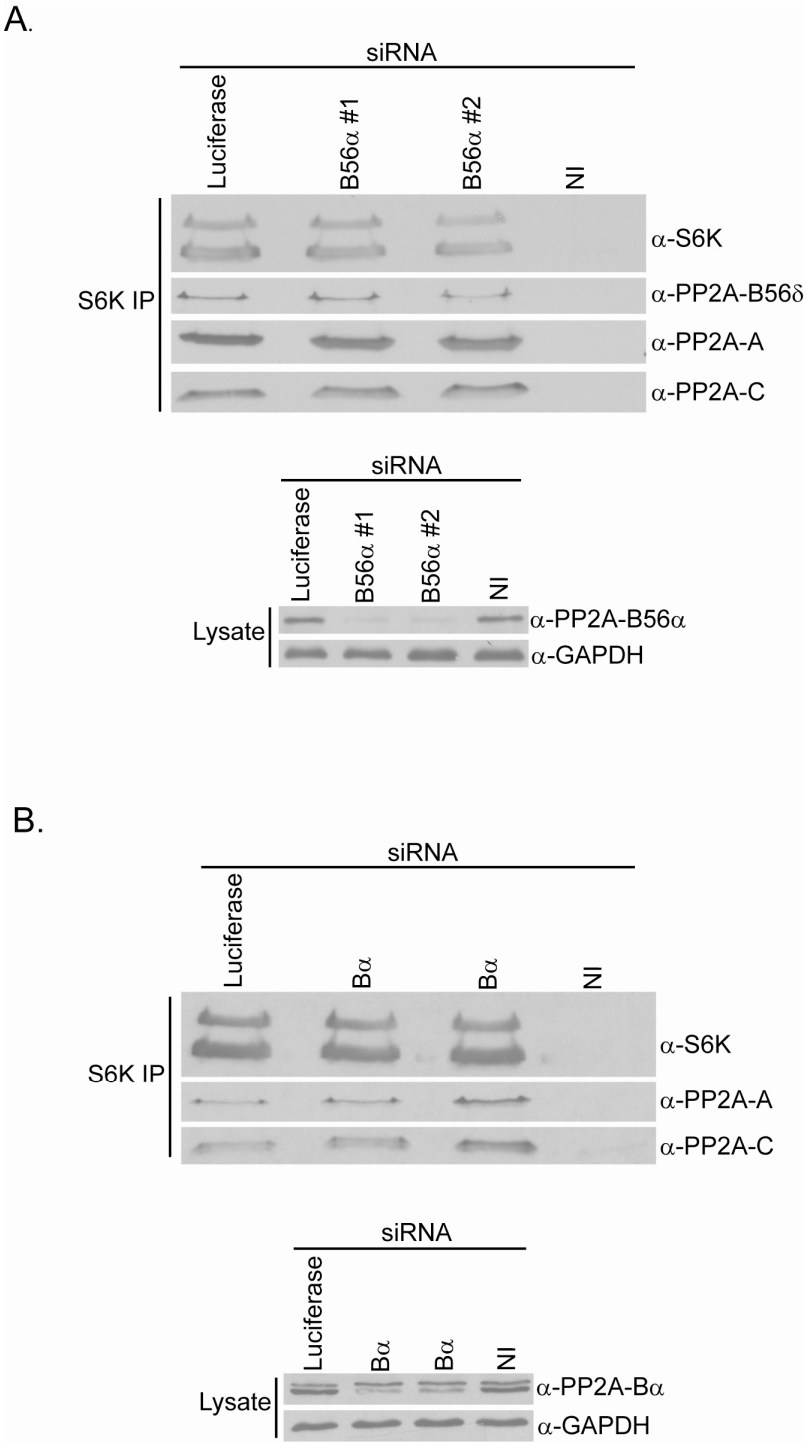

Figure 7. Knockdown of B56α or Bα does not affect association of PP2A with S6K1. Cells were transfected with siRNA targeting (A) B56α or (B) Bα and knockdowns were carried out for 48 hours. S6K was immunoprecipitated and pellets washed 5x with wash buffer. Samples were solubilized in SDS sample buffer and run on 8% SDS-PAGE and Western blot analysis was performed. Non-immune (NI) samples were incubated with purified rabbit IgG.

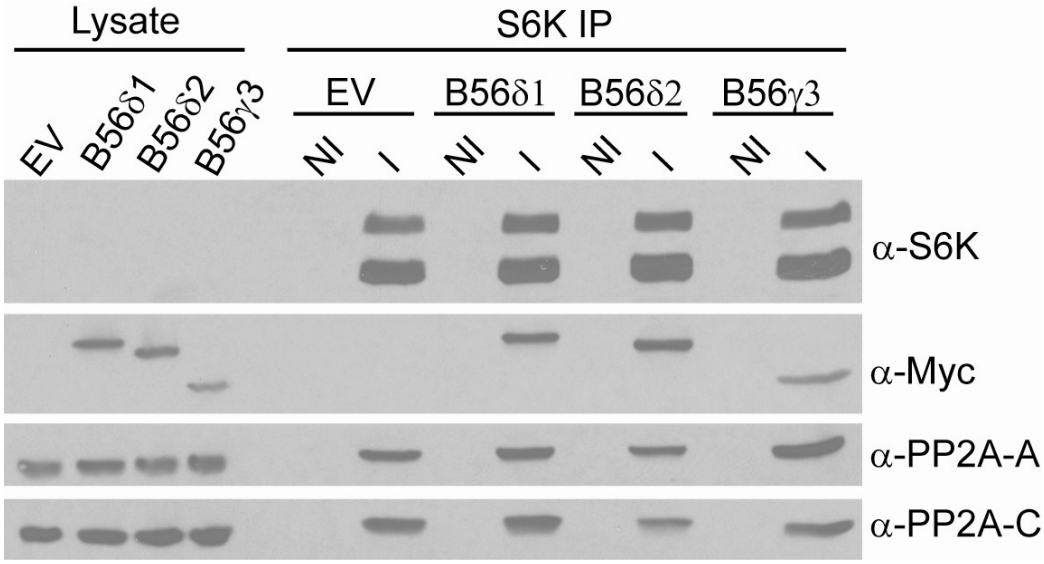

Figure 8. Association of Myc-B56γ and Myc-B56δ with S6K1. Cells were transfected with Myc-tagged B56δ variant 1 (B56δ1), B56δ variant 2 (B56δ2), B56γ variant 3 (B56γ3) or empty vector (EV) and harvested 48 hours post-transfection. Lysates were incubated for 2.5 hours with protein A beads and α-S6K antibody for immune (I), or rabbit IgG for non-immune (NI) samples. S6K-bound beads were washed five times and then solubilized in 2x SDS sample buffer. Samples were run on 8% SDS-PAGE and analyzed by western blotting. Lysates lanes are 1/500 of the total lysate that was split between corresponding immune and non-immune samples.

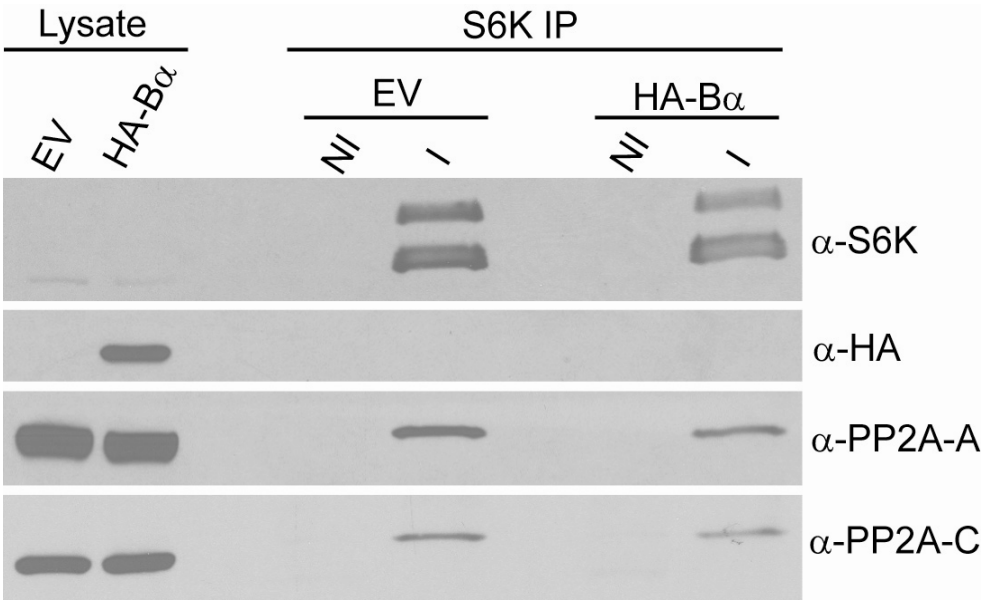

Figure 9. Over-expressed Bα does not associate with S6K1. Cells were transfected with HA-tagged Bα and harvested 48 hours later. Lysates were incubated with protein A beads, a-S6K antibody (I) or rabbit IgG (NI) for 2.5 hours. Samples were washed and solubilized in SDS sample buffer and then run on 8% SDS-PAGE. Samples were transferred to a nitrocellulose membrane and probed using the indicated antibodies.

D. Discussion

The major findings that have emerged from this study demonstrate an association of the PP2A core dimer with S6 kinase 1 that increases upon down-regulation of mTOR activity, and decreases when mTOR is activated. They further identify two related PP2A regulatory subunits that associate with S6K and whose depletion by siRNA disrupts co-immunoprecipitation of the A/C core dimer with S6K1.

Initial studies focused on blocking the dephosphorylation of S6K at T389 using an inhibitor of PP2A-like phosphatases. Rapamycin, sorbitol and amino acid starvation-induced dephosphorylation of T389 was blocked by calyculin A, confirming previous reports that a PP2A-like phosphatase mediates dephosphorylation of this regulatory site in the S6K linker region (Parrott and Templeton, 1999; Grove et al., 1991). Furthermore, the rapid kinetics of dephosphorylation observed in the absence of inhibitor suggests that a phosphatase is actively involved in this process.

To identify the phosphatase that is responsible for dephosphorylation of S6K at T389, immunoprecipitation studies were performed that showed the association of both the scaffolding and catalytic subunits of PP2A with S6K. Co-immunoprecipitation of the core dimer was enhanced when mTOR activity was inhibited by rapamycin, sorbitol or amino acid starvation. However, when mTOR was activated by insulin, co-precipitation of PP2A A and C subunits was diminished. Thus, the association of the PP2A core dimer with S6K is negatively correlated with mTOR kinase activity. The ability of rapamycin to increase

association of PP2A suggests that mTOR activity plays a direct role in this process.

Association of the A and C subunits of PP2A is typically mediated by a regulatory subunit. Treatment of HEK 293T cells with siRNA against PP2A regulatory subunits identified B56δ and B56γ as important components in the co-immunoprecipitation of PP2A with S6K1. Knockdown of B56δ by siRNA had the biggest effect on association of the core dimer with S6K1, suggesting that it is the predominant regulatory subunit involved in this interaction. However, B56γ also plays a role as knockdown of this subunit has a mild effect by itself, but leads to almost complete disruption of the complex when it is knocked-down together with B56δ. It should be noted that knockdown of B56γ is not as efficacious as that of B56δ and this may be a reason why knockdown of B56γ alone does not have as strong an effect on co-immunoprecipitation of A and C subunits. Similar roles of these two regulatory subunits in this interaction are not surprising since B56γ and B56δ are more closed related to one another than to any of the other B56 family members. A high degree of sequence similarity at the C-terminus of these two subunits supports the observation that they could have redundant roles in mediating interaction of PP2A with potential substrates. This specificity was further demonstrated by showing that knockdown of B56α with two different siRNA oligos had no effect on the co-immunoprecipitation of the core dimer. Additionally, knockdown of Bα (PR55), a member of another regulatory subunit family also did not have any effect. These findings confirm a specific role of B56δ and B56γ in this interaction.

Co-immunoprecipitation studies also demonstrated the association of endogenous B56δ with S6K1. Although endogenous B56γ was not seen in these experiments, it is possible that this was due to detection limits of the antibody used and because B56γ runs at approximately the same molecular weight as antibody heavy chain which could be occluding the subunit band. Over-expression of Myc-tagged B56δ and B56γ showed co-immunoprecipitation of both with S6K whereas HA- Bα did not interact with S6K. Association of both Myc-B56δ variants and Myc-B56γ complements the knockdown data and further supports the role of these two subunits in the interaction with S6K.

Chapter 3

Functional Analysis of Phosphatase Regulation of S6 Kinase

A. Introduction

The study of signaling pathways in mammalian cells has benefited greatly from the development of RNA interference. This technology has become a powerful tool that has widespread use as a method to silence pathway components and study the resulting consequences. Many recent studies have used this method to investigate the role of protein phosphatases in regulating the activity and function of important cellular proteins (Ahn et al., 2007; Bielinski and Mumby, 2007; Rocher et al., 2007; Tang et al., 2006). Use of RNAi in *Drosophila* S2 cells has shown that knockdown of the catalytic subunit of PP2A can block the dephosphorylation of S6K caused by depletion of amino acids (Bielinski and Mumby, 2007).

PP2A regulatory subunits play an essential role in regulating the activity of the holoenzyme. In addition to targeting PP2A to specific substrates, regulatory subunits target the enzyme to specific intracellular locations. The many different forms of PP2A that can be achieved by association of regulatory subunits allow for the participation of PP2A in a wide variety of cellular processes. Knockdown of individual regulatory subunits by RNAi has led to a better understanding of the functions of PP2A. For example, ablation of the B56β regulatory subunit demonstrated its role in cell viability (Ma et al., 2007) and knockdown of B56γ demonstrated a role for PP2A in cell proliferation and transformation (Li et al., 2006). Although the B56δ regulatory subunit has not

been studied as well as other members of the B56 family, two recent studies have provided insights into the processes in which this subunit is involved (Ahn et al., 2007; Margolis et al., 2006).

In the previous chapter, a role for the regulatory subunits B56γ and B56δ in mediating association of the PP2A core dimer with S6 kinase was observed. Furthermore, both B56δ and B56γ co-immunoprecipitated with S6K suggesting they may regulate S6K activity and function. The goals of this chapter were to 1) determine if T389 of S6K is dephosphorylated by targeted forms of PP2A, 2) determine if B56δ and B56γ regulate the kinase activity of S6K, 3) test the effect of B56δ and B56γ knockdowns on cell growth, and 4) use an siRNA-based approach to target multiple calyculin A-sensitive phosphatases to determine their role in dephosphorylation of S6K-T389 following rapamycin treatment or amino acid starvation.

B. Experimental Procedures

1. Materials

Rapamycin, insulin, propidium iodide, RNase A, poly-L-lysine, chloroquine, and protein A sepharose were purchased from Sigma (St. Louis, MO). Calyculin A was obtained from Cell Signaling Technology (Danvers, MA) and Lipofectamine 2000 and Opti-MEM Reduced Serum Medium were from Invitrogen (Carlsbad, CA). A GST-S6 fragment was a kind gift from John Blenis (Harvard Medical School, Boston, MA). The Myc-B56δ construct was described previously.

2. Antibodies

Anti-phospho-S6K (T389) (1A5), anti-PARP, anti-GAPDH, and anti-β-actin were purchased from Cell Signaling Technology (Danvers, MA). Anti-PP2A-A subunit antibody and anti-PP4-C subunit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-B56δ was purchased from Novus Biologicals (Littleton, CO), anti-B56γ and anti-PP2A-C antibodies were previously described (Mumby et al., 1987; Mumby, Russell and Garrard, 1985). Anti-PP6-C antibody was from Sigma (St. Louis, MO) and anti-PP5 was from BD Biosciences (San Jose, CA). Anti-Myc was purchased from Roche (Indianapolis, IN). Polyclonal anti-S6K C3 antibody was a kind gift from John Blenis (Harvard Medical School, Boston, MA).

3. Cell Culture

HEK 293T and HeLa cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and incubated at 37^0C with 5% CO_2. Media lacking amino acids was prepared as described by the manufacturer using all components except amino acids. Low glutamine media was prepared according to the manufacturer's directions except only 10% of the total amount of glutamine was added.

4. siRNA

For small interfering RNA (siRNA) studies, 21-23 nucleotide RNA targeted against specific DNA sequences were obtained from Dharmacon (Lafayette, CO). The DNA targeting sequence for the PP2A-C subunit was 5'-CTGAATAATATCCTCTGTC-3' and for the PP2A-A subunit was 5'-GGACCCGAAGTGAGCTTCT-3'. The target sequences for PP2A-B56γ and PP2A-B56δ were 5'-TGATGGCACTTCTCAAATA-3' and 5'-GCTTATATCCGTAGGCAGA-3', respectively. The PP4-C target sequence was 5'-CGCAGCCAATGACATTGAC-3' and the PP6-C sequence was 5'-ATGGCCTGATCGTATTACA-3'. The DNA targets sequences for PP5 and Luciferase were 5'-AACATATTCGAGCTCAACGGT-3' and 5'-TCGAAGTATTCCGCGTACG-3', respectively.

5. Transfection of siRNA and Myc-B56δ

For HEK 293T cells, $2x10^5$ cells were plated on a 35 mm dish coated with poly-L-lysine. The next day, cells were transfected with 100 picomoles of siRNA or 2 μg of Myc-B56δ using Lipofectamine 2000 according to the manufacturer's directions. Assays were performed 48 hours post-transfection. For experiments using HeLa cells, cells were plated at a density of $4x10^5$ on a 60 mm dish. Cells were transfected the following day using the calcium phosphate method. Briefly, cells were incubated in 21.25 μL of 30 μM chloroquine for 15 minutes at 37^0C. Next, 100 picomoles of siRNA was added to 728 μL of H_2O and 105.4 μL of 2 M

$CaCl_2$ was added to this mix in a drop-wise manner. This mixture was then combined with 850 μL of HEPES buffered saline (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4, pH 7.05) and then added drop-wise to cells. Media was replaced after 4-6 hours. Samples were harvested 72 hours after transfection.

6. S6K1 Dephosphorylation and Stimulation Assays

HeLa cells were transfected with siRNA as previously described. 72 hours after transfection, media was removed and new complete media with 10% FBS was added for re-simulations. For rapamycin treatments, complete media containing 5 nM rapamycin was added and for amino acid starvations, media lacking all amino acids was used. Samples were harvested at the indicated times by washing once in 10 mL cold PBS and then 250 μL of 5% SDS sample buffer (5% SDS, 0.5% 2-mercaptoethanol, 50% glycerol, 312.5 mM Tris-HCl pH 6.7, 0.5% bromophenol blue) was added directly to the plate. Lysates were briefly vortexed and then boiled for 3-5 minutes. Samples were then vortexed again and centrifuged at 14,000 rpm for 10 minutes. Equal volumes of each sample were loaded on 8% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was Western blotted using the indicated antibodies.

HEK 293T cells were transfected with siRNA or Myc-B56δ as described earlier. Approximately 30 hours post-transfection, cells were put in media without serum overnight to allow all samples to reach a low basal level of S6K activation. The following day, 48 hours post-transfection, new complete media with 10% FBS was added for two hours to stimulate S6K-T389 phosphorylation.

10nM Rapamycin in complete media or media lacking amino acids was then added for indicated times. For re-stimulation studies, cells were serum starved overnight and then re-stimulated with DMEM + 10% FBS for indicated times. For calyculin A experiments, cells were serum-starved overnight, then put in complete media for one hour, followed by complete media with 5 nM calyculin A for an additional hour. Cells were then put in complete media with 10 nM rapamycin and 5 nM calyculin A for indicated time-points. All samples were harvested by washing once with 5 mL cold PBS and then 500 µL of 5% SDS sample buffer was added directly to the plate. Samples were vortexed, boiled, and then vortexed again. After centrifugation at 14,000 rpm for 10 minutes, an amido black protein concentration assay was performed. Equal amounts of protein from each sample were run on 8% SDS-PAGE and analyzed by Western blotting.

7. S6K1 Immunoprecipitation Kinase Assays

HEK 293T cells were seeded at a density of $4x10^5$ on 60 mm plates coated with poly-L-lysine. The following day, 200 picomoles of each siRNA or 4 µg of DNA were transfected using Lipofectamine 2000 according to the manufacturer's instructions. 18 hours before harvesting, cells were put in media without serum. The next day, 48 hours after transfection, cells were stimulated with DMEM containing 10% FBS and the indicated concentrations of insulin (5 nM, 10 nM or 25 nM). Samples were washed once in 5 mL cold PBS and 250 µL of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor

cocktails 1 and 2 (Sigma, St. Louis, MO) was added directly to the plate. Cells were scraped, transferred to an Eppendorf tube and incubated on ice for 30 minutes. Lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4^0C and then protein concentration was determined using the BCA method (Pierce, Rockford, IL). Immunoprecipitations were carried out by incubating 2 µL of anti-S6K1 C3 antibody with 300 µg of lysate and 20 µL of protein A sepharose beads for 2 hours at 4^0C. Samples were then washed three times with cold lysis buffer and the final wash was done in kinase buffer (20 mM HEPES pH 7.2, 10 mM $MgCl_2$, 0.1 mg/mL BSA). Residual wash buffer was aspirated using a 27-guage needle. Kinase cocktail (20 mM HEPES pH 7.2, 10 mM $MGCl_2$, 0.1 mg/mL BSA, 50 mM β-Glycerophosphate, 100 µM ATP, 0.25 µg GST-S6, 10 µCi ^{32}P-ATP) was then added to each sample followed by incubation at 30^0C for 15 minutes. The kinase reaction was terminated by adding 2x SDS sample buffer and boiled for 5 minutes. 30 µL of each reaction was run on 8% SDS-PAGE and stained with coomassie brilliant blue. After destaining, gels were dried using a Gel Dryer (Biorad, Hercules, CA) and exposed to x-ray film.

8. Fluorescence Activated Cell Sorting (FACS)

For cell size analysis studies, $8x10^5$ HEK 293T cells were plated on 60 mm dishes and transfected with siRNA the following day as previously described. Twenty-four hours after transfection, cells were washed once with PBS and replated onto 10 cm dishes in the absence or presence of 20 ng/mL rapamycin. Samples were harvested after 72 hours by washing once in PBS and cells were

resuspended in 1 mL of growth media. $3x10^6$ cells were transferred to a 15 mL tube and centrifuged at 200 x *g* for 5 minutes. Cell pellets were washed once in 5 mL PBS and centrifuged as before. Cells were then fixed by resuspending the pellet in 0.5 mL PBS and then added to 4.5 mL of 70% ethanol on ice. The cell suspension was then stored at 4^0C overnight for analysis the next day, or stored at -20^0C for analysis at a later time. For propidium iodide (PI) staining, the ethanol-suspended cells were centrifuged for 5min at 200 x *g*. The ethanol was decanted and the cell pellet suspended in 5 mL PBS and incubated on ice for 60 seconds. Cells were once again centrifuged and the cell pellet was resuspended in 1 mL PI staining solution (0.1% Triton X-100 in PBS, 0.2 mg/mL RNase A, 0.02 mg/mL PI) and incubated at 37^0C for 15 minutes. DNA content and cell size were determined using a Beckton Dickinson FACScan flow cytometer and data analyzed using Cell Quest software. Forward Scatter Height (FSC-H) of the G_1-phase population was determined as a measure of the relative cell size.

9. Cell Proliferation Assay

HEK 293T cells were plated in 60 mm dishes at a density of $8x10^5$ cells per dish. After 24 hours, cells were transfected with 10 μL of B56γ and B56δ siRNA using Lipofectamine 2000 according to the manufacturer's instructions. 24 hours post-transfection, cells were washed once with PBS and resuspended in growth media. Cells were then replated at a density of 30,000 cells per well in a 6 well plate. 2 mL of medium was added per well. The following media conditions were performed in duplicate; 1) complete media, 2) low glutamine and, 3) 20

ng/mL rapamycin. Cells were harvested 48, 72, and 96 hours after replating by resuspension in growth media and then cell counts were taken using a hemocytometer. The data were plotted using GraphPad Prism 5 Software. Samples were also harvested in 5% SDS sample buffer (5% SDS, 0.5% 2-mercaptoethanol, 50% glycerol, 312.5 mM Tris-HCl pH 6.7, 0.5% bromophenol blue) and run on SDS-PAGE for Western blotting as previously described.

C. Results

1. Depletion of the B56γ and B56δ Subunits Does Not Alter Dephosphorylation of S6K1 at T389.

To determine the functional role of B56γ and B56δ containing PP2A holoenzymes in regulating mTOR signaling, changes in phosphorylation of S6K-T389 were monitored when these subunits were knocked-down by siRNA. Depletion of B56γ and B56δ did not alter the kinetics of dephosphorylation of T389 following addition of rapamycin (Fig. 10A). When compared to cells treated with control siRNA, S6K was still dephosphorylated at T389 when mTOR was inhibited by rapamycin. This dephosphorylation appeared to occur at a similar rate as control cells and was unaffected by B56γ/δ knockdown. B56δ siRNA treated cells were also serum starved overnight and then re-stimulated with complete medium containing FBS (Fig. 10B). Although the increase in S6K phosphorylation caused by serum stimulation was not robust even in control cells, T389 phosphorylation did not appear to be enhanced in B56δ knockdowns. Results from figure 10 suggested that B56γ and B56δ do not regulate

dephosphorylation of T389. Additionally, in both Fig. 10A and Fig. 10B, the knockdown efficiency was very high suggesting that most of the B56γ and B56δ had been depleted. Re-stimulation assays were also performed using siRNA against B56δ and B56γ in combination (data not shown). The results obtained from these studies were similar to those seen when B56δ was knocked-down by itself.

Immunoprecipitation experiments conducted earlier showed a decrease in the associated A and C subunits that co-immunoprecipitated with S6K when cells were depleted of B56γ and B56δ. However, there was still some core dimer that co-immunoprecipitated with S6K and it was possible that this residual PP2A may be sufficient to fully dephosphorylate the kinase. To detect an effect due to depletion of B56δ, a low concentration of calyculin A was used in an attempt to inhibit any residual C subunit that maybe associated with S6K in cells treated with siRNA. Samples were then treated with rapamycin to block mTOR and changes in the phosphorylation of S6K-T389 were monitored (Fig. 10C). The results showed that dephosphorylation of T389 was not altered under these conditions as the rate of dephosphorylation of T389 by rapamycin in knockdown and calyculin A treated samples appeared similar to that in Luciferase siRNA-treated cells. This suggests that either the calyculin A treatment was not sufficient to block PP2A activity, or that B56δ is likely not involved in mediating dephosphorylation of S6K at T389. It should be noted that the concentration of calyculin A used in these experiments was not sufficient to prevent S6K dephosphorylation.

The effects of over-expressing B56δ on S6 kinase T389 phosphorylation were also monitored. Myc-B56δ variant 1 was over-expressed in HEK 293T cells for 48 hours and S6K dephosphorylation was induced by rapamycin treatment (Fig. 11A). Over-expression of B56δ did not affect rapamycin-induced dephosphorylation of T389. When compared to control samples transfected with empty vector, there did not appear to be any difference in the rate of dephosphorylation. Similarly, there was no change in the re-phosphorylation of T389 caused by serum stimulation (Fig. 11B). Although serum stimulation caused a modest effect, there was no noticeable difference in the rate of phosphorylation between control and B56δ over-expressing cells. This result indicates that over-expression of B56δ does not act in a dominant-negative manner to block dephosphorylation of S6K.

2. Depletion of B56δ and B56γ Does Not Alter S6K Activity.

The previous section showed that knockdown or over-expression of B56δ had no effect on phosphorylation of S6K at T389. Although full activation of the kinase requires phosphorylation at this site, it is possible that PP2A may be controlling kinase activity through regulation of other phosphorylation sites. To determine if there was any effect on S6K kinase activity, immunoprecipitation kinase assays were performed from cells treated with a combination of siRNA targeting B56δ and B56γ. Knockdowns were carried out for 48 hours, cells were stimulated with various doses of insulin, and S6K1 kinase activity determined (Fig. 12). In Luciferase siRNA treated lanes there was a dose-dependent increase

in ^{32}P incorporation with increasing concentrations of insulin. In samples treated with siRNA against the B56 subunits, there was also a dose dependent increase in ^{32}P incorporation and kinase activity that was similar to that observed in control cells suggesting that knockdown of these subunits had little or no effect on activation of S6K. These results confirm that while B56δ and B56γ mediate association of A/C core dimer with S6K, they do not appear to regulate S6K activity.

3. Analysis of cell size by FACS.

Although B56δ and B56γ did not appear to regulate S6K dephosphorylation or activity, their association with S6K1 may play a role in controlling cell growth. Both subunits were knocked-down and cells were replated 24 hours later in the presence or absence of rapamycin. After 72 hours, Forward Scatter Height (FSC-H) was determined on cells gated in G_1 phase as a measure of relative cell size. As shown previously, rapamycin treatment caused a decrease in cell size, confirming that suppression of mTOR decreases cell growth (Fig. 13A). Samples that were depleted of both B56γ and B56δ did not have a significant difference in cell size compared to control cells (Fig. 13B). FSC-H values obtained from these experiments showed that there was not sufficient difference between samples treated with B56 siRNA versus Luciferase siRNA that could be detected with this assay. These results indicate that B56δ and B56γ did not have a significant affect on the ability of 293T cells to grow in a low concentration of rapamycin.

4. Cell proliferation is Decreased in B56γ and B56δ Double Knockdowns.

Knockdown of B56γ and B56δ did not have an effect on cell size. However, it was possible that these subunits have other effects on cell growth including regulation of the cell cycle. Therefore, the proliferation of cells depleted of these two subunits was examined. Cells depleted of both B56γ and B56δ grew at a much slower rate than the control Luciferase siRNA-treated cells (Fig. 14). Proliferation of cells depleted of both subunits was considerably lower than proliferation of control cells in complete media (Fig. 14A). Addition of rapamycin reduced cell growth in both knockdown and control samples. Proliferation of cells in rapamycin was also lower for knockdown samples. Cells treated with siRNA were also maintained in DMEM containing 10% of the normal amount of the amino acid, glutamine. Cell growth was decreased in low glutamine media for both control and knockdown samples (Fig. 14B). Similar to the effects seen in complete media, knockdown samples had decreased proliferation compared to control cells under low glutamine conditions suggesting that these subunits are necessary for cells to proliferate at a normal rate. These studies were performed twice and reduced growth rate due to knockdown of B56δ and B56γ was seen on both occasions. The effects of B56γ and B56δ co-knockdown appeared to be due to lengthening of the cell cycle and not to apoptosis. FACS analysis revealed that co-depleted cells had an increased G_0/G_1 population relative to control cells (data not shown).

5. Depletion of the PP2A Catalytic Subunit Does Not Block Dephosphorylation of T389.

Time course studies were performed to monitor dephosphorylation of S6K at T389 in cells depleted of the PP2A catalytic (C) subunit by siRNA. In HeLa cells treated with control (Luciferase) siRNA, rapamycin caused a time-dependent dephosphorylation at T389 as observed by western blotting (Fig. 15A). In PP2A-C knockdown samples, T389 was still dephosphorylated although the phospho-signal was much lower than in control cells. Comparison of basal phosphorylation (0 min) between the two siRNA treatments shows that PP2A-C knockdowns have a lower initial phospho-signal that decreases over time upon rapamycin treatment. When PP2A-C siRNA treated cells were starved of amino acids, T389 is dephosphorylated and once again, phosphorylation levels are much lower than in control samples (Fig. 15B). Basal levels of T389 were barely detectable by Western blotting and are similar to those observed in Fig. 15A. Results suggest that PP2A is a positive regulator of mTOR signaling and that without it, S6K cannot be activated. However, knockdown of the catalytic subunit of PP2A is harmful to cells and can trigger apoptosis. It is likely that in these cells, low levels of phosphorylation may be due to lower amounts of total protein as cells are dying. This was investigated further by depleting cells of the scaffolding subunit of PP2A; a condition that is less toxic to cells. HeLa cells were transfected with siRNA targeted against PP2A-A and samples were treated with rapamycin after 48 hours. Dephosphorylation of S6K observed in these cells was similar to that seen in control siRNA samples (Fig. 16). However, levels of

phospho-signal in PP2A-A knockdowns was lower than that observed in control cells. Although this was similar to results in PP2A-C siRNA treatments (Fig. 15), phosphorylation levels in PP2A-A siRNA-treated samples were higher. There was also less cell death observed in these samples suggesting that reduction in PP2A-A levels was not as harmful to cells. It is still likely that the lower phosphorylation of S6K in PP2A siRNA-treated cells is due to cell death and lower protein synthesis, which is supported by an increase in cleaved PARP (lower band) that is seen in PP2A-A knockdowns (Fig. 16; lower panel). While interpretation of these data are complicated, they suggest that knockdown of the catalytic or scaffolding subunits of PP2A does not cause a decrease in dephosphorylation of S6K following rapamycin treatment.

6. Dephosphorylation of T389 is Partially Regulated by PP4 and PP6.

Knockdown of both the catalytic and scaffolding subunits of PP2A did not block rapamycin- or amino acid starvation-induced dephosphorylation of S6K. The next calyculin A-sensitive phosphatases studied were PP4 and PP6. Knockdown of PP4 by siRNA resulted in greater than fifty percent reduction in protein levels while knockdown of PP6 was even more efficacious (Fig. 17A and Fig. 17B). Although, rapamycin treatment of cells depleted of PP4 resulted in dephosphorylation of S6K-T389, there was a higher phospho-signal in PP4 siRNA treated samples compared to control siRNA lanes (Fig. 17A). Basal phosphorylation was higher (time = 0 min) in the PP4 knockdown samples and at the 40 min time-point phospho-S6K was still detectable whereas the equivalent

time-point in Luciferase samples had no detectable phosphorylation. Similarly, knockdown of PP6 also led to an increase in S6K phosphorylation (Fig. 17B; upper panel). Although S6K was still dephosphorylated in a time-dependent manner following addition of rapamycin, levels of phospho-signal were higher than in control samples. When PP6 knockdown cells were starved of amino acids, dephosphorylation of S6K-T389 was not as complete as in Luciferase siRNA-treated cells (Fig 17B; lower panel). Unlike control cells that showed nearly complete dephosphorylation by 30 minutes, PP6 knockdown samples still had a significant phospho-signal at this time-point. Additionally, phosphorylation levels of other time-points were also higher in the PP6 knockdown cells. Both phosphatases appear to play a role in regulating S6K and cause a delay in T389 dephosphorylation; although siRNA-mediated depletion of either did not completely block dephosphorylation.

To test if there was a combinatorial role for PP4 and PP6 in S6K-T389 dephosphorylation, HeLa cells were transfected with siRNA targeting both phosphatases. Rapamycin treatment as well as amino acid starvation caused dephosphorylation of S6K-T389 over time in the double-knockdown samples (Fig. 18A and Fig. 18B). Under both conditions the basal levels of S6K phosphorylation were elevated in the double-knockdowns compared to control samples (time = 0 min). In addition, co-depletion of PP4 and PP6 caused a significant delay in dephosphorylation as evidenced by a higher phospho-signal in the latter time points. However, S6K was still dephosphorylated in cells depleted of both PP4 and PP6. Due to the non-quantitative nature of the assay, it was not

possible to determine if co-depletion of PP4 and PP6 caused a greater reduction in S6K phosphorylation than seen with depletion of each phosphatase separately.

7. Depletion of PP5 Does Not Inhibit T389 Dephosphorylation.

Protein phosphatase 5 (PP5) is the other known calyculin A sensitive phosphatase and its potential role in regulating S6K dephosphorylation was also examined using siRNA. Transfection of cells with siRNA targeting PP5 resulted in a dramatic reduction in PP5 protein levels (Fig. 19). When these samples were treated with rapamycin in a time-course experiment, S6K was dephosphorylated at the same rate as in control cells (Fig. 19). Knockdown of PP5 had no effect in the kinetics of dephosphorylation or basal phosphorylation levels suggesting that this phosphatase does not play a direct role in regulating phosphorylation of T389.

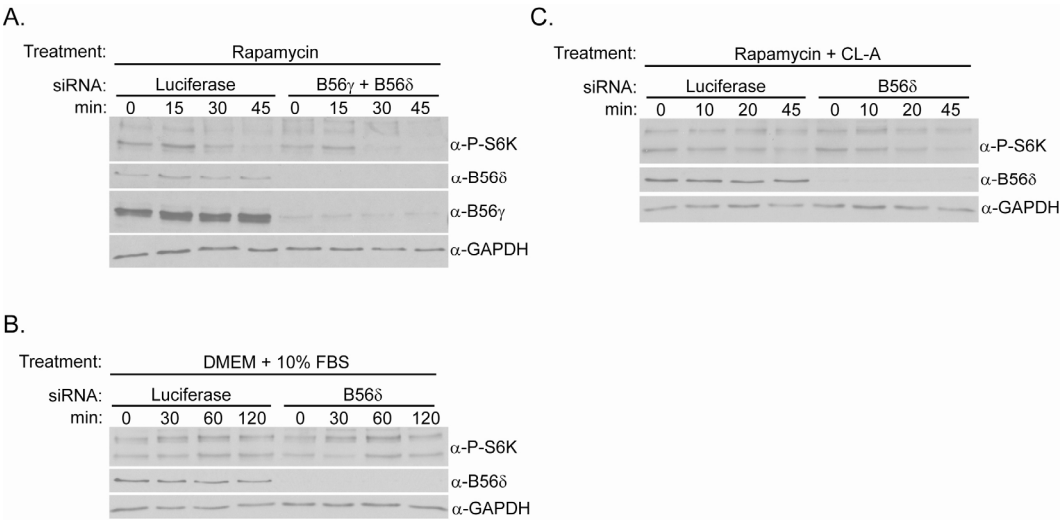

Figure 10. Knockdown of B56γ and B56δ does not affect phosphorylation of S6K at T389. HEK 293T cells were transfected with siRNA and harvested after 48 hours. (A) Cells were serum-starved for 18 hours prior to harvesting and then treated with 10 nM rapamycin in complete media with 10% FBS. (B) For complete media stimulations, serum-starved cells were treated with complete media containing 10% FBS for indicated times. (C) For treatments with low a concentration of inhibitor, serum-starved cells were re-stimulated with complete media for 1 hour and then treated with 5 nM calyculin A (CL-A) for an additional hour. Cells were then treated with 10 nM rapamycin in the presence of 5 nM CL-A for indicated times. All samples were washed once with PBS and then lysed in 5% SDS sample buffer. Lysed samples were separated by SDS-PAGE and Western blotted using the indicated antibodies.

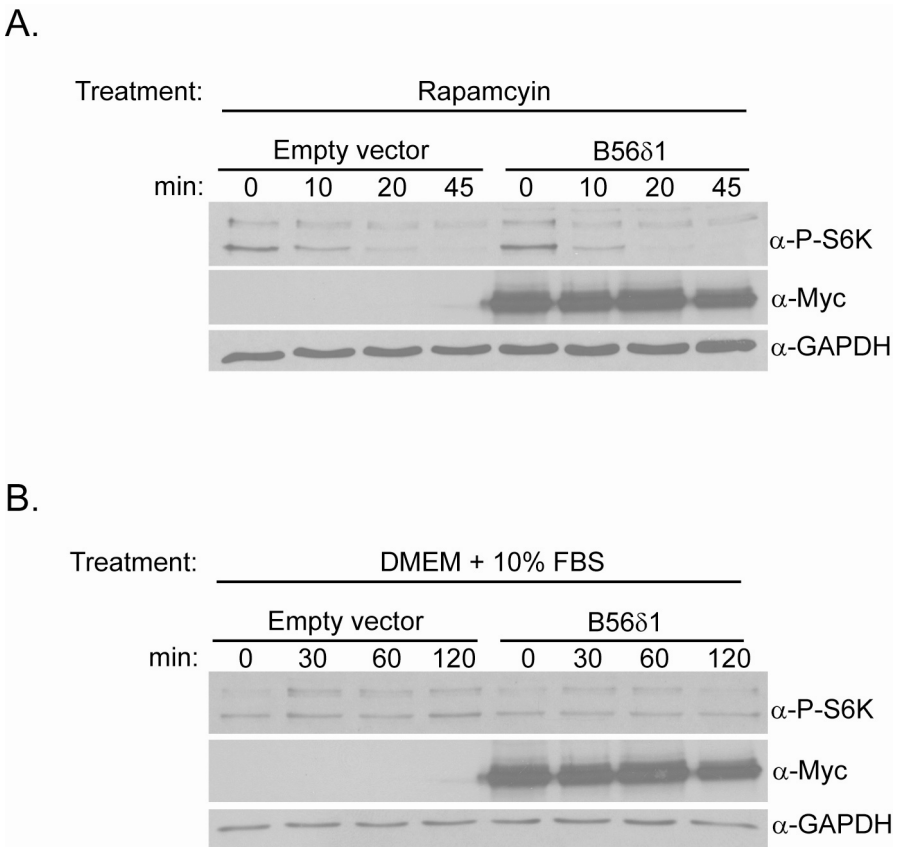

Figure 11. Over-expression of B56δ does not affect S6K phosphorylation at T389. Cells were transfected with Myc-B56δ and harvested 48 hours after transfection. (A) For rapamycin studies, cells were re-stimulated with complete media for 2 hours and then treated with 10 nM rapamycin for indicated times. (B) Cells were serum-starved for 18 hours prior to harvesting and then re-stimulated with complete media containing 10% FBS. All samples were harvested by washing in PBS and then SDS sample buffer was added directly to the plate. Samples were run on 8% SDS-PAGE, transferred to a nitrocellulose membrane and Western blotted using the indicated antibodies.

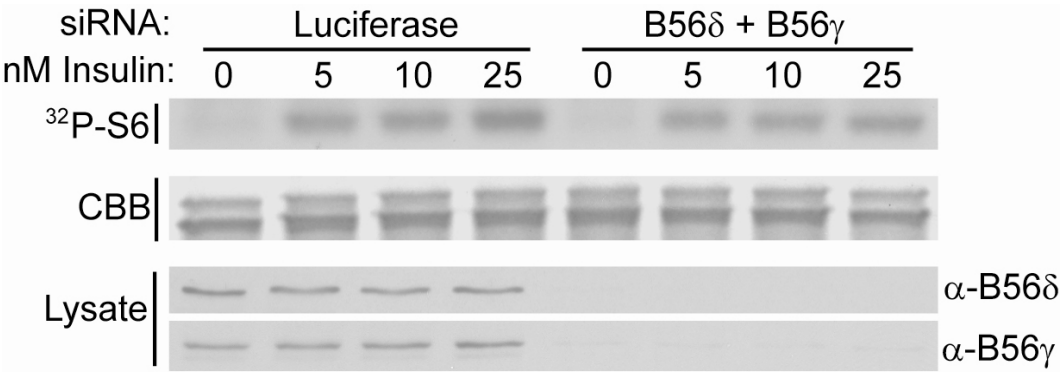

Figure 12. Knockdown of B56γ and B56δ does not affect S6K1 activity. Cells were treated with siRNA and harvested 48 hours post-transfection. Cells were placed in media lacking serum for 18 hours. Cells were then put in 1x PBS for an additional 30 minutes prior to stimulation with indicated concentrations of insulin. Cells were lysed for 30 minutes on ice and then cleared by centrifugation for 15 minutes. Lysates were incubated with protein A sepharose beads and α-S6K antibody for 2 hours. Beads were washed three times with lysis buffer and once with kinase buffer and then incubated with kinase cocktail containing ^{32}P-ATP and GST-S6 for 15 minutes at 30^{0}C. The reaction was stopped by boiling in SDS sample buffer and samples were loaded on 8% SDS-PAGE. The gel was dried and exposed to x-ray film to determine ^{32}P incorporation.

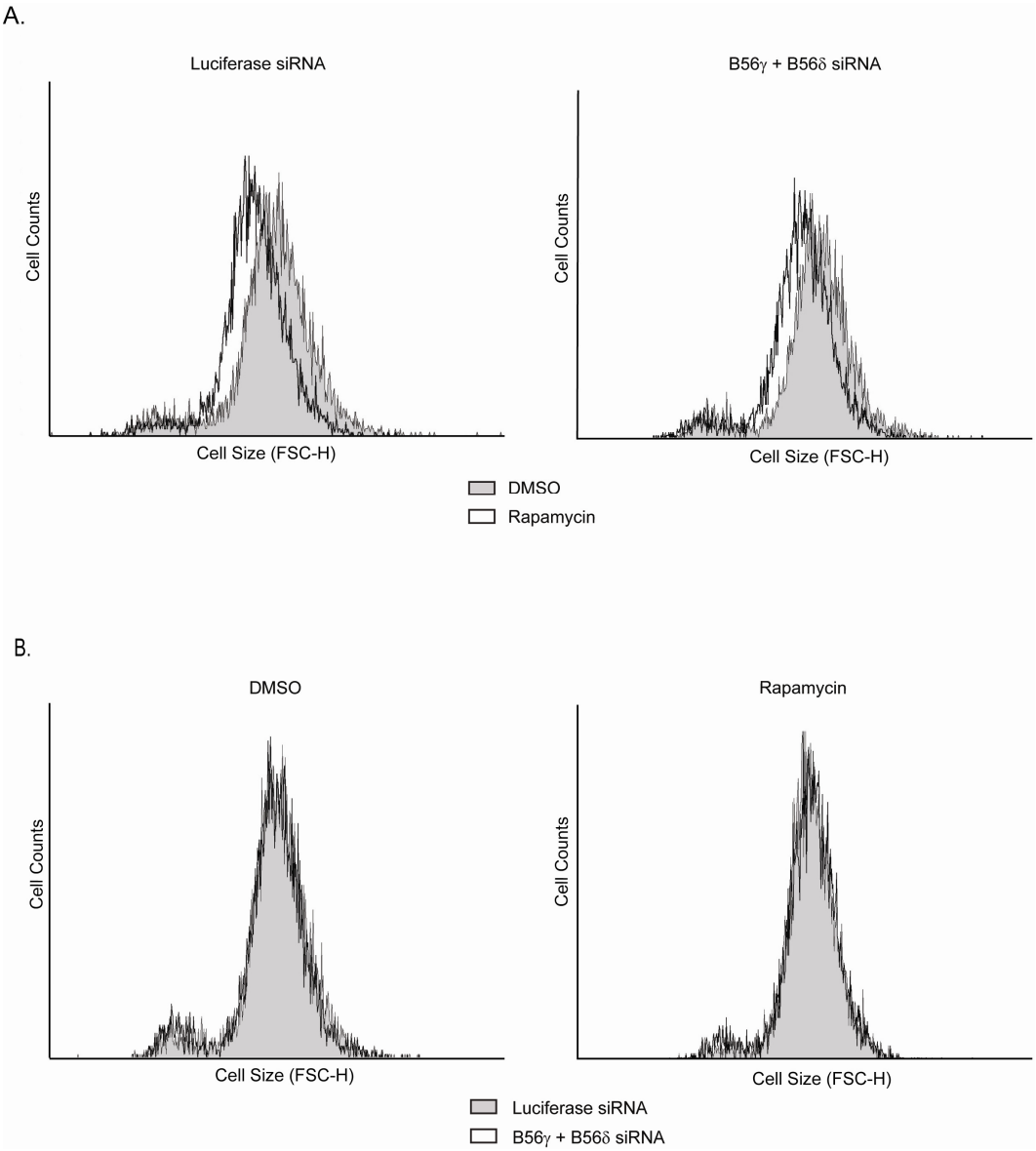

Figure 13. Knockdown of B56δ and B56γ does not alter cell size. HEK 293T cells were transfected with control (Luciferase) siRNA or siRNA against both B56γ and B56δ. 24 hours after transfection, cells were replated and grown for an additional 72 hours with or without 20 ng/mL rapamycin. Cells were fixed in 70% ethanol overnight and then stained with propidium iodide. Forward scatter height (FSC-H) of G_1-phase gated cells was measured.

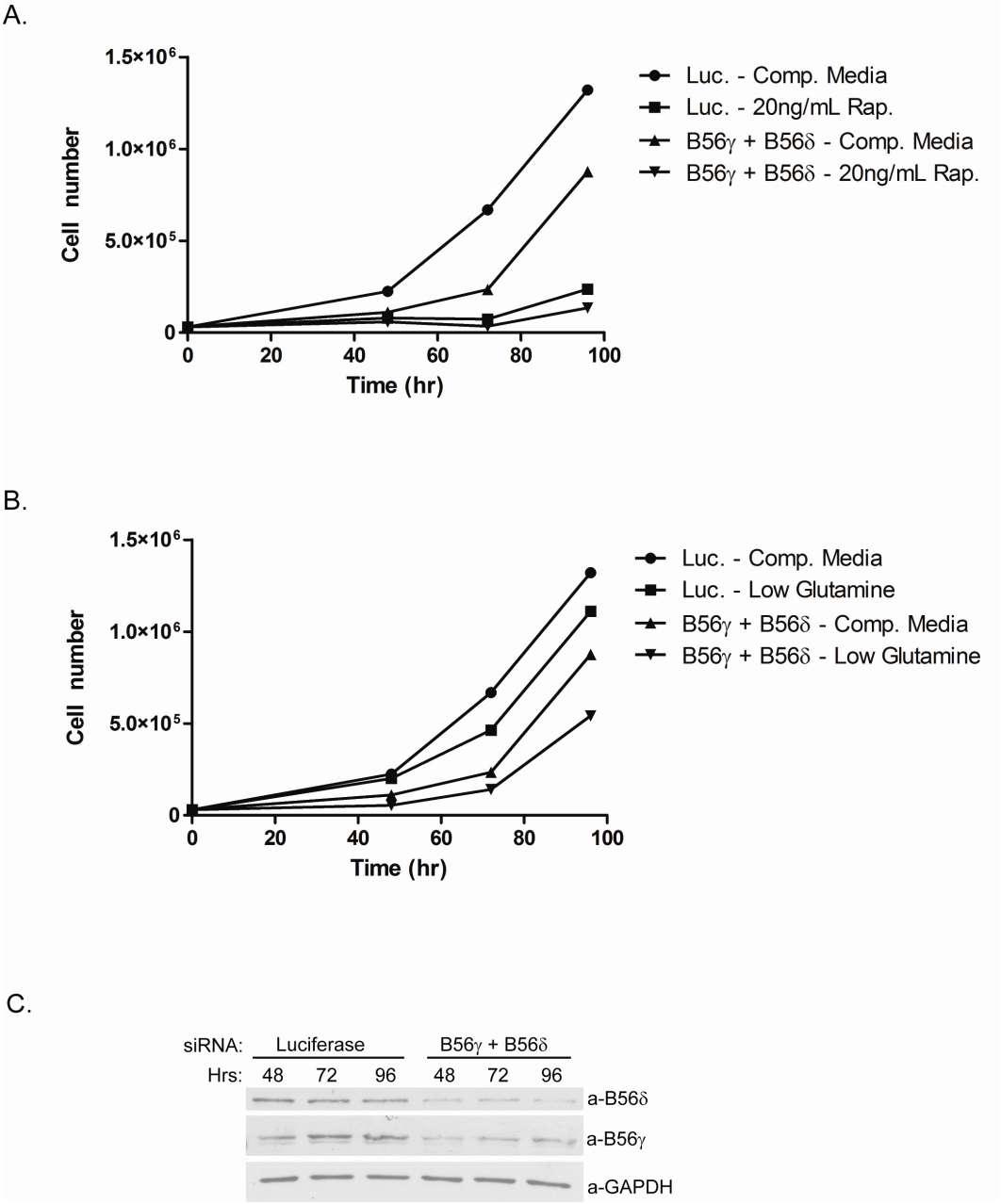

Figure 14. Knockdown of B56γ and B56δ decreases proliferation of HEK 293T cells. $8x10^5$ cells were plated in a 60 mm dish and transfected with siRNA against both B56δ and B56γ or Luciferase (Luc) after 24 hours. 24 hours after transfection, cell were replated at a density of 30,000 cells per well in complete media, media with 20 ng/mL rapamycin (A), or media with low glutamine (B). Cells were harvested after 48, 72, and 96 hours and counted using a hemocytometer. Samples at each time point were also harvested by lysing directly in 5% SDS sample buffer and run on SDS-PAGE. Gels were transferred to a nitrocellulose membrane and Western blotted using the indicated antibodies (C).

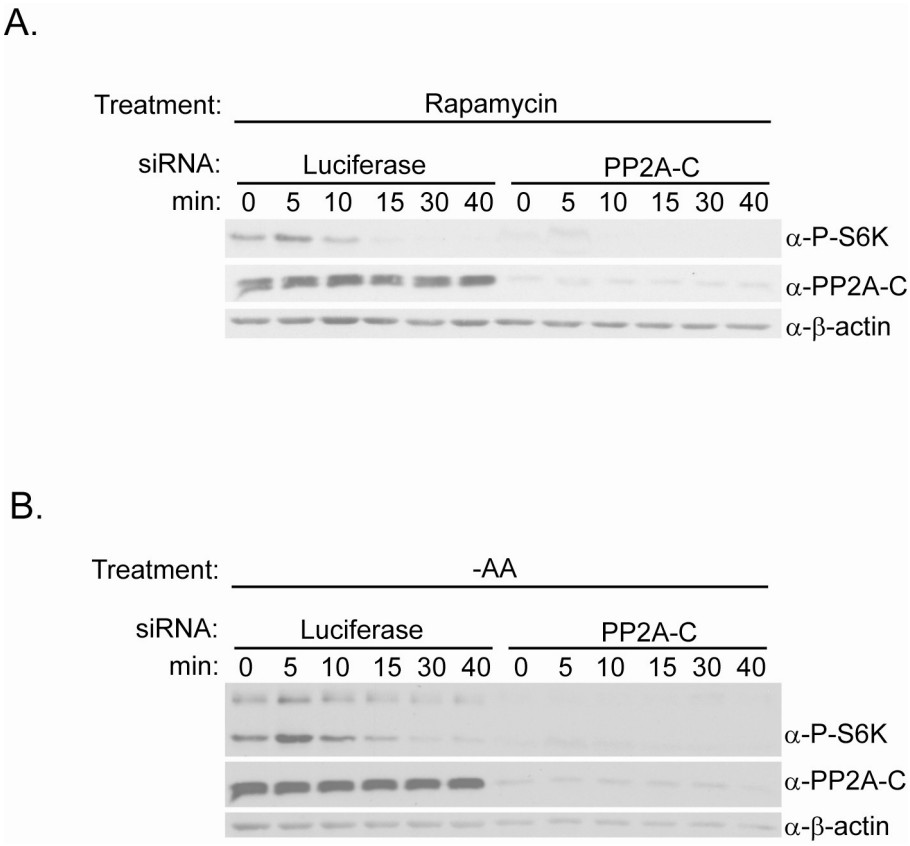

Figure 15. Knockdown of PP2A-C does not block dephosphorylation at T389. HeLa cells were treated with siRNA against the catalytic subunit of PP2A (PP2A-C). 72 hours after transfection, cells were treated with (A) 5 nM rapamycin or put in (B) media lacking amino acids (-AA) for indicated times. Samples were harvested by washing with PBS and then 5% SDS sample buffer was added directly to the plate. Lysates were scraped, vortexed and then boiled. Samples were then centrifuged and equal volumes loaded on SDS-PAGE. Membranes were then Western blotted with the indicated antibodies. β–actin was used as a loading control.

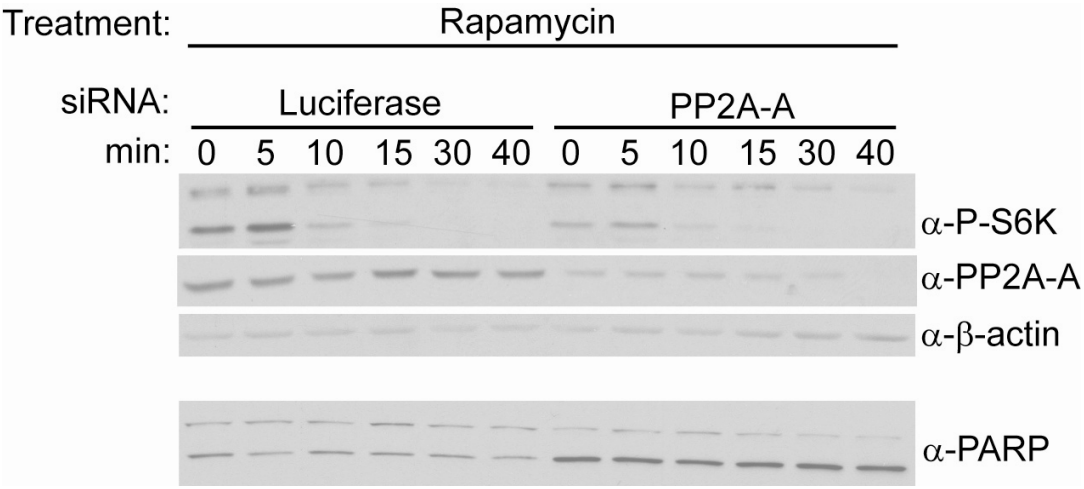

Figure 16. PP2A-A subunit knockdown does not block dephosphorylation at T389. siRNA targeting the scaffolding subunit of PP2A (PP2A-A) was transfected in HeLa cells and samples were harvested 72 hours post-transfection. Cells were treated with 5 nM rapamycin for indicated times and then washed once with PBS before being solubilized in SDS sample buffer. Boiled samples were loaded on 8% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was probed using indicated antibodies.

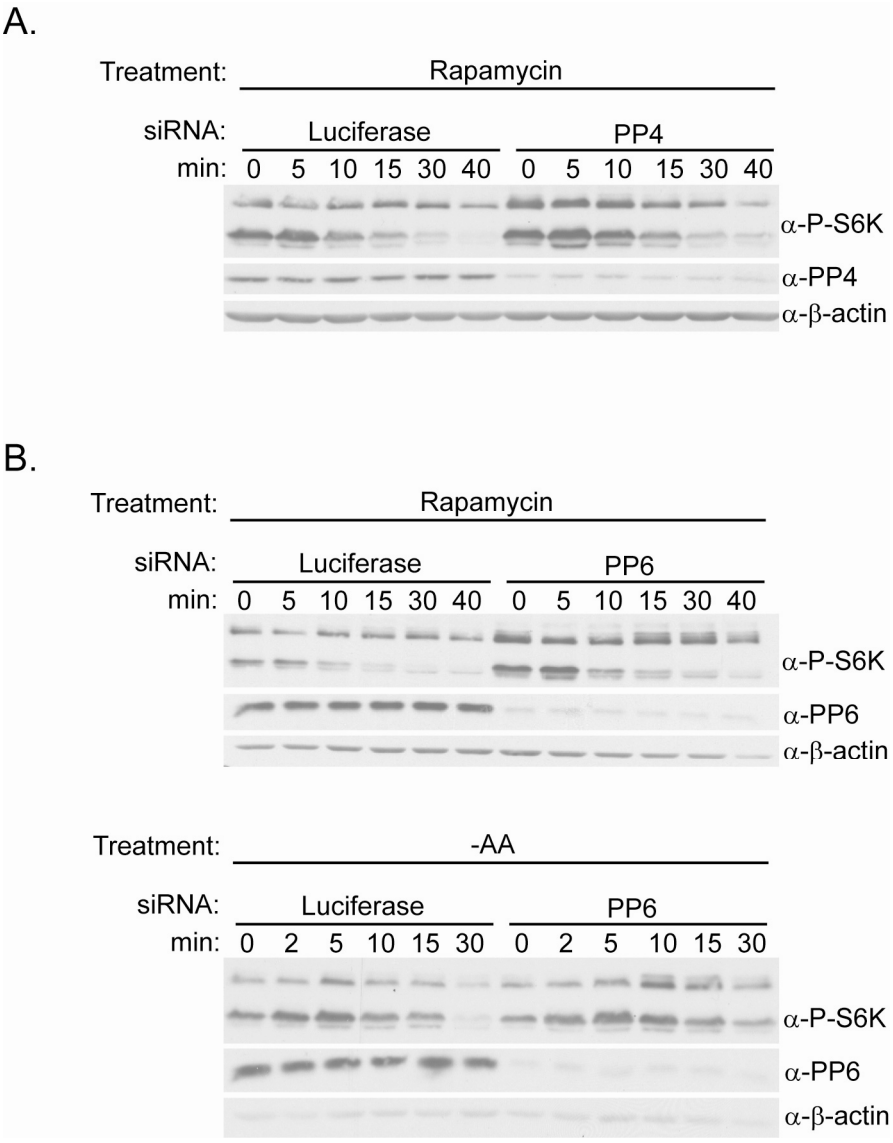

Figure 17. Knockdown of PP4 and PP6 results in a delay in S6K dephosphorylation. HeLa cells were transfected with siRNA targeting (A) PP4 or (B) PP6. 72 hours after transfection, cells were treated with 5 nM rapamycin or put in media lacking all amino acids for indicated times. Cells were harvested by washing once with PBS and then lysed directly on plates with 5% SDS sample buffer.

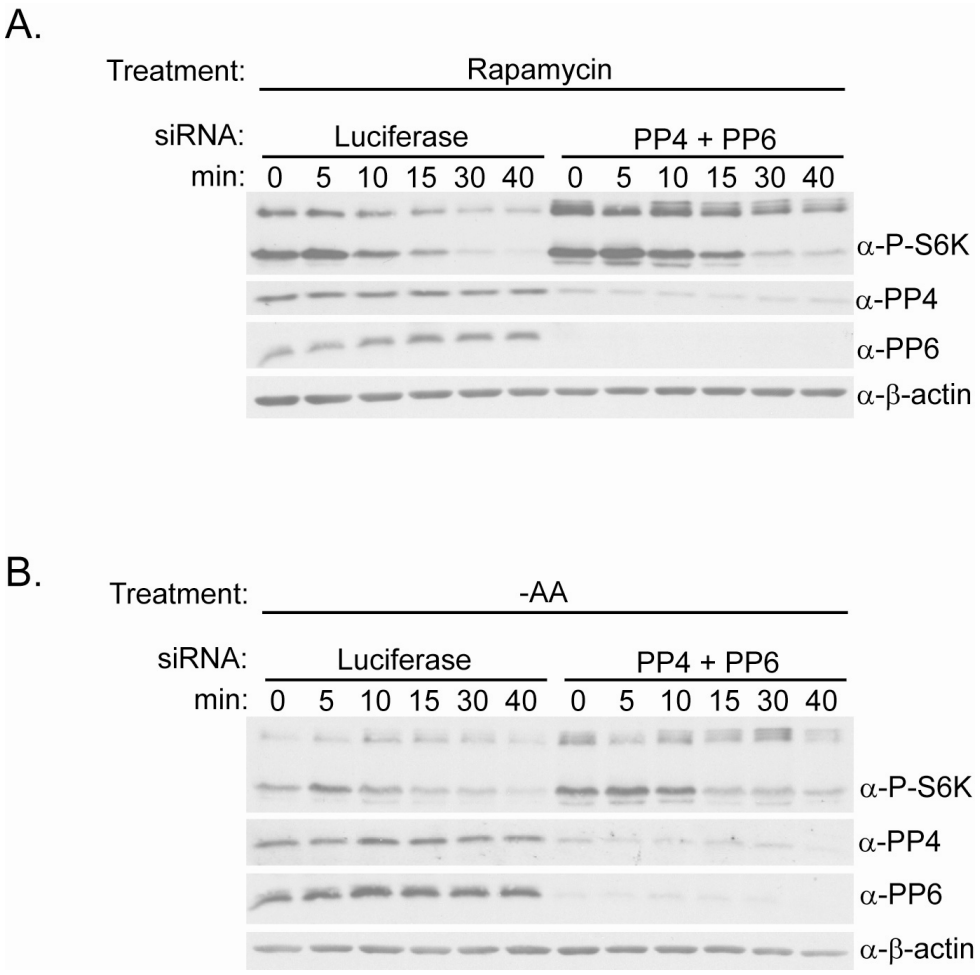

Figure 18. PP4 and PP6 combined knockdown results in delayed S6K dephosphorylation. HeLa cells were transfected with siRNA using the calcium phosphate method. After 72 hours, cells were treated with fresh media containing (A) 5 nM rapamycin or with (B) media lacking amino acids (-AA) for various times. Samples were harvested by washing once with PBS and then lysed by directly adding 5% SDS sample buffer to the plate. Boiled samples were separated by SDS-PAGE and Western blotted using the indicated antibodies.

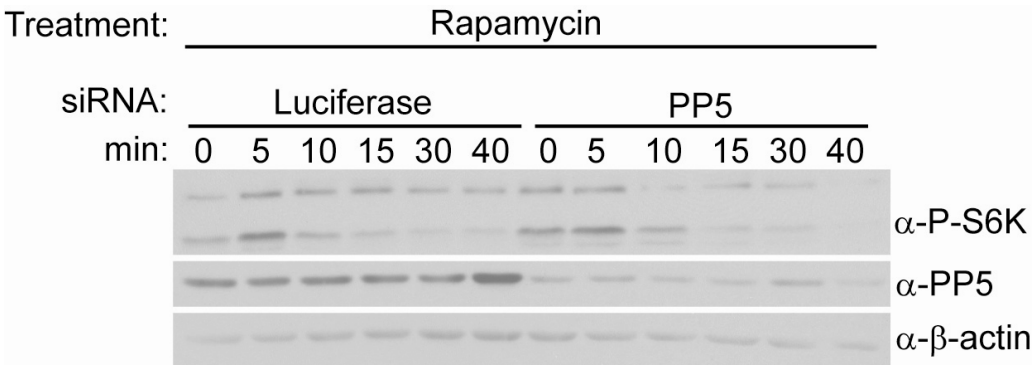

Figure 19. Knockdown of PP5 does not block dephosphorylation of S6K. Cells were transfected with siRNA against PP5 and then treated with fresh media containing 5 nM rapamycin after 72 hours. Samples were harvested at the indicated time points by washing with PBS and then 5% SDS sample buffer was added directly to the plate. Cells were scraped, boiled and then vortexed. After centrifugation for 10 minutes, samples were loaded on 8% SDS-PAGE. Samples were then transferred to a nitrocellulose membrane and probed with the indicated antibodies.

D. Discussion

The results from this chapter suggest that PP2A holoenzymes containing the B56δ or B56γ regulatory subunits are not directly involved in the dephosphorylation of S6K1 at T389 induced by rapamycin or amino acid starvation or the regulation of S6K kinase activity. Furthermore, knockdown of these regulatory subunits did not have a demonstrable effect on cell size. When other PP2A-like phosphatases were ablated by siRNA treatment, only PP4 and PP6 showed a modest effect to increase T389 phosphorylation levels.

Initial studies examined the role of PP2A regulatory subunits identified to associate with S6K1 in controlling phosphorylation at T389 and kinase activity. Knockdown of B56δ and B56γ in HEK 293T cells did not have any effect on dephosphorylation of T389 by rapamycin or activation of S6K following insulin stimulation. Even when siRNA treatments were combined with a low concentration of calyculin A to try to inhibit any residual catalytic subunit that may have still been associated with S6K1, there was no effect on rapamycin-induced dephosphorylation of T389. To complement the knockdown studies and to implement a different approach to look at B56δ function, rapamycin treatments and insulin stimulations were performed in cells over-expressing a Myc-tagged variant of B56δ. These studies also failed to demonstrate an effect on S6K-T389 phosphorylation. Thus it appears that neither B56δ nor B56γ play a significant role in regulating S6K1 activity by dephosphorylating T389.

To determine if there was a role for these subunits in controlling S6 kinase activity, immunoprecipitation kinase assays were performed from cells depleted

of B56δ and B56γ. S6K1 activity was measured in response to increasing doses of insulin in control and knockdown cells. Although there was a dose-dependent increase in ^{32}P incorporation, there was no significant difference between Luciferase and B56 siRNA-treated samples. These results show that B56γ and B56δ do not regulate S6K activity and are consistent with the observations that these subunits do not regulate dephosphorylation of T389. Thus, it seems most likely that although PP2A co-immunoprecipitates with S6K1, it is not a direct interaction and that both proteins are components of a larger complex. It is still possible that PP2A is regulating cell size through association with S6K1. This was addressed using FACS analysis to measure relative cell size in B56δ and B56γ knockdowns. Control experiments showed that rapamycin treatment causes the expected decrease in cell size. However, knockdown samples did not show any difference in size compared to control cells. The lack of effect on cell size is also consistent with normal regulation of activity in cells depleted of B56γ and B56δ.

Proliferation of cells treated with siRNA against B56γ and B56δ showed that these cells grew at a much slower rate than control siRNA-treated cells. Growth in complete media as well as media with low glutamine or rapamycin was reduced for knockdown samples. This was a surprising observation since previous studies utilizing these siRNA treatments did not show any effect on S6K1 regulation or on cell size. Furthermore, based on immunoprecipitation data it was expected that depletion of these subunits would lead to hyperactivation of mTOR signaling and increased cell growth. The fact that the double knockdown

causes a reduction in cell proliferation suggests that these subunits are positive regulators of growth in 293T cells. It is possible that the effect on cell proliferation due to knockdown of B56γ and B56δ is not related to S6K1 or mTOR and may be due to the roles these subunits play in other pathways. B56δ has been shown to be an important component of a cell cycle checkpoint that regulates mitotic entry (Margolis et al., 2006). When it is depleted, cells may be unable to enter mitosis. It is unclear how B56γ knockdown may be contributing to this effect since it has been reported to be important for p53-mediated apoptosis and its depletion by RNAi can cause transformation of cells (Li et al., 2006).

It is clear that dephosphorylation of S6K1 at T389 is mediated by a serine/threonine phosphatase that is sensitive to the inhibitor, calyculin A. Although PP2A holoenzymes containing the B56δ or B56γ regulatory subunits may not be regulating this dephosphorylation, it was possible that PP2A is still involved in this process. To test this possibility, the catalytic subunit of PP2A was knocked-down using siRNA in HeLa cells. Initial results were surprising and showed that S6K1 was still dephosphorylated by rapamycin and amino acid starvation in knockdown samples, but levels of phosphorylation were actually lower than in control cells. This was a result of activation of the apoptotic pathway due to depletion of PP2A-C. Lower phosphorylation levels were caused by a decrease in total protein. To circumvent this effect, PP2A-A was knocked-down, which is not as harmful to cells. However, similar results were obtained and further analysis showed PARP cleavage suggesting that even these cells were undergoing programmed cell death. It was clear that depletion of either the A or

C subunit of PP2A did not block dephosphorylation of S6K1 at T389 suggesting that another phosphatase may be important in this process.

The next phosphatase studied was PP5. Cells depleted of PP5 by siRNA were treated with rapamycin and showed no change in the kinetics of S6K-T389 dephosphorylation compared to control cells. These results clearly demonstrate that PP5 is not involved in T389 dephosphorylation. The catalytic subunits of two other calyculin A-sensitive phosphatases, PP4 and PP6 were also knocked-down by siRNA. Upon treatment of these cells with rapamycin or amino acid starvation, T389 dephosphorylation was delayed. This suggests that PP4 and PP6 may have roles in regulating S6K1 activity. Knockdowns of both phosphatases in combination showed a greater delay in dephosphorylation than either one by itself. The effects on S6K-T389 phosphorylation observed by knockdown of PP4 and PP6 are the most dramatic so far. It is also possible that both phosphatases may be dephosphorylating S6K1 at other sites or even an upstream component of the pathway that regulates S6K1.

Chapter 4

Conclusions and Future Directions

Dephosphorylation of S6K1 at T389 was blocked using the serine/threonine phosphatase inhibitor, calyculin A. This confirms previous reports that dephosphorylation of this site is controlled by a PP2A-like phosphatase (Grove et al., 1991; Parrott and Templeton, 1999). Immunoprecipitation studies were then carried out that identified an association between S6K1 and the A/C core dimer of PP2A. This interaction was enhanced when mTOR was inactivated by rapamycin, osmotic stress, or amino acid withdrawal. However, when mTOR activity was stimulated by insulin, this association was diminished. Previous studies have reported an association between the catalytic subunit of PP2A and S6K but this interaction was not affected by rapamycin treatment and appeared to be very weak (Peterson et al., 1999). In another study, Westphal et al. were only able to see an association between PP2A and S6K when cell lysates were cross-linked to stabilize interacting proteins. A third report by Petritsch et al. showed a TGF-β-induced association of a PP2A holoenzyme with S6K which was again a very weak interaction and was not affected by rapamycin treatment. These data support the hypothesis that rapid dephosphorylation of T389 by rapamycin, sorbitol, or amino acid starvation is due to increased association of S6K with the PP2A core dimer. When mTOR is activated and T389 is phosphorylated and phosphatase activity

needs to be decreased, this association is reduced. This suggests that regulation of phosphatase association with S6K1 is regulated by mTOR.

An siRNA screen against a number of different regulatory subunits was conducted to identify the specific subunit that was mediating interaction of the core dimer with S6K1. These results showed that depletion of the B56δ subunit greatly diminished association and this was enhanced when it was knocked-down in combination with B56γ. Endogenous B56δ also co-immunoprecipitated with S6K1 in these experiments further supporting its role in this interaction. Overexpression studies confirmed this interaction by showing association of Myc-tagged B56γ with S6K1. The specificity of this association was demonstrated by the observation that knockdown of another B56 family member, B56α, did not affect co-immunoprecipitation of PP2A with S6K1. Furthermore, depletion of Bα, which belongs to a different family of regulatory subunits also had no effect and epitope-tagged Bα did not co-immunoprecipitate with S6K1. These observations were particularly interesting since Bα association with S6K was observed by Petritsch et al. The cell line used in their report was derived from mammary epithelial (EpH4) cells whereas immortalized human embryonic kidney (HEK 293T) cells were used in this study. It is possible that association of PP2A with S6K is differentially regulated depending on the cell type. Expression of B56γ and B56δ is high in muscle and brain, respectively, so it is not likely that this difference is due to higher expression of a subunit in a particular cell type. Additionally, regulation of the association of PP2A with S6K appears to be different as Petritsch et al. did not see an increase in core dimer co-

immunoprecipitation following rapamycin treatment. Taken together, this suggests that there may be different mechanisms regulating phosphatase association with S6K in different cell types.

The data presented in this report provide evidence that mTOR activity controls binding of S6K1 to PP2A. In addition, the identification of regulatory subunits that mediate this interaction could be used to perform functional studies to determine if S6K1 is indeed a PP2A substrate. The first experiments conducted looked at the effect of regulatory subunit knockdown on the phosphorylation state of T389. There was no affect on S6K-T389 dephosphorylation by rapamycin or re-stimulation by insulin following depletion of B56γ and B56δ by siRNA. Overexpression of B56δ also had no effect on T389 phosphorylation levels. These results were unexpected since knockdown of these regulatory subunits decreases association of the catalytic subunit with S6K1, and should therefore reduce its dephosphorylation. However, it is possible that PP2A regulates another site on S6K1 and knockdown of B56γ and B56δ may not affect T389 phosphorylation. Another possibility is that since knockdown is not depleting all of the protein, there is still some residual phosphatase left that is associated with S6K1 and is able to dephosphorylate it. This was tested by performing knockdowns with low levels of calyculin A to inhibit any C subunit that was still present on the complex. However, this approach did not reveal an effect of knockdown on T389 phosphorylation.

Despite the lack of effect on the regulation of T389, the idea that PP2A may still be regulating activity of S6K1 via another regulatory site was

investigated by looking at activity of the kinase. S6K1 immunoprecipitation kinase assays from double knockdown samples showed no effect on kinase activity, further supporting previous observations that B56γ and B56δ containing PP2A holoenzymes do not regulate T389 dephosphorylation. These subunits were also not involved in regulating cell size as FACS analysis of B56δ and B56γ siRNA-treated cells showed no difference in forward scatter height (FSC-H) compared to control. Additionally, B56δ immunoprecipitations did not pull-down detectable S6K raising the possibility that this is not a direct interaction. Although as previously discussed, this may be due to the fact that only a small fraction of the total Myc-B56δ was immunoprecipitated. Taken together, these data indicate that S6K1 is not regulated by these PP2A regulatory subunits despite the fact that they appear to mediate association of PP2A with S6K. It is still possible that PP2A is the S6 kinase phosphatase, but that interaction is mediated by other proteins or regulatory subunits.

The eIF3 preinitiation complex (eIF3-PIC) acts as a scaffold for assembly of the translation initiation complex and its interaction with S6K1 is regulated by phosphorylation of T389 (Holz et al., 2005). This model suggests that an activating signal such as insulin or amino acids causes recruitment of mTOR and raptor to the eIF3-PIC, leading to phosphorylation of S6K-T389 that is also associated with the complex. Phosphorylated S6K1 is then released from the complex and can be further phosphorylated and activated by PDK1. While this study did not look at the effect of rapamycin treatment alone on S6K1 binding to the eIF3-PIC, pre-treatment with rapamycin did block insulin induced

dissociation. Similarly, amino acid starvation induced association of S6K with the eIF3-PIC that was then disrupted when amino acids were added back. The effects of mTOR activation on this association of S6K with the eIF3-PIC are similar to those seen with the A/C core dimer and S6K1. This brings up the possibility that PP2A is also a part of the preinitiation complex. The observed interaction between S6K and PP2A may be mediated by another protein in the complex. Holz et al. have shown that there are numerous components involved in the assembly of the eIF3-PIC. B56γ/δ PP2A holoenzymes may already be situated on the complex either regulating the dephosphorylation of a protein other than S6K1 or even as a scaffold. Under basal conditions, S6K1 is part of the eIF3-PIC and when S6K1 is immunoprecipitated, it may pull-down other proteins including PP2A. However, when mTOR is inactivated, a larger pool of dephosphorylated S6K1 will be at the complex, resulting in the co-immunoprecipitation of more associated proteins. Conversely, upon mTOR activation, most of the S6K1 dissociates from the complex and less PP2A co-immunoprecipitated. A model of this alternative explanation is depicted in Figure 20.

It is important to note that if B56γ/δ-containing PP2A is indeed part of the eIF3-PIC, it does not appear to play a major role in regulating S6K or cell size as shown by this report. It is difficult to say what its role would be in the complex, considering the number of potential substrates. One way to further investigate this proposal is to immunoprecipitate other proteins of the eIF3-PIC and look at PP2A association. This can be combined with siRNA treatments against the B56

subunits to see if the complex disassembles when B56γ or B56δ are depleted. Another useful experiment would be to immunoprecipitate S6K1 from B56γ/δ RNAi-treated cells that are ^{32}P-labeled and see if there is greater ^{32}P incorporation in immunoprecipitated samples from knockdown cells versus control samples. This would determine whether these subunits are mediating phosphorylation of any proteins that are in the complex with S6K1.

Further studies performed on cells in which B56γ and B56δ were knocked-down showed a surprising decrease in cell proliferation. If any effect on proliferation was expected, it would have been that knockdown of these subunits would increase proliferation due to over-activation of S6K. Since no functional effects were observed in previous experiments, this result was highly unexpected. It suggests that these subunits normally are positive regulators of cell growth and proliferation, and that dephosphorylation of other substrates is important for this process. However, since cell size was not affected by depletion of B56γ and B56δ, the most likely explanation is that these subunits are also involved in other pathways that control proliferation. This is supported by work done by Margolis et al. where they show a role for B56δ in regulating mitotic entry. Without this subunit, cells would most likely proliferate at a much lower rate.

The discovery that both B56δ and B56γ associate with S6K1 is not surprising since these subunits share more sequence similarity with one another than with any of the other B56 isoforms. In particular, B56γ variant 3, which was used in these studies is the largest of the B56γ proteins and is most similar to B56δ. The fact that another isoform (B56α) does not appear to associate

suggests that association with S6K1 is mediated by a conserved region in the C-terminus of these isoforms. Truncation mutants of both B56γ and B56δ could be used to identify this region and determine specific amino acids that are required for this interaction. Also contained in this B56γ/δ common domain is a nuclear localization signal which is not found in other B56 isoforms. This brings up the possibility that association with S6K occurs in the nucleus, which would suggest that PP2A is in fact interacting with p85-S6K1 rather than p70-S6K1 (Fig. 21) (Coffer and Woodgett, 1994; Reinhard et al., 1994; Grove et al., 1991). Unfortunately, testing this hypothesis with siRNA specific to p85-S6K1 is not possible since these isoforms are products of different translation start sites rather than alternative splicing and therefore have the same mRNA sequence. However, each isoform can be cloned into a vector with a different epitope tag to immunoprecipitate the specific isoform from cells using antibodies against the epitope. Alternatively, S6K1 could be immunoprecipitated from nuclear extracts to selectively pull-down p85. These immunoprecipitates could then be checked for co-immunoprecipitation of the PP2A core dimer. Calyculin A inhibitor studies also show a dramatic increase in phosphorylation levels of the corresponding residue of p85-S6K1. This effect is indeed more pronounced than that seen for p70-S6K1. However, determining a functional role for a possible interaction with p85-S6K1 is more complicated since very little is known about its role in protein synthesis and cell growth.

Knockdown of different calyculin A-sensitive phosphatases in HeLa cells did not definitively identify one as the S6K phosphatase. While PP4 and PP6

may partially regulate dephosphorylation of S6K at T389, they do not appear to be the primary phosphatases at that site since T389 was still dephosphorylated in cells depleted of both phosphatases. It would be important to complete this screen by focusing on PP1 next. Although this enzyme has three catalytic subunit isoforms, cells can be treated with siRNA against all three to see if there is any effect on T389 dephosphorylation. If there is an effect, then siRNA against each isoform can be used individually to identify the specific isoform involved. However, if PP1 knockdown does not alter T389 phosphorylation levels, then other serine/threonine phosphatases such as PP2B/calcineurin could be investigated.

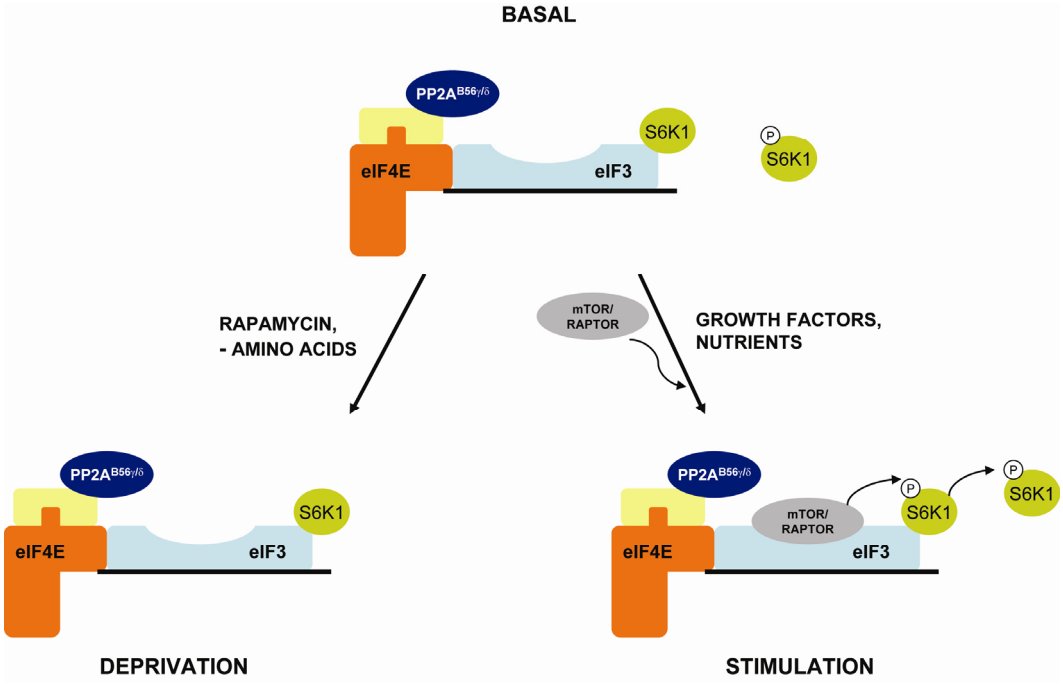

Figure 20. Co-immunoprecipitation of PP2A depends on association of S6K1 with the eIF3 preinitiation complex. In growing cells, S6K1 is both bound to the complex and also dissociated due to basal activation of mTOR. Rapamycin treatment or amino acid starvation inhibits mTOR activity, thereby maintaining almost all S6K1 in a dephosphorylated state at the complex. When S6K1 is immunoprecipitated, more PP2A associated with the complex co-immunoprecipitates since more S6K1 is present. Stimulation of mTOR causes the phosphorylation and subsequent dissociation of S6K1 from the complex. Immunoprecipitation of phosphorylated S6K1 results in less co-immunoprecipitation of PP2A and other complex components. Adapted from Holz et al., 2005.

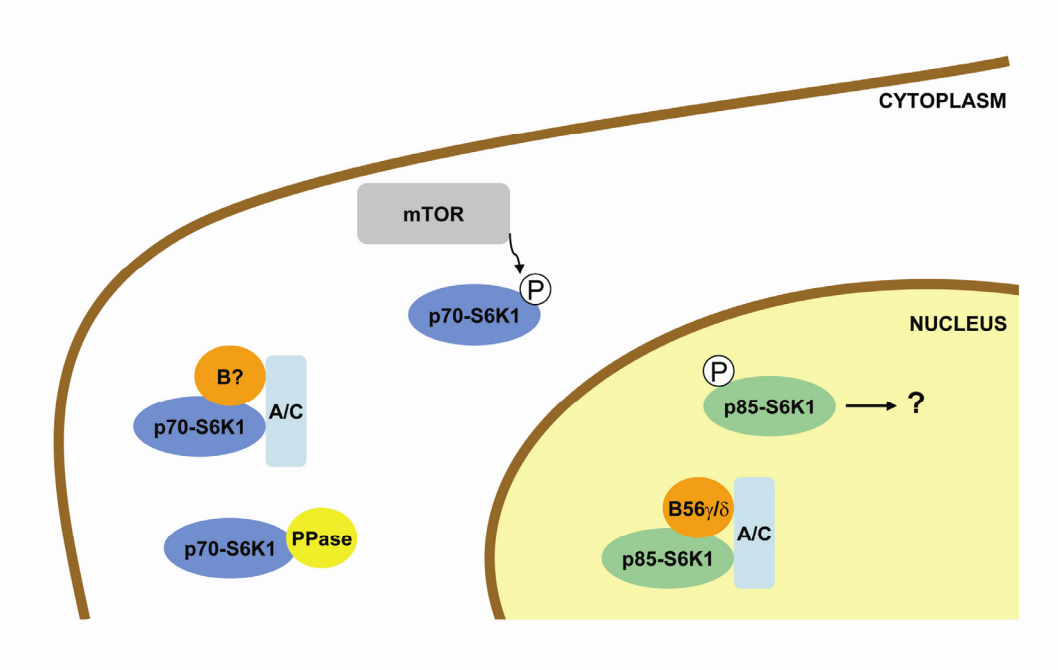

Figure 21. B56γ and B56δ PP2A holoenzymes may specifically associate with p85-S6K1 in the nucleus. Known substrates and regulation of p85-S6K1 activity remain unknown, but its localization is predominantly nuclear. The γ and δ isoforms of the B56 regulatory subunit family have nuclear localization sequences that allow them to be localized to both the cytoplasm and the nucleus whereas other B56 isoforms do not have an NLS. The antibody against S6K1 used in these studies immunoprecipitates both p70- and p85-S6K1. Additionally, calyculin A treatments show increased phosphorylation of p85-S6K1 at S412 in the linker region. This suggests the possibility that PP2A may not be associated with p70-S6K1, but rather with p85-S6K1 in the nucleus.

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VITAE

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