Insights into the regulation of mTOR signaling and the consequences of pharmacological inhibition

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Abstract

Cells have evolved a highly tuned system for driving growth in response to the right cues. Permissive signals initiate a cascade of events that send nutrient transporters to the membrane, suppress apoptosis, boost protein synthesis, and adjust metabolic processes to fuel the cell's energy demands. Increases in cell growth are often coordinated with cell division, though the two programs can be decoupled. The TOR complexes, TORC1 and TORC2, are central regulators of cell growth and share the serine/threonine TOR kinase as their catalytic domain. In mammals, the TORC2 homolog mTORC2 is activated by growth factors through the lipid kinase PI3K, and is a primary effector for many of its functions, including regulation of the proliferation and survival kinase Akt/PKB. Activation of PI3K also leads to activation of mTORC1. Unlike mTORC2, mTORC1 is equally dependent on nutrient availability, and connects to the protein translation machinery through its substrates S6K and 4E-BP1. Additionally, S6K can suppress insulin signaling, establishing a negative feedback loop to PI3K. Consistent with its role in cell growth, derangements in mTOR signaling are increasingly associated with cancer and, more surprisingly, metabolic diseases. In the work described here, we have investigated the mechanism through which insulin activates mTORC1 and identified the protein PRAS40 as a growth factor-regulated inhibitor and mTORC1 component. PRAS40 cooperates with rheb, an mTORC1 activator, to regulate growth factor signaling through the pathway. We have also developed a potent and selective mTORC1/2 small molecule inhibitor and used this to probe the role of mTOR signaling in tumor cell growth and proliferation. Through this, we have identified common genetic mutations that determine sensitivity to mTOR inhibition and suggest a novel therapeutic anti-cancer strategy.

Thesis supervisor: David M. Sabatini
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Introduction

I. Introduction

Proliferating cells have two difficult challenges: splitting one cell into two (cell division) and synthesizing enough protein, lipids, and other molecules to satisfy demand (cell growth). After decades in the spotlight, the processes that control cell division are now understood in great detail. Comparatively, our understanding of cell growth is still in its infancy. Cell growth collectively refers to many processes that include protein translation, ribosome biogenesis, nutrient metabolism and nutrient uptake and exacts a substantial energetic cost. Consequently, cells work hard to coordinate these processes with fluctuating energy availability. The difficulty of this challenge is reflected in the elaborate systems that have evolved to ensure a steady nutrient supply and adjust growth and division to suit changing environments. In many organisms, including yeast, flies, worms and mammals, the TOR signaling pathway is an essential coordinator of these processes. A diverse collection of energy indicators, such as nutrient availability and energetic stress, directly influence TOR activity. TOR integrates this information and, in turn, wields broad control over growth related processes, coupling energy utilization with energy availability and maintaining metabolic homeostasis.

The mammalian TOR homolog (mTOR) is a large serine/threonine kinase and the principal and founding component of this pathway. It was originally discovered as the target of the anti-fungal and immunosuppressive compound rapamycin, which offered the first clues to the pathway's role in cell growth. In cells, mTOR serves as the catalytic domain in two functionally distinct but complementary protein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Insulin and other growth factors stimulate both complexes, while nutrient signals, such as amino acid and glucose availability, uniquely regulate mTORC1. Under permissive conditions, activating signals from both complexes converge on the machinery that regulates protein

translation initiation. Additionally, mTORC1 suppresses insulin signaling through a negative feedback loop, establishing an upper limit on insulin's effects, while mTORC2 drives nutrient transporters to the plasma membrane and suppresses apoptosis. Starvation conditions provoke an opposite response. In addition to stalling translation initiation, loss of mTORC1 activity initiates autophagy, a process that recycles the contents of the cell to maintain a pool of basic nutrients.

Not surprisingly, deranged mTOR signaling is strongly associated with disease. Mutations in PTEN, a tumor suppressor that normally opposes mTORC1/2 activity, are one of the most common cancer-initiating events. Inactivation of TSC, an mTORC1 negative regulator, causes the formation of hamartomous tumors that can severely impair neurologic function. There is also increasing support for the idea that excessively high levels of nutrients, as might occur in obesity, leads to insulin insensitivity by overloading the nutrient input to mTORC1. The implication of mTOR in each of these diseases has spurred enthusiasm for the application and development of mTOR inhibitors. Rapamycin is already in clinical trials for many of these disorders, but is limited in its utility because it preferentially inhibits mTORC1. Hence, there is there is growing demand for a general mTORC1/2 or mTORC2-specific inhibitor. The following introduction summarizes the history of TOR signaling, and outlines its role in normal cell physiology and disease.

II. The discovery of rapamycin and its mechanism of inhibition

A. A family of unusual immunosuppressant molecules

Interest in TOR originated in attempts to understand the molecular basis of the anti-fungal and anti-tumor properties of the lipophilic macrohalide rapamycin. Rapamycin had been isolated from the bacteria *Streptomyces hygroscopicus* out of soil samples taken from Easter Island (Rapa nui) in the 1970s and later identified as an anti-fungal compound in a screen at Ayerst Research Laboratories(Abraham and Wiederrecht,

1996). The drug had no obvious anti-bacterial activity, but caused a profound G1 growth arrest in yeast at even low nanomolar concentrations. Rapamycin also caused a similar arrest in several NCI tumor cell lines, generating hope that it might be used as an anti-cancer agent, though substantial toxicity in some animal models and problems with solubility dampened early enthusiasm (Eng et al., 1984).

Rapamycin acts through an unusual mechanism that is similar to the compounds FK506 and Cyclosporin A (CsA). All three compounds bind to a class of small intracellular proteins called immunophilins. CsA binds to the 18 kDa cyclophilin A (CyPA), while rapamycin and FK506 bind to the 12 kDa FK506 binding protein (FKBP12). Both CyPA and FKBP12 are enzymes, termed prolyl-isomerases, that catalyze cis-trans isomerization of peptidyl-prolyl bonds in peptides and proteins (Abraham and Wiederrecht, 1996). However, it was quickly determined that inhibition of the isomerase activity failed to explain the effects of all three of these drugs. Instead, the binding of these molecules to their immunophilin partners potentiates their ability to inhibit a second target. For FK506 and Cyclosporin A, that target is the Ca++-dependent serine-threonine phosphatase calcineurin. The target for rapamycin was not identified at the time.

Although rapamycin was isolated first, it was ignored until discovery of the FK506, nearly a decade later. FK506 was discovered in a screen for natural products that inhibit IL-2 production at Fujisawa Pharmaceutical Laboratories and showed exceptionally strong inhibitory activity in a variety of immune function assays(Abraham and Wiederrecht, 1996). Most importantly, it was nearly 100-fold more potent than CsA, which had become a standard therapy in preventing transplant rejection. The chemical similarities between FK506 and rapamycin prompted the hope that rapamycin might be similarly useful, and this turned out to be true(Abraham and Wiederrecht, 1996). Since then, numerous variations, sometimes called rapalogs, have been clinically approved for use in kidney transplant patients (1999), and are at various stages of testing for autoimmune, chronic inflammatory and anti-cancer applications (Abraham, 2002).

Many of the solubility and stability problems that hindered early use of the drug have been essentially resolved in the more recent analogues CCI-779 (Wyeth) and RAD001 (Novartis)(Easton and Houghton, 2006).

B. Identification of the cellular target of rapamycin

Many of the early insights into the molecular effects of rapamycin came from work in yeast. In wild-type cells, rapamycin triggers a reversible growth arrest and initiates a stress program that closely resembles the response to nutrient starvation. To identify the molecular rapamycin targets, two groups conducted a screen for mutations that conferred resistance to this arrest (Heitman et al., 1991; Koltin et al., 1991). The most common mutations were found in FPR1, the homologue for the mammalian FKBP12, though the observation that loss of FPR1 had no obvious consequences ruled it out as the cytostatic target of rapamycin. Instead, just as FKBP12 is required for FK506 to inhibit calcineurin, this confirmed that FPR1 is a requirement for rapamycin toxicity. The other two classes of mutations were dominant gain-of-function defects in two similar genes, which were named the target of rapamycin (TOR1 and TOR2). Both TOR1 and TOR2 genes produce large 280 kDa products and are highly homologous to each other (67% identical). For both TOR proteins, single missense mutations (Ser1972Arg in TOR1 and Ser1975Ile in TOR2) conferred resistance to rapamycin by preventing the binding of the rapamycin-FPR1 complex (Cafferkey et al., 1994).

These early genetic studies of TOR function also hinted at the complexity of the pathway. For instance, deletion of the TOR proteins alone or together caused a phenotype that was similar, but not identical, to rapamycin treatment. Loss of TOR1 severely hindered cell growth, but it failed to recapitulate the G1 arrest caused by rapamycin treatment. By contrast, TOR2 was essential and deletion caused a random cell cycle arrest (Cafferkey et al., 1994). Strangely, the combined deletion of TOR1 and TOR2 caused

a G1 arrest and was the most similar to rapamycin treatment (Kunz et al., 1993). The conclusion at the time was that TOR1 and TOR2 were partly redundant, but that TOR2 also had other roles. Later work showing that rapamycin-resistant alleles of TOR1 could support normal growth in the presence of rapamycin demonstrated that the essential functionality of TOR2 was rapamycin-insensitive (Zheng et al., 1995).

Although work in yeast established a solid rationale for the TOR proteins as the direct targets of rapamycin, that hypothesis wasn't proven until several years later. Using a biochemical approach in mammalian cells, several groups identified a large protein with homology to the yeast TORs as that binding target of the FKBP12-rapamycin complex (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). This protein was christened with several names upon its discovery, including FRAP and RAFT1, but is now referred to simply as mammalian TOR, or mTOR. The high degree of sequence similarity between the mammalian and yeast homologues indicated correctly that many of features of TOR signaling were preserved between these highly diverged species.

C. TOR proteins belong to the PIKK family of kinases

The TOR proteins belong to a class of serine/threonine kinases that includes ATM, ATR, DNA-PK and hSMG1. Each of these is giant by protein standards, ranging from 289 kDa (TOR) to greater than 500 kDa (DNA-PK). The similarity between them is concentrated in their C-terminal kinase domains, which is similar to the kinase domain of phosphoinositide-3-kinase (PI3K), earning them the name PI3K-related kinases (PIKKs). However, the kinase domain only represents a small fraction of the overall protein, and the rest is composed of a series of N-terminal domains that are thought to mediate protein-protein interactions (Figure 1). The N-terminus of TOR begins with at least 20 tandemly-repeated HEAT domains, which are named based on their presence in Huntingtin, Elongation factor 3, the A subunit of PP2A and TOR(Andrade and Bork, 1995). Each repeat is 40-50 amino acids long and are thought to form anti-parallel alpha-

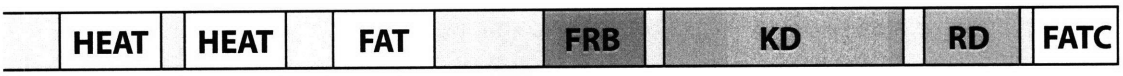

Figure 1. TOR domain structure

The TOR protein contains several conserved domains, including N-terminal HEAT repeats and FAT (FRAP, ATM and TRRAP), FRB (FKBP12-rapamycin binding), KD (kinase domain), RD (repressor domain) and FATC (FAT C-terminal) domains.

helices(Abraham, 2002; Abraham and Wiederrecht, 1996; Hemmings et al., 1990). TOR, and several other PIKKs, also contain a FAT domain, named for its presence in FRAP (mTOR), ATM and TRRAP, that immediately follows the HEAT repeats and is invariably accompanied by a C-terminal domain called the FATC(Bosotti et al., 2000). Although neither of these domains has a clear function, the FATC is required for kinase activity, suggesting a possible structural role (Bosotti et al., 2000). The final domain, situated between the FAT and kinase domains, is known as the FKBP12-rapamycin binding (FRB) domain and contains the sites of both rapamycin-resistance mutations (Chen et al., 1995; Stan et al., 1994). Interestingly, the only other occurrence of the FRB domain is found in hSMG1(Yamashita et al., 2001).

A final point that distinguishes mTOR from other PIKKs is substrate specificity. ATM, ATR, DNA-PK and SMG1 all show a strong preference for a serine/threonine followed by a glutamine, and are thus often referred to as S/T-Q-directed kinases. These kinases are also all involved in the response to DNA damage or the detection of aberrant nucleotides. mTOR, on the other hand, is not known to respond to genotoxic stress and additionally prefers a less-defined motif that consists of a serine/threonine followed by a proline, or a serine/threonine surrounded by bulky hydrophobic residues(Brunn et al.,

1997; Burnett et al., 1998; Isotani et al., 1999). It has been suggested that this may reflect a functional evolutionary divergence between TOR and the rest of the PIKKs, which are all involved in the response to genotoxic stress(Abraham, 2002).

D. The two sides of TOR signaling: TORC1 and TORC2

Understanding of TOR signaling has progressed at an accelerating pace since its discovery over a decade ago. Since then, the most revealing finding has been that TOR acts as the catalytic domain in two functionally distinct complexes, called TORC1 and TORC2 (mTORC1 and mTORC2 in mammalian systems). The early hints of rapamycin-insensitive functionality had implied two distinct roles for TOR, and the biochemical identification of TORC1- and TORC2-specific binding partners confirmed this suspicion. Not surprisingly, only one complex, TORC1, is inhibited by rapamycin, and these two complexes are often referred to as the rapamycin-sensitive and rapamycin-insensitive pathways. Each complex responds to different upstream signals and are tied to different downstream pathways. In particular, TORC1 is tightly regulated by the intracellular availability of nutrients, such as amino acids, and is frequently called the nutrient-sensitive pathway. TORC2 is insensitive to these signals, and, at least in mammalian systems, appears to be entirely regulated by growth factor signaling. The following sections describe their composition, downstream effectors and physiologic roles and are summarized in Figure 2.

III. TORC1: a regulator of protein translation and cell growth

A. Discovery of the TORC1 complex

The TOR pathway has classically been considered a regulator of cell growth, beginning with the finding that rapamycin treatment diminished cell size. Although growth is often confused with proliferation, the two are functionally separable processes:

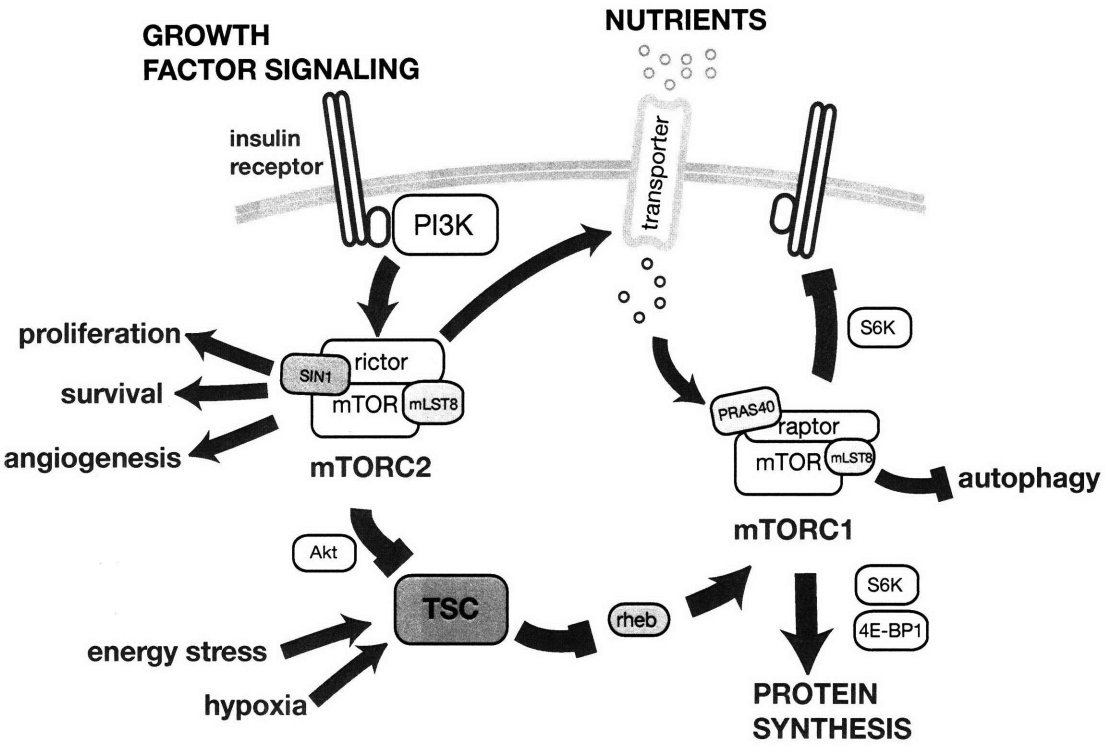

Figure 2. mTORC1 and mTORC2 complexes in growth factor and nutrient signaling

Growth factors bind to their receptors and initiate a cascade of events that results in activation of PI3K and mTORC2. mTORC2 then phosphorylates and activates Akt, which drives translocation of nutrient transporters to the plasma membrane, inactivation of pro-apoptotic molecules and stimulation other growth related programs. Akt also inactivates the mTORC1 inhibitor tuberous sclerosis complex (TSC), which is an integration point for many stress and energy signals. Under permissive growth conditions, TSC is inactivated and downstream inhibition of mTORC1 is relieved. When sufficient nutrients are available, mTORC1 then promotes protein translation by activating S6K and suppressing the translational inhibitor 4E-BP1. Completing the loop, S6K inhibits insulin signaling by marking IRS1 for degradation.

proliferation refers to cell division, while growth is the accumulation of mass. In both mammals and yeast, TORC1 exerts influence by coupling the intracellular availability of nutrients, such as amino acids and glucose, to regulation of protein translation and ribosome biogenesis. As these two processes place substantial demand on cellular resources, TOR activity can have a profound influence on cellular metabolism energy requirements. In mammals, insulin signaling also influences mTORC1, ensuring coordination between organismal nutrient availability and cellular demand.

The defining member of the TORC1 complex is kog1/mip1 in yeast and raptor in mammals. It was originally characterized in a screen for high-copy suppressors of ectopic meiosis caused by expression of the meiotic regulator mei2 in Schizosacchromyces pompe(Shinozaki-Yabana et al., 2000). Mei2p normally induces meiosis only in response to starvation conditions, and loss of mip1p interfered with this process. However, it was not until the identification of the human homologue, raptor, by immunoprecipitation and mass-spectrometry that this protein was associated with TOR signaling(Hara et al., 2002; Kim et al., 2002). Soon after, the raptor homolog kog1p was found to associate with both TOR1 and TOR2, defining TORC1 in budding yeast (Loewith et al., 2002).

Raptor, like mTOR, is a large 150 kDa protein scattered with domains that reveal little about its function. The N-terminus begins with the unique raptor-N-conservered (RNC) domain, followed by a series of three HEAT repeats and seven WD domains, all of which are thought to mediate protein-protein interactions (Kim et al., 2002). In cells, raptor has a clear positive role in mTORC1 activity, and depletion of it by RNAi leads to inhibition of downstream targets similarly to rapamycin treatment (Hara et al., 2002; Kim et al., 2002). Interestingly, raptor is not required for TORC1 activity in vitro and paradoxically seems to be inhibitory. Although a potential artifact, this observation suggests that raptor activates mTOR in cells through a mechanism that doesn't directly alter its kinase activity, such as localization or substrate recognition. Along these lines, several groups have suggested that raptor is involved in recognizing a 5-amino acid

TOR-signaling (TOS) motif that appears in many TORC1 substrates (Schalm and Blenis, 2002).

The association between raptor and mTOR is a dynamic one that can be influenced by nutrient availability. Under appropriate cell lysis conditions, amino acid starvation or other kinds of metabolic stress increases the strength of the mTOR-raptor interaction (Kim et al., 2002). Nutrient stimulation had the opposite effect, though treatment with reversible cross-linkers showed that the two didn't dissociate completely. Kim et al used this observation to develop a model where mTOR and raptor are in a "weak-binding" state during nutrient stimulation and switch to a "tight-binding" state under starvation conditions. Although the molecular basis of this change remains unclear, these findings demonstrated that mTORC1 was the direct target of some sort of nutrient signal. Interestingly, rapamycin also disrupts the association between mTOR and raptor, and some have suggested that this is the mechanistic basis for mTORC1 inhibition (Kim et al., 2002; Oshiro et al., 2004).

mTORC1 also contains two other components: mLST8 (also known as Gbeta-like, or Gbl), which binds directly to TOR, and PRAS40. mLST8/Gbl was first identified as a TORC1 component in budding yeast(Loewith et al., 2002) and shortly after in mammalian cells (Kim et al., 2003). It is a small 36 kDa protein consisting almost entirely of 7 WD-40 repeats with high sequence similarity to those in the beta subunits of heterotrimeric G-proteins, hence it's name (Rodgers et al., 2001). Ironically, mLST8 was probably first identified as RAFT2 in the experiment that identified mTOR/RAFT1 as the binding target of FKBP12-rapamycin, though it wasn't further characterized at the time (Sabatini et al., 1994). There is some debate concerning the function of mLST8. Although it was originally considered a positive regulator of mTORC1, recent analysis of cells derived from an mLST8-/- mouse found that mTORC1 signaling remains intact (Guertin et al., 2006; Kim et al., 2003). Unexpectedly, this same work showed that mLST8 is instead required for mTORC2 signaling (Guertin et al., 2006).

PRAS40 is the final mTORC1 component. It was recently identified as a raptor-binding protein and a negative regulator of the pathway (Sancak et al., 2007; Vander Haar et al., 2007). PRAS40 will be discussed in a later chapter.

B. TORC1 controls the translational machinery and nutrient uptake

In both yeast and metazoans, TORC1 activation drives protein synthesis by promoting ribosome biogenesis and translational initiation. Conversely, under starvation conditions, TORC1 inhibition shifts the balance towards catabolic processes such as autophagy and, at least in yeast, drives high-affinity nutrient transporters to the plasma membrane. The important TORC1 effectors are less conserved between yeast and mammals than other components of the pathway, though this may reflect different experimental approaches between the two systems. In yeast, TORC1 acts primarily by controlling different transcriptional programs, while mTORC1 drives cell growth almost exclusively through post-translational signaling cascades. Nonetheless, the types of processes that are regulated between the two organisms are still very similar, reflecting the overall conservation of the pathway.

In budding yeast, TORC1 controls a collection of nutrient-regulated processes that can be loosely categorized as nitrogen scavenging transcriptional programs (Gln3, Ncr1, MSN2/4, and RTG1/3), ribosomal biogenesis (RP genes), translation initiation (eIF4E/G, eIF2) and autophagy (ATG) (reviewed in De Virgilio and Loewith, 2006; Schmelzle and Hall, 2000). Of these processes, the best understood is the regulation of the nitrogen scavenging response, which is mediated by TOR control of the SIT4 PP2A phosphatase. Under nutrient-rich conditions, SIT4 is tightly bound to the protein TAP42 (Di Como and Arndt, 1996). Upon TORC1 inactivation by rapamycin treatment or starvation, TAP42 is dephosphorylated and dissociates from SIT4 (Di Como and Arndt, 1996; Jiang and Broach, 1999). Active SIT4 then initiates nitrogen scavenging and stress

response programs by dephosphorylating Gln3, RTG1/3, GCN2 and NPR1 (reviewed in De Virgilio and Loewith, 2006).

However, many consequences of TORC1 signaling are independent of TAP42/ SIT4. For instance, TORC1 inhibition dramatically represses expression of both ribosomal proteins (RP genes) and rRNA independently of SIT4 (Cardenas et al., 1999; Powers and Walter, 1999). TORC1 inhibition also stimulates the recycling of cellular material through the process of macroautophagy, in which double-membraned vesicles, called autophagosomes, engulf parts of the cytoplasm and various organelles (Kamada et al., 2000). These vesicles then fuse with lysosomes and the contents are degraded to base nutrients that can be reused. TORC1 regulates this process by directly phosphorylating components of the autophagic signaling pathway(Kamada et al., 2000). Finally, TORC1 can influence translation initiation by regulating the 5'-mRNA cap binding proteins eIF4E and eIF4G, and eIF2 (De Virgilio and Loewith, 2006). Thus, by coordinating anabolic and catabolic processes in response to nutrient availability, TORC1 plays a key role in maintaining energy homeostasis.

One note of caution is that many of these connections to TORC1 were inferred from either rapamycin-treated or TOR-deficient cells, leaving the possibility that the association is indirect. For instance, the AGC kinase Sch9 was recently identified as a bona fide TORC1 target that may mediate many of these effects (Urban et al., 2007). In particular, Sch9 was required for control of ribosome biogenesis and translation initiation, but not for the expression of any Gln3-dependent genes. As Sch9 is homologous to the mammalian mTORC1 target S6K (described in the next section), these findings suggest that the molecular mechanisms of TORC1 signaling might be more conserved than previously appreciated.

C. Effectors of mammalian TORC1 signaling: S6K and 4E-BP1

The downstream effectors of mTORC1 appear to have diverged from those in yeast more than other features of the pathay. For instance, there is no obvious mammalian SIT4 homologue, perhaps reflecting the difference between metazoan and single-celled nutrient requirements. Instead, the classical mTORC1 substrates are the eIF4E inhibitor 4E-BP1/PHAS-1 and the ribosomal S6 kinases (S6K1/2). Before the discovery of mTOR, both proteins had already been identified as downstream targets of a rapamycin sensitive pathway (Beretta et al., 1996; Chung et al., 1992; Kuo et al., 1992; von Manteuffel et al., 1996). Several groups later showed them to be direct mTOR substrates(Brunn et al., 1997; Burnett et al., 1998; Hara et al., 1997; Isotani et al., 1999). Both substrates associate with and, in varying capacities, regulate the formation of the translational pre-initiation complex, which also includes eIF4E, eIF4G and eIF3 (Holz et al., 2005). 4E-BP1 interferes with the complex formation by binding to eIF4E, which is normally bound to the 7-methylguanosine mRNA 5' cap, and preventing its association with the larger eIF4G and inhibiting translation. Phosphorylation of 4E-BP1 by mTORC1 and potentially other kinases diminishes its affinity for eIF4E, which is then able to assemble into a competent initiation complex.

The S6 kinases have a more complicated role. S6K1 was identified first, and is produced as two splice variants (70 and 85 kDa) that differ by the addition of 23 N-terminal amino acids containing a nuclear localization sequence(Reinhard et al., 1992). They were originally discovered as the kinases responsible for phosphorylating a collection of sites on the 40S ribosomal subunit protein S6, which was strongly associated with activation of protein translation (Jeno et al., 1988; Sturgill and Wu, 1991). Mechanistically, phosphorylation of these sites permits association with the pre-initiation complex and subsequent translation(Holz et al., 2005). Recently, S6K was also implicated in the phosphorylation of PDCD4, marking it for degradation and relieving its inhibition of eIF4A (Dorrello et al., 2006). Other targets include SKAR, BAD, and mTOR itself, though the significance of these events is still unclear(Dann and Thomas, 2006).

Deletion of the S6K homologue in Drosophila is semi-lethal and causes a severe reduction in organismal size(Montagne et al., 1999). Mutant flies have the same number of cells as their wild-type counterparts, but the size of each is severely reduced, demonstrating a decoupling of growth and proliferation. Deletion of S6K1 from mice also results in animals that are approximately 20% smaller than wild-type and are mildly glucose intolerant, though the authors concluded that this might be explained by smaller pancreatic beta-cell size and reduced insulin production (Shima et al., 1998). The S6K1-knockout mouse also revealed the existence of a second highly similar and functionally redundant isoform, called S6K2, that could compensate for the S6K1 deficiency(Pende et al., 2004). Combined deletion of S6K1 and S6K2 showed a profound impact on viability, underscoring the importance of these genes in normal physiology(Pende et al., 2004).

S6K1 also influences cell growth through a second, and perhaps more profound, feedback mechanism that modulates insulin signaling. The binding of insulin to the insulin receptor stimulates its tyrosine kinase activity, causing it to phosphorylate a family of proteins called insulin receptor substrates (IRS). IRS proteins are normally localized to the plasma membrane, and phosphorylation causes them to associate with and activate the p85alpha subunit of PI3K, initiating the activation of many downstream kinases that include S6K, Akt and PKC (Zick, 2005). However, sustained activation of S6K1 eventually suppresses PI3K activation by phosphorylating key residues on IRS with the following consequences: dissociation of IRS from the insulin receptor; mark IRS for degradation; block Tyr phosphorylation sites on IRS; dissociate IRS from the plasma membrane; or turn IRS proteins into inhibitors of the insulin receptor kinase (Zick, 2005). The physiologic relevance of the role of S6K in this mechanism is further underscored by work from Um et al, who showed that S6K1-/- mice were resistant to obesity, primarily because they maintained abnormally high insulin sensitivity(Um et al., 2004).

There are some indications the S6K and/or mTORC1 can suppress growth factor signaling through a more general mechanism. For instance, constitutive activation of

S6K leads to downregulation of the PDGF-receptor, though the mechanism is not entirely clear (Zhang et al., 2003). Other groups have proposed that over-stimulation of protein translation causes ER-stress, thereby clogging the conduit that leads growth factor signaling components, such as PDGFR and IRS, to the plasma membrane (Ozcan et al., 2008). The strength of this negative feedback signaling has been a surprise, and it will be interesting to better understand its physiologic relevance, particularly in metabolic diseases.

D. Upstream regulators: many roads lead to mTORC1

mTORC1 is controlled by a constellation of signals that reflect the overall metabolic state of the cell. In yeast, TORC1 acts as a sensor of nitrogen and carbon availability and is activated by amino acid and glucose sufficiency. In metazoans, the pathway has many other inputs, including oxygen availability, ATP levels, growth factor signals and other indicators of energy status. These metabolic cues signal to mTORC1 either directly, or through two primary avenues. The best characterized of these involves the small GTPase rheb and the large heterodimeric TSC complex, which integrates most of these signals. A notable exception is the amino acid signal, which is transmitted through a parallel pathway that involves a family of small GTPases known in yeast as Gtr1/2 and in mammals as Rag proteins.

The TSC complex is a heterodimer of the proteins tuberin (TSC1) and hamartin (TSC2). Before their association with TOR signaling, they had already been characterized as tumor suppressor genes, and inactivation of either causes a familial autosomal dominant disorder tuberous sclerosis complex (TSC) that affects approximately 1:6000 individuals (Montagne et al., 2001). A defining feature of TSC is the development of hamartomous tumors in multiple tissues that can be severely disrupt tissue function but are rarely metastatic. Loss of TSC2 is also strongly associated with lymphangioleiomyomatosis (LAM), a rare multi-systemic disease that causes cystic

destruction of the lung parenchyma and abdominal tumors(Chorianopoulos and Stratakos, 2008). LAM occurs almost exclusively in young women, and the mechanistic connection to TSC is still not well understood.

The TSC proteins were first associated with cell growth in drosophila, where selective deletion in the eye and wing led to the formation of giant cells (Montagne et al., 2001). They were not connected to the TOR pathway, however, until it was identified as the GTPase activating protein (GAP) for the small G-protein rheb (ras homolog enriched in brain)(Manning and Cantley, 2003). Rheb binds only weakly to mTORC1, but can profoundly activate its kinase activity both in vivo and in vitro (Manning and Cantley, 2003; Sancak et al., 2007). Only the TSC2 protein contains a GAP domain, but its activity is dependent on its association with TSC1. Generally, upstream signals that act through the TSC complex do so by disrupting the association between the two subunits and inhibiting TSC2 GAP activity. A putative GTP-exchange-factor (GEF) for rheb has also been identified in drosophila, though the knock-out mouse has no mTOR related phenotype(Hsu et al., 2007).

Kinases from many signaling pathways phosphorylate a collection of sites on TSC, presumably either stabilizing or disrupting its integrity. Erk, a downstream effector of the Ras/MAPK pathway phosphorylates many sites, promoting destabilization of TSC(Ballif et al., 2005; Ma et al., 2005; Roux et al., 2004). Akt, which is activated upon insulin stimulation, also phosphorylates and destabilizes TSC(Inoki et al., 2002; Manning et al., 2002). AMPK, which is activated by rising intracellular AMP and energetic stress, phosphorylates and stabilizes TSC(Inoki et al., 2006). The Drosophila hypoxia-induced proteins Scylla and charybdis and their mammlian homolog Redd1 also stabilize TSC to inhibit mTORC1.(Brugarolas et al., 2004; Reiling and Hafen, 2004). The assumption has been that the sum effect of these phosphorylations determines the final stability of the TSC1/TSC2 interaction, and hence its activity.

An interesting and final note concerns the effect of TSC inactivation on the S6K-

mediated regulation of insulin signaling. Loss of TSC1/2 function in mouse embryonic fibroblasts constitutively induces S6K phosphorylation, but, by engaging the negative feedback loop and suppressing insulin signaling, also suppresses Akt activation (Zhang et al., 2003). Thus it has been hypothesized that TSC tumors are less aggressive because of impaired PI3K/Akt signaling. The finding that other growth factor receptors, such as PDGFR, are similarly inactivated further supports this hypothesis. Recent work suggests that TSC can also influence insulin signaling independently of its effect on S6K, indicating that the coordination between these two pathways is more complicated than previously appreciated (Huang et al., 2008).

E. TORC1 and amino acid metabolism

The connection between TORC1 signaling and amino acid availability has historically been a fascinating and poorly understood feature of the pathway. In yeast, rapamycin treatment and amino acid deprivation elicit remarkably similar responses, including induction of macroautophagy and expression of TOR-regulated starvation response genes. Indeed, TORC1 may be the primary mediator of the amino acid starvation response. In mammalian cells, both S6K and 4E-BP1 are rapidly dephosphorylated by amino acid starvation, indicating that the amino acid sensing machinery has been evolutionarily conserved (Dann and Thomas, 2006). Re-addition of amino acids reverses these effects in a rapamycin-sensitive manner. Despite considerable effort by many groups, there is still little understanding of the underlying mechanics. Specifically, it is unknown which particular metabolite is actually sensed, and it is unknown how this signal is relayed to TORC1.

It is widely believed that the amino acid signal originates from an intracellular receptor. The best evidence for this is that inhibition of protein synthesis (ie. Cycloheximide treatment) can activate mTORC1 even the absence of extracellular amino

acids, presumably by creating a surplus of unused intracellular amino acids(Beugnet et al., 2003). Secondly, leucine and glutamine appear to play a privileged role in TORC1 activation and together can nearly compensate for the lack of all other amino acids (Xu et al., 2001). Leucine is also known to stimulate protein synthesis in animals, and many have argued that this is partly due to activation of mTORC1 (Kimball and Jefferson, 2006).

Leucine influences cells in three ways: as a translational building block for protein synthesis; as a catabolic energy source; and as an allosteric activator of glutamate dehydrogenase (GDH), an important enzyme that converts glutamine to alpha-ketoglutarate for use in the TCA cycle. Many groups have probed each of these branches in an attempt to identify the one that leads mTORC1. Iiboshi et al proposed early on that mTORC1 might by inhibited by the accumulation of uncharged tRNAs, though other groups have shown that inhibition of leucyl-tRNA synthetases using leucine alcohol analogues had no effect on mTOR activity(Dennis et al., 2001; Gao et al., 2002; Iiboshi et al., 1999).

Other groups have proposed that leucine itself or a catabolic product is responsible for activating mTORC1. Leucine is primarily degraded in the mitochondria, beginning with an initial reversible deamination by Branched chain amino acid transferase (BCAT) to produce keto-isocaproate (KIC). KIC is then irreversibly dehydrogenated by the rate-limiting branched chain alpha-keto acid dehydrogenase (BCKDH) complex and further oxidized. KIC alone is a potent activator of mTORC1, and rapidly induces 4EBP1 and S6K phosphorylation in cell culture(Fox et al., 1998). However, this effect is prevented by the addition of (aminooxy)acetic acid, a general inhibitor of amino acid transferases, suggesting that the effect of KIC depends on its conversion back to leucine and not it catabolic degradation. In mouse embryonic fibroblasts where the predominant mitochondrial BCAT variant (BCATm) has been deleted, TORC1 is also no longer stimulated by KIC, supporting the conclusion that

catabolism of leucine does not influence mTORC1 (She et al., 2007). Leucine-mediated activation of GDH is also an unlikely mechanism, as BCH, a non-metabolizable leucine analog that is still capable of activating GDH, has no effect on mTORC1 signaling (Kanazawa et al., 2004; Lynch et al., 2000).

The only clear conclusion from this work is that the effects of leucine are not due to its use in any of these well-characterized pathways. A second possibility is that intracellular leucine is sensed directly. Several groups have proposed that high-affinity low-capacity amino acid transporters might serve as amino acid sensors in additional to their transport roles. In particular, Goberdhan et al have proposed that the proton-assisted transporter PATH is essential for TORC1 activity while Columbani et al have identified an important role for the transporter slimfast in regulating drosophila body size(Colombani et al., 2003; Goberdhan et al., 2005). However, it is not clear whether either of these transporters signal directly to TORC1 or simply alter the intracellular leucine availability. It has also been suggested that L-amino acid transporters, which also have a particularly high affinity for leucine and are transcriptionally regulated by mTORC1, might play a role in amino acid sensing, though this hypothesis has not yet been explored (Dann and Thomas, 2006).

The role of glutamine in mTORC1 activation has been received more attention in yeast. Unlike leucine, glutamine is known to be a fundamental building block for many pathways and can be rapidly converted into TCA cycle intermediates, precursors for other amino acids, nucleotides and is generally considered a key indicator of the cell's overall nitrogen status (De Virgilio and Loewith, 2006). Starvation for glutamine causes a response that closely resembles rapamycin treatment or TOR deficiency, as does treatment with the glutamine synthetase inhibitor methionine-sulfoximine (MSX), though this molecule is known to have many non-specific effects(Crespo et al., 2002). In yeast, TOR is also known to negatively regulate the expression of many genes involved in glutamine synthesis through its control of Gln3, potentially indicating a homeostatic

negative feedback loop that maintains intracellular glutamine levels (Crespo et al., 2002). Regardless, there is no clear connection to TORC1, and the identity of the relevant metabolite remains an open question.

F. Mechanisms that connect mTORC1 to amino acid availability

Despite many attempts to connect amino acids to TORC1 through known upstream regulators, none have been convincing. For instance, some groups had proposed that loss of Tsc1 and Tsc2 in mammalian and drosophila cells rendered TORC1 signaling resistant to amino acid deprivation (Gao et al., 2002). However, it is now widely believed that, although loss of TSC hyperactivates TORC1 signaling and can overcome the effects of growth factor withdrawal, it cannot overcome the effects of amino acid deprivation (Nobukuni et al., 2005; Smith et al., 2005). Other groups have shown that overexpression of rheb in cell culture can overcome the effects of amino acid starvation on mTORC1 signaling and that overexpression in drosophila embryonic tissue causes overgrowth regardless of amino acid availability (Saucedo et al., 2003). However, these results are complicated by the possibility that ectopic overexpression of rheb overwhelms the endogenous regulatory machinery and overcomes the effects of amino acid deprivation artificially. It also is worth noting that TORC1 signaling is robustly regulated by amino acids in budding yeast despite the lack of TSC homologs, further suggesting that this conserved sensory mechanism works through other pathways.

A model that as been recently proposed is that amino acids signal to TORC1 through the class III PI3K hVps34 (vacuolar protein sorting 34). Unlike its well-known class I PI3K relative, which produces PI(3,4,5)P in response to growth factor signaling, hVps34 only generates the monophosphorylated PI(3)P. Vps34 was originally implicated in endosmal/lysosomal vesicular trafficking and the recruitment of proteins containing PI(3)P binding domains in yeast, and shown to have a similar role in mammalian

cells (Backer, 2008). Vps34 is also required for macroautophagy and has been shown to interact directly with regulators of autophagy in both yeast and mammalian cells, suggesting that it may have a more general role in vescicular trafficking (Backer, 2008).

The hypothesis that hVps34 participates in amino acid regulation of TORC1 signaling is primarily based on three observations from Nobunki et al. The first is that wortmannin, an irreversible potent pan-PI3K inhibitor, blocks mTORC1 signaling in tsc2-/- cells (Nobukuni et al., 2005). Although wortmannin is known to inhibit class I PI3K-mediated growth factor signaling to mTORC1, tsc2-null cells are insensitive to this input, suggesting that wortmannin also suppresses a second PI3K-mediated TSC-independent signal. Secondly, hVps34 activity is regulated by amino acid availability. Finally, RNAi-mediated depletion of hVps34 suppresses mTORC1 activation despite of amino acid or growth factor sufficiency. Together, these findings support the argument that hVps34 activity is required for mTORC1 signaling. However, it has not yet been shown that overexpression or ectopic activation of hVps34 can overcome the effects of amino acid starvation on mTORC1 activity. In the absence of this data, it remains possible that hVps34 might be required for mTORC1 function without direct involvement in relaying the amino acid signal. Additionallyl, recent work in Drosophila supports the hypothesis that vps34 functions downstream of TORC1(Juhasz et al., 2008).

Regardless, the finding that deregulation of vesicular trafficking might influence TORC1 signaling is an interesting one that is also supported by work from other groups. Dubouloz et al identified a vacuolar membrane-associated protein complex (EGO), composed of EGO1, EGO3 and the GTPases Gtr1 and Gtr2, which was required for the resumption of growth following a rapamycin-induced G1 arrest (Dubouloz et al., 2005). Gtr1 and Gtr2, in particular, are believed to have a role in the intracellular sorting of the general amnio acid permease Gap1p(Gao and Kaiser, 2006). Two observations suggested that the EGO proteins might act somewhere in the TORC1 pathway: deletion of EGO components elicited a response that resembled TOR deficiency or rapamycin treatment

and overexpression of some EGO components confered rapamycin resistance (Dubouloz et al., 2005). Furthermore, a molecule that was identified in a screen for compounds that confer rapamycin resistance targets the Ego3 protein, apparently causing a gain of function (Huang et al., 2004). The interpretation of these results was that EGO proteins acted primarily downstream of TORC1, though that it might influence upstream signaling as well.

The Gtr1/2 proteins are highly homologous to four mammlian small GTPases called Rags (RagA, B, C and D). Work from our lab has recently shown that Rag proteins can bind directly to raptor in a manner that is regulated by amino acids (Sancak et al., 2008). Moreover, expression of a constitutively GTP-bound mutant of RagB activates mTORC1 and renders it insensitive to amino acid starvation, while RNAi-mediated depletion suppresses the pathway. Wild-type RagB GTP loading is regulated by amino acid availability. Sancak et al also showed that re-addition of amino acids to starved cells caused a dramatic rapamycin-insensitive re-localization of mTOR from diffuse cytoplasmic structures to much larger Rab7-positive vesicles regardless of the presence of absence of growth factors. Expression of the RagB-GTP mutant causes the same change in localization. Although it is not yet clear how amino acids affect Rag activity, the evidence strongly supports their role as a key amino acid regulated input to mTORC1. Interestingly, rheb has also been shown to localize to endosomal membranes (Buerger et al., 2006; Saito et al., 2005; Takahashi et al., 2005). An attractive though still hypothetical model is that Rag proteins mediate a nutrient-dependent mTORC1 translocation to a cellular compartment that also contains rheb, which can then activate mTORC1 directly (Figure 3).

IV. TORC2: connections to proliferation, survival and cytoskeletal structure

A. The rapamycin-insensitive complex

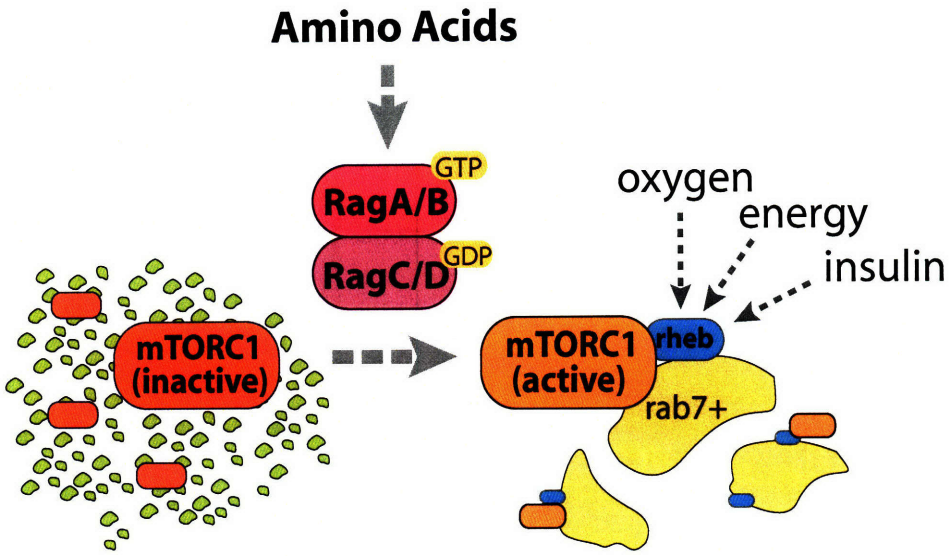

Figure 3. mTORC1 regulation by amino acids

mTORC1 is normally distributed throughout the cytoplasm where it is likely bound to small endosomal membranes. In the presence of amino acids, the Rag proteins drive mTORC1 to larger rab7+ vesicles. Rheb is also localized to these vesicles and, when activated by growth factors, energy, and oxygen availability, stimulates mTORC1.

An early mystery in the TOR field was that TOR2, but not TOR1, had some functionality that was sensitive to rapamycin and some that wasn't. The discovery of two distinct TOR-containing complexes, first described in a landmark paper by Loewith et al, resolved many of these questions (Loewith et al., 2002). Using immunopurification of epitope-tagged TOR1 and TOR2 and mass-spectrometry, these authors showed that all TOR1-interacting proteins also associated TOR2. These included the proteins LST8 and KOG1, which, together with TOR1 or TOR2, defined the rapamycin-sensitive TORC1. As described above, KOG1 is homologous to mammalian raptor and LST8 is homologous to mammalian GbL/mLST8. TOR2 additionally associated with the proteins AVO1 (homologous to human SIN1), AVO2 and AVO3, defining the rapamycin-insensitive TORC2. TORC2 also contains LST8, but not KOG1. Deletion of TORC2 components caused a defect in actin cytoskeleton assembly that was attributed to inhibition of ROM2, RHO1 and PKC1 signaling. Loewith et al also noted that Avo1 and Avo3 were orthologous to the Dictyostelium RIP3 and pianissimo, both of which had been implicated, albeit weakly, in mediating RAS signaling.

The discovery and characterization of mammalian TORC2 (mTORC2) came soon after with the identification of rictor as an mTOR-interacting protein (Sarbassov dos et al., 2004). Rictor is a 200 kDa protein that shows weak but definite homology to the budding yeast Avo3 and Dictyostelium pianissimo and, together with mTOR and mLST8, define mTORC2. Like yeast TORC2, disruption of mTORC2 using rictor-targeted RNAi caused a defect in actin cytoskeleton assembly that appeared to involve PKC. More recent work has since identified SIN1, the mammalian Avo1 ortholog, and protor/PRRL5 as additional mTORC2 components (Frias et al., 2006; Jacinto et al., 2006; Pearce et al., 2007; Thedieck et al., 2007). Although protor has no apparent function, mSIN1, like Avo1, is necessary for mTORC2 integrity. Interestingly, mSIN1 is expressed as three different isoforms that can all associate with mTORC2 and define three distinct mTORC2 complexes(Frias et al., 2006). Two of these contain a divergent, putative PH domain

at their C-terminus, suggesting that they may play a role in connecting mTORC2 to PI3K activity(Schroder et al., 2007). The third isoform lacks this domain and defines an mTORC2 that remains active in the absence of PI3K signaling, suggesting that it might have a unique function (Frias et al., 2006).

B. mTORC2 regulates the proliferation and survival kinase Akt/PKB

Aside from its connection to actin cytoskeleton assembly, very little was known about the role of mTORC2. This changed with the discovery that the serine/threonine kinase Akt/PKB was an mTORC2 substrate (Sarbassov et al., 2005). Akt/PKB is best known as the primary downstream effector of the PI3K/PTEN pathway, and mediates many of the consequences of insulin signaling. It is also hyper-activated in a wide variety of cancers, most commonly by loss of PTEN or mutational activation of PI3K, and has consequently been a protein of intense interest for many cancer researchers. Like S6K, Akt belongs to the AGC family of kinases and is expressed as three highly similar and functionally redundant isoforms. Akt1 and Akt2 are expressed ubiquitously, while Akt3 is preferentially expressed in the brain and testis (Hanada et al., 2004). S6K and Akt share several structural similarities and are both regulated by mTOR by phosphorylation of C-terminal site known as the hydrophobic motif (T389 for S6K and S473 for Akt) (Figure 4). An additional C-terminal domain in S6K ensures that each mTOR complex only phosphorylates the proper substrate; deletion of this domain permits phosphorylation of S6K by both complexes (Ali and Sabatini, 2005).

Like most other AGC kinases, Akt activity is controlled through a two-part mechanism that requires phosphorylation of the hydrophobic motif, followed by phosphorylation of an internal "activation loop" site (T308) by the kinase PDK1. In the AGC kinases S6K, Rsk, SGK and typical PKCs, phosphorylation of the hydrophobic site is a prerequisite for phosphorylation of the activation loop site (Biondi et al., 2001). The

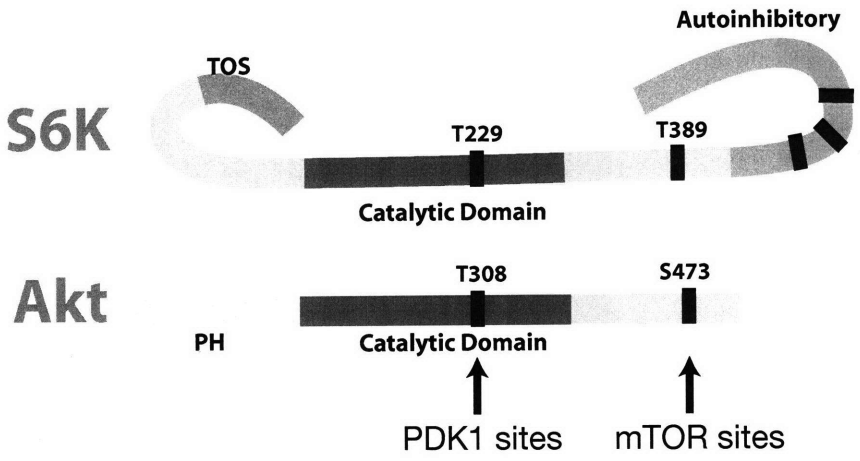

Figure 4. Structural homology between the mTOR substrates S6K and Akt

S6K and Akt are both members of the AGC family of kinases and substrates of the mTORC1 and mTORC2 complexes, respectively. mTOR phosphorylates these kinases at a C-terminal site known as the hydrophobic motif (T389 for S6K and S473 for Akt), while PDK1 phosphorylates the T-loop site within the catalytic domain. S6K contains an additional C-terminal domain that is thought to be an important determinant of its substrate selectivity for mTORC1.

situation is less clear in Akt: although S473 phosphorylation is required for full kinase activity and is generally co-regulated with T308, phosphorylation of either site appears to occur independently of the other (Alessi et al., 1996; Biondi et al., 2001). The physiologic consequence of this relationship is unclear.

The existence of three redundant Akt isoforms has complicated efforts to unravel its particular physiologic role. Generally, the evidence supports a diverse role in mediating the effects of PI3K activation by influencing metabolic regulation, cell survival and cell growth (Greer and Brunet, 2005). Akt accomplishes this primarily by phosphorylating and inhibiting several key targets, including the kinase GSK3, the pro-apoptotic protein BAD, the mTORC1 negative regulator TSC2 and the FoxO

transcription factors (Greer and Brunet, 2005). The FoxO proteins, in particular, are responsible for many of the phenotypes associated with suppressed PI3K signaling, and promote transcription of genes regulating cell cycle progression, apoptosis, metabolism and angiogenesis. Moreover, combined deletion of foxO1, foxO3 and foxO4 in mice leads to the development of lymphomas and hemangiomas, underscoring the role of this pathway in tumorigenesis (Paik et al., 2007; Tothova et al., 2007). However, the tumor spectrum is more constrained than what is caused by loss of PTEN, suggesting that Akt and/or mTORC2 can drive cancers through other downstream pathways as well (Guertin and Sabatini, 2007).

How mTORC2 is activated by PI3K remains an entirely open question. When mTORC2 is purified from insulin-stimulated cells, it retains increased activity in vitro towards Akt, suggesting a regulatory mechanism that involves a stable modification (Sarbassov et al., 2005). One hypothesis is that mSIN1, which contains a putative divergent C-terminal PH domain, binds to PIP3 following growth factor stimulation and coordinates activation of mTORC2 by another membrane-bound protein (Frias et al., 2006). However, there is currently no evidence that such a change in localization occurs. RIP3, the Dictyosetlium SIN1 ortholog, has also been shown to interact with RAS and contains a RAS-binding domain, though this remains to be shown whether this feature can influence mTORC2 activity (Lee et al., 2005; Lee et al., 1999; Schroder et al., 2007). Clarifying this connection between PI3K and mTORC2 is undoubtedly of great interest to those hoping to understand how growth factor signaling regulates cell processes, and may also present a new target for anti-cancer intervention.

C. The physiologic relationship between Akt/PKB and mTORC2

The physiologic role of mTORC2 is currently being explored using mice where various mTORC2 components have been knocked out. While deletion of mTOR or raptor

causes early embryonic lethality around the time of implantation, embryos lacking a functional rictor or mLST8 survive until mid-gestation, reflecting different developmental requirements for mTORC1 and mTORC2 (Guertin et al., 2006). Consistent with the connection between mTORC2 and angiogenesis, both rictor- and mLST8-null embryos appear to suffer from a severe vascular defect (Guertin et al., 2006). As expected, in mouse embryonic fibroblasts (MEFs) derived from these embryos, Akt S473 phosphorylation was completely ablated and its in vitro kinase activity was reduced to levels seen in wild-type serum-starved cells (Guertin et al., 2006). However, T308 remained phosphorylated at normal levels, suggesting that Akt might maintain a basal level activity. Indeed, while phosphorylation of some verified Akt targets such as FoxO was suppressed, others, such as TSC2 and GSK3, were unaffected.

The finding that loss of mTORC2 doesn't suppress many Akt-regulated pathways indicates a much more complicated relationship between these PI3K effectors than was previously understood. There are two probable explanations, neither of which is mutually exclusive. The first is that Akt might maintain a basal level of activity in the absence of S473 phosphorylation that allows it to phosphorylate some targets, but not others. The observation that Akt, but not TORC2, is required for viability in drosophila supports this idea insofar as it confirms a role for Akt that is independent of S473 phosphorylation (Hietakangas and Cohen, 2007; Staveley et al., 1998). Moreover, although drosophila TORC2 isn't required for viability, it can completely block the tissue overgrowth caused by a concomitant deletion of PTEN (Hietakangas and Cohen, 2007). Thus it is possible that mTORC2, and implicitly S473 phosphorylation, is only required for maximal Akt activation, and that PI3K and PDK1 alone are capable for maintaining a basal level of regulation.

A second possibility is that other AGC kinases that are stimulated by serum factors but don't depend on mTORC2 can redundantly activate many of the same pathways. For instance, Rsk, which is activated through an Erk-dependent mechanism

that is independent of PI3K, and S6K can both phosphorylate GSK3 at the S9 Akt site (Sutherland et al., 1993; Zhang et al., 2006). Likewise, SGK phosphorylates the same sites on FOXO that are also regulated by Akt(Greer and Brunet, 2005). The physiologic consequence of these redundant pathways isn't entirely clear, and it is puzzling that cells appear to compensate for mTORC2 inhibition in so many ways.

V. mTOR signaling in cancer

A. mTOR is a core member of the PI3K/PTEN signaling pathway

The mTOR complexes are at the crossroads of many classical oncogenic signaling pathways. The most prominent connection is to the PTEN/PI3K pathway, which is activated by growth factor signaling and is mutationally deregulated in a wide spectrum of cancers. PTEN alone is disrupted in 50-80% of sporadic cancers, which includes endometrial carcinoma, glioblastoma and prostate, and 30-50% of breast, colon and lung cancers (Salmena et al., 2008). Both mTOR complexes are hyper-activated by PTEN inactivation and are increasingly blamed for many of the subsequent signaling defects, such as dysregulation of Akt/PKB. Consequently, there has been much interest in mTOR inhibition as an anti-cancer strategy. Indeed, loss of PTEN has been associated with an increased sensitivity to mTORC1 inhibition (Neshat et al., 2001). Much of our current understanding of the anti-cancer efficacy of mTOR inhibition has come from use of rapamycin. Although rapamycin preferentially acts an mTORC1-specific inhibitor, a discussion of its successes and failures offers valuable insight into the effectiveness of mTOR inhibition as a more general strategy.

The best rationale for therapeutic inhibition of mTOR is in hamartoma syndromes such as tuberous sclerosis complex (TSC). A defining feature of TSC is the development of cerebral cortical tubers that are characterized by a disorganized structure and the presence of large astrocytes and a unique type of cell known as a giant cell (Crino et al.,

2006). These tubers are considered benign, but can nonetheless cause severe neurological dysfunction. Linkage analysis of multigenerational families established a connection to the TSC1 and TSC2 genes, which, as described above, are key repressors of mTORC1 signaling (Crino et al., 2006). The constitutive activation of mTORC1 that results from loss of TSC1/TSC2 function is thought to drive many of the pathological features of TSC, thus establishing a compelling argument for the therapeutic use of rapamycin. Clinical trials are currently underway and early results have been encouraging (Franz et al., 2006). Other pathologically similar syndromes (Cowden, Peutz-Jeghers, Neurofibromatosis, and Birt-Hogg-Dube synrome) are also characterized by the inactivation of genes that suppress mTORC1 signaling (pten, lkb1, nf1 and flcn, respectively) and might also respond well to treatments that target mTOR (Guertin and Sabatini, 2007).

A second class of cancers that offer a good molecular rationale for targeting mTORC1 are those characterized by overexpression of the eukaryotic initiation factor 4E (eIF4E). eIF4E coordinates the translation of a subset of mRNAs with extensive secondary structure in the 5' untranslated region and is frequently amplified in a diverse variety of cancers (Bjornsti and Houghton, 2004). Mild overexpression is sufficient to transform cells in culture, but, curiously, only in the presence of functional RAS signaling (Lazaris-Karatzas et al., 1992). Moreover, transgenic overexpression in mice causes a wide-spectrum of tumors, including lymphomas, angiosarcomas, lung adenocarcinomas, and hepatocellular adenomas (Ruggero et al., 2004). mTORC1, along with ERK1/2 and possibly other kinases, regulates eIF4E-dependant translation by phosphorylating and inactivating the eIF4E repressor, 4EBP1. Clinically, 4EBP1 phosphorylation negatively correlates with survival rates (Armengol et al., 2007).

eIF4E drives cancer growth by promoting the translation of several oncogenes, including cyclin D1 and c-myc. Interestingly, two cancers that are already known to respond well to rapamycin treatment, mantle cell lymphoma (MCL) and the familial cancer syndrome neurofibromatosis type-1(NF1), are characterized by overproduction

of cyclin D1(Johannessen et al., 2008; Williams and Densmore, 2005). MCL is a particularly aggressive non-Hodgkins lymphoma with the poorest prognosis among its subtypes (Williams and Densmore, 2005). Rapamycin causes a cell-cycle arrest in many MCL cell lines that appears to be mediated by nuclear accumulation of p27(Kip1) and, in some but not all cases, suppression of cyclin D1(Dal Col et al., 2008). NF1, which is caused by mutations in the RasGAP NF1 gene, is characterized by the development of highly metastatic malignant peripheral nerve sheath tumors (MPNSTs). Like MCL, rapamycin induces a nearly complete cell-cycle arrest in NF1 tumor cells that is accompanied by a suppression in cyclin D1 levels(Johannessen et al., 2008).

mTORC1 can also contribute to tumor growth by promoting angiogenesis. Activation of mTORC1 increases levels of the transcription factor hypoxia-induciple factor 1 alpha (HIF1alpha) by driving its expression and suppressing its degradation (Bernardi et al., 2006; Hudson et al., 2002). As HIF1a accumulates, it drives angiogenesis through expression of the vascular endothelial growth factor (VEGF). Rapamycin can reverse these effects, and has been shown to interfere with angiogenesis in mouse tumor models (Guba et al., 2002). This relationship predicts that rapamycin should be particularly effective in highly vascularized cancers, which appears to be true in several situations. Kaposi's sarcoma and certain sporadic kidney cancers, which show elevated VEGF signaling and increased HIF1a levels, respectively, are both responsive to rapamycin therapy (Guertin and Sabatini, 2007).

B. Shortcomings of rapamycin therapy and future hopes

Despite these successes, the most surprising conclusion from rapamycin clinical trials is that it has not been more effective. There are no clear explanations, but several hypotheses have been put forward. The first is that rapamycin might paradoxically drive tumor growth by disengaging the negative feedback loop that normally squelches insulin

signaling (Manning et al., 2005). The resulting hyper-activation of PI3K and mTORC2 could potentially overcome the suppressive effects of mTORC1 inhibition and, in the worst-case scenario, enhance tumor growth. A second possibility is that mTORC1 inhibition doesn't lead to apoptosis, and instead causes a cytostatic growth arrest that is more easily overcome by compensatory signaling pathways. In NF1 cells and tumor models, rapamycin causes a profound growth arrest, but cells rapidly re-enter the cell cycle when rapamycin is removed (Johannessen et al., 2008). It isn't a stretch to believe that other tumor types might resist mTORC1 inhibition by activating other pathways that overcome this arrest.

A second hypothesis was prompted by the finding that rapamycin can inhibit mTORC2 in certain situations, and that this might be the true explanation for its success against some cancers and not others. Although acute rapamycin treatment is selective for mTORC1, prolonged treatment can also inhibit mTORC2 in some, but not all, cell types (Sarbassov et al., 2006). The mechanism of inhibition appears to involve disruption of mTORC2 assembly. In sensitive cell lines, prolonged rapamycin treatment causes a complete depletion of functional mTORC2 and a total loss of Akt phosphorylation at S473. Insensitive cell lines also suffer a substantial reduction in functional mTORC2, but retain a small amount of complex that, surprisingly, is capable of maintaining wild-type levels of pathway activity. Two important unanswered questions are why does rapamycin completely inhibit mTORC2 in only some cell lines and why is such a small amount of mTORC2 able to maintain normal levels of activity in insensitive cell lines?

Additional insight has come from the recent discovery that CCI-779, a rapamycin analog, can inhibit both complexes directly in an FKBP12-independent manner at high but clinically relevant concentrations (Shor et al., 2008). This high concentration had remarkably enhanced anti-proliferative activity against a broad panel of tumor cell lines, including cell lines that were resistant to low dose rapamycin treatment. Like low dose rapamycin treatment, high-doses were generally cytostatic, although pten-null tumor

cell lines showed a small but significant increase in apoptosis. Interestingly, the authors observed a profound global reduction in protein synthesis that correlated with inactivation of elongation factor eEF2 and reasoned that the combined effect of this and inhibition of S6K and eIF4E might explain the enhanced anti-proliferative effect.

Together, these clues suggest that mTORC2 might be the more effective clinical target. In many respects, there is a clearer molecular rationale for targeting mTORC2 because of its role as a primary effector of PTEN/PI3K signaling and a regulator of Akt/PKB. Pharmacologically, the easiest approach to inhibiting both complexes is with a general mTOR ATP-competitive kinase inhibitor. A caveat is that, as with rapamycin, mTORC1 inhibition disengages the feedback loop and results in hyper-activation of PI3K, which may interfere with inhibition of mTORC2. A more ideal solution would be to create an mTORC2-specific inhibitor, though this is a much more difficult task. Possible strategies include identifying and targeting the function of mTORC2 components and inhibiting mTORC2 assembly. It is also possible that dual inhibition of mTOR and PI3K can further blunt the effects of feedback inhibition, and such inhibitors are already in clinical trials. Given the high levels of cross-talk between the mTOR pathways and the RAS/MAPK pathway, it will also be interesting to see whether combined inhibition can elicit a synergistic response.

VI. Conclusions

For an evolutionarily conserved regulator of cell growth, the role of mTOR signaling in normal physiology is a surprisingly subtle one. In mice, both complexes are clearly essential during development. However, the mild side effects of rapamycin treatment in both mice and humans and the viability of rictor-null drosophila suggest that mTOR inhibition might be well tolerated in adult tissues. There is some data to suggest that drosophila TORC2 is only required for transmitting amplified PI3K signaling, and

that, in its absence, Akt maintains a limited but sufficient basal activity (Hietakangas and Cohen, 2007). Obviously, flies and mammals are not the same thing, but the observation that many Akt substrates remain phosphorylated in rictor-null MEFs supports this model (Guertin et al., 2006). The implication is that mTOR signaling would be essential during periods of rapid growth, such as development and hematopoeitic expansion, but less important in most relatively quiescent adult tissues.

In contrast with this role in normal tissue, deranged amplification of mTORC1 or mTORC2 signaling has clear pathological consequences. Activation of mTORC1 by loss of TSC proteins and activation of mTORC2 by loss of PTEN are fundamental initiating events in many cancers. As the defining feature of tumors cells is unmitigated growth and proliferation, it is not surprising that hyper-activation of pathways that drive nutrient uptake, increases in translational capacity and angiogenesis would confer a substantial advantage. Moreover, the proposition that excessive levels of nutrients cause insulin insensitivity by activating mTORC1 and engaging the S6K-dependent feedback loop suggests a role in metabolic diseases as well. From a therapeutic perspective, a pathway that is normally dispensable but essential for aberrant growth is a promising target.

As the molecular composition and regulation of mTORC1 and mTORC2 are understood more completely, the challenge is shifting to discovering the physiologic roles of these pathways. What are the important downstream effectors and what processes do they regulate? In animals, is this a tissue specific arrangement? What metabolites does mTOR actually sense, and do unnaturally high concentrations sufficiently alter mTOR signaling to cause disease? If mTOR mediates the balance between nutrient availability and nutrient demand, how does it cooperate with pathways that regulate cell proliferation, like Ras/MAPK? In cancers, can these relationships be exploited to identify inhibitors that synergistically cause apoptosis? Creative use of new tools, such as conditional knockout mice and mTORC1/2 and mTORC2-specific inhibitors, will be key to answering these questions.

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Chapter 2

PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase

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Experiments in Figure 6 were performed by R.A.L.
Experiments in Figures 7A, 7B, and 7C were performed by C.C.T.
Experiments in Figure 7D were performed by T.R.P.

Summary

The heterotrimeric mTORC1 protein kinase nucleates a signaling network that promotes cell growth in response to insulin and becomes constitutively active in cells missing the TSC1 or TSC2 tumor suppressors. Insulin stimulates the phosphorylation of S6K1, an mTORC1 substrate, but it is not known how mTORC1 kinase activity is regulated. We identify PRAS40 as a raptor-interacting protein that binds to mTORC1 in insulin-deprived cells and whose in vitro interaction with mTORC1 is disrupted by high salt concentrations. PRAS40 inhibits cell growth, S6K1 phosphorylation, and rheb-induced activation of the mTORC1 pathway, and in vitro prevents the great increase in mTORC1 kinase activity induced by rheb1-GTP. Insulin stimulates Akt/PKB-mediated phosphorylation of PRAS40, which prevents its inhibition of mTORC1 in cells and in vitro. We propose that the relative strengths of the rheb- and PRAS40-mediated inputs to mTORC1 set overall pathway activity and that insulin activates mTORC1 through the coordinated regulation of both.

Introduction

The evolutionarily conserved TOR pathway is a critical controller of growth in eukaryotes, regulating cell as well as organ and body size in a variety of organisms (reviewed in (Sarbassov et al., 2005; Wullschleger et al., 2006)). The pathway was discovered in studies into the mechanism of action of rapamycin, an immunosuppressive and anti-restenosis drug that is also in clinical trials as a cancer therapy. The mammalian TOR (mTOR) pathway integrates signals from growth factors, nutrients, and stresses to regulate many growth-related processes, including mRNA translation, small molecule metabolism, cell survival, and autophagy. Emerging evidence indicates that deregulation of the mTOR pathway occurs in common diseases, including cancer and diabetes, underscoring the importance of identifying and understanding the function of the components of the mTOR signaling network.

The central component of the pathway, the large protein kinase mTOR, nucleates two distinct multi-protein complexes called mTOR Complex 1 (mTORC1) and 2 (mTORC2) (reviewed in (Sabatini, 2006)). mTORC1 is a heterotrimer consisting of the mTOR catalytic subunit and two associated proteins, raptor and mLST8/GβL. mTORC2 also contains mTOR and mLST8/GβL, but, instead of raptor, the rictor and mSin1 proteins. The molecular functions of most mTOR-associated proteins are not understood, but raptor has been proposed to be a docking site for substrates on mTORC1 and to regulate mTOR kinase activity (Hara et al., 2002; Kim et al., 2002; Nojima et al., 2003; Schalm et al., 2003). The best-characterized downstream effectors of mTORC1 are S6 Kinase 1 (S6K1) and 4E-BP1, two translational regulators that mTORC1 directly phosphorylates (Brunn et al., 1997; Burnett et al., 1998; Gingras et al., 2001).

As judged by the phosphorylation state of S6K1 or 4E-BP1, the mTORC1 pathway senses many upstream signals, including growth factors like insulin as well as environmental nutrient levels. The mechanisms underlying mTORC1 regulation are

not well understood but a key upstream player is clearly the GTP-binding protein rheb, which is negatively regulated by the dimeric TSC1-TSC2 GTPase Activating Protein (GAP) (Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003a; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2002; Tee et al., 2003b; Zhang et al., 2003). When TSC1 or TSC2 is lost, such as in the cancer-prone syndrome Tuberous Sclerosis Complex (TSC), rheb becomes constitutively loaded with GTP, rendering the mTORC1 pathway active and insensitive to insulin. It is now appreciated that several pathways in addition to the insulin-stimulated PI3K/Akt pathway signal to mTORC1 by modulating TSC1-TSC2 activity towards rheb. For example, energy deprivation sensed by AMPK (Inoki et al., 2003b), MAPK signaling (Ma et al., 2005; Roux et al., 2004; Tee et al., 2003a), and hypoxia and stress sensing (Brugarolas et al., 2004; Corradetti et al., 2005; Reiling and Hafen, 2004) all regulate TSC1-TSC2 activity and the GTP-loading of rheb. Within cells rheb overexpression strongly activates the mTORC1 pathway and the overexpressed protein binds to the mTOR kinase domain (Long et al., 2005). However, it is still not clear if endogenous rheb acts by binding directly to mTORC1 or if it requires an unknown intermediate.

Here, we identify PRAS40 as a raptor-binding protein that potently inhibits mTORC1 kinase activity in vitro and mTORC1 signaling within cells. Insulin-stimulated phosphorylation of PRAS40 by Akt/PKB suppresses its mTORC1 inhibitory activity. Thus, we propose that insulin activates mTORC1 through the coordinated regulation of rheb, an mTORC1 activator, and PRAS40, an mTORC1 inhibitor.

Results

A salt-sensitive factor inhibits the kinase activity of mTORC1

We previously developed protocols for immunopurifying intact mTORC1 and an in vitro assay for measuring its kinase activity towards full length S6K1 (Guertin et al., 2006). Surprisingly, this assay does not detect any difference between the activity of mTORC1 from serum-deprived and insulin-stimulated HEK-293E cells, even

though insulin robustly increases S6K1 phosphorylation in these cells (Figure 1A). We speculated that an unknown factor important for conferring insulin sensitivity is lost from mTORC1 during the washing of immunoprecipitates with buffers containing high salt concentrations. Indeed, when washed with buffers containing 150 but not 300 or 400 mM NaCl, mTORC1 from insulin-stimulated cells has much higher activity than that from serum-deprived cells (Figure 1B). By avoiding high salt buffers we showed that insulin also activates endogenous mTORC1 from HeLa cells and mouse embryonic fibroblasts (MEFs) (Figure 1C), as well as mTORC1 containing recombinant HA-raptor stably expressed in HEK-293E cells (Figure 1D). High salt washing eliminates the insulin-induced difference in mTORC1 activity by increasing the activity of mTORC1 from serum-deprived cells (Figure 1B). This indicates that a salt-sensitive factor inhibits mTORC1 during insulin deprivation and suggests that loss or repression of this factor participates in activating mTORC1 in response to insulin.

Inactivation of the TSC1 or TSC2 tumor suppressor leads to insulin-insensitive and constitutively high S6K1 phosphorylation (Garami et al., 2003; Inoki et al., 2002; Manning et al., 2002; Zhang et al., 2003). Paradoxically, however, we find that the in vitro kinase activity of mTORC1 from TSC2-null MEFs treated with or without insulin is very repressed, being similar in magnitude to that of mTORC1 from wild-type MEFs deprived of insulin (Figure 1E). Substantial evidence (Harrington et al., 2004; Shah et al., 2004) indicates that hyperactive mTORC1 signaling down-regulates the insulin/PI3K pathway so that TSC2-null MEFs are in a state equivalent to insulin-deprivation. Consistent with this, high salt washes activate mTORC1 from TSC2-null MEFs (Figure 1E), just as they do mTORC1 from insulin-deprived wild-type MEFs (Figure 1E) or HEK-293E cells (Figure 1B). Thus, in TSC2-null MEFs an mTORC1 activator must exist that overcomes repression of mTORC1 by the salt-sensitive inhibitor and leads to the hyperactive mTORC1 signaling characteristic of these cells. Such an activator is likely lost from mTORC1 during its purification, explaining the low in vitro

activity of mTORC1 from TSC2-null cells.

An obvious candidate for such an mTORC1 activator is rheb—the small GTPase that becomes constitutively loaded with GTP in the absence of TSC1 or TSC2. Previous studies show that overexpressed rheb1 strongly activates the mTORC1 pathway and binds to the mTOR kinase domain (Long et al., 2005). We confirmed these studies (data not shown) but, despite considerable effort, we were unable to detect endogenous rheb bound to immunopurified mTORC1 (data not shown), probably because the rheb-mTORC1 interaction is transient and too weak to survive even the gentlest immunopurifications. Thus, to determine if rheb can activate mTORC1 in vitro, we added GTP-loaded rheb1 or control proteins to mTORC1 immunopurified under low salt conditions and measured its kinase activity towards S6K1. Rheb1-GTP, but not rheb1-GDP, rap2a-GTP, or rap2a-GDP, dramatically activated endogenous (Figure 2A) or recombinant (Figure 2B) mTORC1 from serum-deprived HEK-293E cells and also boosted the activity of mTORC1 from insulin-stimulated cells (Figure 2C). Rheb1-GTP also strongly activated mTORC1 from TSC2-null MEFs (Figure 2D). Lastly, rheb1-GTP also activated mTORC1 kinase activity towards 4E-BP1 (Figure 2E). To our knowledge these are the first demonstrations that soluble GTP-loaded rheb1 directly activates mTORC1 in vitro. We conclude that rheb1-GTP can overcome, like high salt washes, the suppression of in vitro mTORC1 activity caused by insulin-deprivation or TSC2-null status, suggesting that rheb1-GTP can counter the actions of the salt-sensitive inhibitor.

PRAS40 is a raptor-binding protein that interacts with mTORC1

To identify the salt-sensitive inhibitor of mTORC1 we searched for proteins that co-purify with mTORC1 when raptor or mTOR immunoprecipitates are washed with buffers containing low but not high salt concentrations. This led to the identification by mass spectrometry of PRAS40, a 28 kDa proline-rich protein lacking named domains but which is known to be phosphorylated near its C-terminus by Akt/PKB (Kovacina et al.,

2003) and potentially other kinases (Huang and Porter, 2005) (Figure 3A). Interestingly, the gene for PRAS40 (called AKT1S1) is located at chromosome 19q13.33, a region thought to contain an unknown tumor suppressor associated with gliomas (Hartmann et al., 2002). PRAS40 is not conserved in budding or fission yeast but has a putative *Drosophila* orthologue named Lobe (Chern and Choi, 2002).

Using immunoblot analyses of raptor immunoprecipitates we confirmed that PRAS40 co-purifies with endogenous mTORC1, but not a control protein, when it is isolated under low salt conditions and that high salt washes greatly reduce the amount of PRAS40 bound to mTORC1 (Figure 3B). In HEK-293E cells, acute insulin stimulation of serum-deprived cells decreases the amount of PRAS40 bound to mTORC1 without affecting its expression level (Figure 3B). mTOR is part of two distinct complexes within cells, mTORC1 and mTORC2, but PRAS40 only interacts with mTORC1 (Figure 3C).

Endogenous PRAS40 interacts, in an insulin-sensitive fashion, with mTORC1 containing recombinant raptor (Figure 3D), suggesting that recombinant proteins can be used to identify the PRAS40 binding site on mTORC1. To do so, we co-expressed flag-PRAS40 with HA-raptor and/or myc-mTOR and isolated mTORC1 using anti-HA or anti-myc immunoprecipitations (Figure 3E). PRAS40 robustly co-immunoprecipititates with HA-raptor and the co-expression of myc-mTOR did not significantly increase the amount of PRAS40 recovered with HA-raptor. While relatively small amounts of PRAS40 co-immunoprecipitate with myc-mTOR, the co-expression of HA-raptor significantly increases the amount of PRAS40 recovered with myc-mTOR. These results indicate that PRAS40 preferentially binds the raptor component of mTORC1 and are consistent with PRAS40 not binding to mTORC2, which does not contain raptor. PRAS40 binds better to the N- than C-terminal half of raptor but the degree of binding obtained to all raptor fragments tested is much less than that to full-length raptor (data not shown). Consistent with PRAS40 binding to raptor, in cells with RNAi-induced partial decreases in raptor or mTOR expression, the amount of PRAS40 recovered in raptor or

mTOR immunoprecipitates correlates with the amount of raptor but not mTOR in the immunoprecipitates (Figure 3F).

Because previous work (Kovacina et al., 2003) indicates that PRAS40 is phosphorylated on T246 in an Akt/PKB-dependent fashion we asked how insulin affects the phosphorylation status of PRAS40 bound to mTORC1 (Figure 3G). As expected, lysates of HEK-293E and HeLa cells treated with insulin had much greater levels of phosphorylated PRAS40 than lysates of serum-deprived cells. Despite the fact that insulin decreases the amount of PRAS40 bound to mTORC1 in HEK-293E and HeLa cells, the PRAS40 that remains bound is highly phosphorylated. Thus, PRAS40 phosphorylation does not preclude the binding of PRAS40 to mTORC1 although it may weaken the interaction. This is consistent with the results of similar experiments in wild-type MEFs, where insulin causes a large increase in the phosphorylation of the PRAS40 that is bound to mTORC1 but only a modest decrease in the amount of total PRAS40 bound to mTORC1 (Figure 3G). In TSC2 null MEFs insulin does not increase PRAS40 phosphorylation, as expected from the profound inhibition of insulin signaling and Akt/PKB activity in these cells (Figure 3G). The fact that PRAS40 phosphorylation on T246 positively correlates with the in vitro activation of mTORC1 by insulin suggests that this phosphorylation event may promote mTORC1 activation.

Phosphorylation of PRAS40 at T246 is unlikely to be a major component of the mechanism through which amino acids signal to mTORC1. Leucine stimulation of wild-type and TSC2 null MEFs and HEK-293E cells only had small effects on the phosphorylation of PRAS40 at T246 and the amount of PRAS40 bound to mTORC1 (Supplemental Figure S1).

PRAS40 is an inhibitor of mTORC1 in vitro

To begin to investigate potential roles for PRAS40 in the regulation of the mTORC1 pathway we asked if it affects mTORC1 kinase activity in vitro. We

have described three ways of obtaining active mTORC1: (1) by washing mTORC1 from serum-deprived cells with buffers containing high salt concentrations; (2) by immunopurifying mTORC1 from cells stimulated with insulin; or (3) by adding rheb1-GTP to mTORC1 from serum-deprived cells. In the experiments described below we tested the in vitro effect of PRAS40 on mTORC1 activity using recombinant PRAS40 overexpressed in and purified from HEK-293T cells. In a dose sensitive fashion PRAS40, but not a control protein, inhibited the in vitro kinase activity of mTORC1 activated with high salt washes (Figure 4A) or insulin stimulation (Figures 4B and 4F). PRAS40 is a potent inhibitor, with half maximal inhibition occurring at or below 20 nM. PRAS40 also blocks the massive activation of mTORC1 caused by 100 nM GTP-loaded rheb1 (Figure 4C), and, as is the case with activation caused by high salt washes or insulin stimulation, half maximal inhibition occurred at around 20 nM PRAS40 (Figure 4D). Thus, PRAS40 is a potent inhibitor of mTORC1 and accounts for the salt-sensitive inhibition of mTORC1 we observe.

It is clear that PRAS40 can inhibit rheb1-GTP activation of mTORC1, but several of the findings we have presented also strongly suggest that at high concentrations rheb1-GTP can overcome PRAS40-mediated inhibition of mTORC1. For example, the addition of rheb1-GTP to mTORC1 from serum-deprived (Figures 3ABC) or TSC2 null (Figure 3D) cells—two situations where PRAS40 is bound to mTORC1—stimulates mTORC1 activity. To formally prove that rheb1-GTP can reverse PRAS40-mediated inhibition of mTORC1, we incubated mTORC1 with a constant amount of PRAS40 (40 nM) and increasing amounts of rheb1-GTP and measured mTORC1 kinase activity. Indeed, in a dose sensitive fashion rheb1-GTP re-activates PRAS40-inhibited mTORC1 (Figure 4E). 40 nM PRAS40 completely inhibits mTORC1 obtained from insulin-stimulated cells and a 9-fold higher concentration (360 nM) of rheb1-GTP is required to restore mTORC1 activity. These results indicate that at elevated levels of rheb1-GTP—as would be expected in TSC2 null cells—the molar ratio of rheb1-GTP to PRAS40 is sufficiently

high to overcome inhibition of mTORC1 by PRAS40.

PRAS40 is an inhibitor of the mTORC1 pathway in mammalian cells

To determine if the in vivo correlates of our in vitro findings are true, we began by using overexpression to test the effects of PRAS40 on the mTORC1 pathway within cells. In HEK-293E cells transient overexpression of PRAS40, but not a control protein, blocks the insulin-induced phosphorylation of T389 on co-expressed S6K1 (Figure 5A). Furthermore, co-expression of PRAS40 with rheb1 eliminates the very large increase in S6K1 phosphorylation that is normally caused by the overexpression of rheb1 in HEK-293E and HEK-293T cells (Figure 5B). When stably overexpressed using retroviral transduction, PRAS40 reduces cell size in HEK-293E cells (Figure 5C). Moreover, stable overexpression of PRAS40 in TSC2 null MEFs reduces the enlarged cell phenotype of these cells (Figure 5C). Thus, as in vitro, PRAS40 overexpression within cells strongly inhibits the mTORC1 pathway.

Because PRAS40 overexpression is sufficient to inhibit the mTORC1 pathway, we asked if endogenous PRAS40 normally has an inhibitory function within cells. Our expectation was that if PRAS40 plays a role in the mTORC1 pathway inhibition that occurs in insulin-deprived cells, a loss of PRAS40 expression should boost the phosphorylation state of S6K1 even in the absence of insulin. Indeed, using two independent PRAS40-directed lentivirally expressed shRNAs, knockdowns of PRAS40 in wild-type MEFs and in human HT-29 colon cancer cells increased the phosphorylation of S6K1 in serum-deprived cells (Figures 5D and 5E). The increase in S6K1 phosphorylation caused by the PRAS40 knockdown was not as great as that caused by insulin stimulation, but this is not surprising because in the absence of insulin cells are missing the mTORC1-activating input coming from rheb. In addition, the mTORC1 and S6K1 activation caused by the PRAS40 knockdown should trigger feedback inhibition of PI3K/Akt signaling, which will suppress rheb and thus limit mTORC1 activation. A

knockdown of PRAS40 expression only slightly increased the phosphorylation of S6K1 in cells growing in the presence of insulin (Figure 5E), suggesting that insulin represses the inhibitory properties of PRAS40.

Lobe is an inhibitor of the dTORC1 pathway in *Drosophila* cells

The inhibitory function of PRAS40 on the mTORC1 pathway is conserved in *Drosophila* tissue culture cells. Transfection into S2 cells of two distinct non-overlapping RNAi-inducing dsRNAs against Lobe, the PRAS40 orthologue, increased dS6K phosphorylation and cell diameter, although not to as large of an extent as a dsRNA targeting dTSC2 (Figures 6AB). In Kc167 cells, the sequential knockdown of dTOR and then Lobe blocks the increase in dS6K phosphorylation caused by the solo knockdown of Lobe, consistent with Lobe being upstream of dTOR (Figure 6C). In contrast, Lobe function does not depend on dRheb, as the sequential knockdown of Lobe and then dRheb partially restores the levels of dS6K phosphorylation compared to cells having a knockdown of dRheb alone. Using cell size as a phenotype, we next used the most active Lobe dsRNA to place within the dTOR pathway Lobe with respect to dRheb and dTOR (Figure 6D). The sequential knockdown of dRheb and then Lobe did not alter the approximately 10% increase in mean cell volume caused by the knockdown of Lobe alone, which is consistent with Lobe function not depending on dRheb. On the other hand, the sequential knockdown of dRheb and then dTSC2 completely blocked the increase in cell size caused by the knockdown of dTSC2 alone, in accord with the established placement of dRheb downstream of dTSC2 (Gao et al., 2002; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003). The sequential knockdown of dTOR and then either Lobe or dTSC2 eliminated the increase in cell size caused by the knockdown of Lobe or dTSC2 alone (Figure 6D). This is consistent with dTOR being downstream of both Lobe and dTSC2 and required for the cell size increases caused by the Lobe and dTSC2 knockdowns. We conclude that in mammalian and *Drosophila* cells PRAS40

negatively regulates the TORC1 pathway.

Akt/PKB-mediated phosphorylation of PRAS40 blocks its inhibitory activity towards mTORC1

Insulin-stimulated activation of the in vitro kinase activity of mTORC1 correlates with an increase in the T246 phosphorylation of the PRAS40 bound to mTORC1, suggesting that this phosphorylation event may relieve the inhibitory action of PRAS40 on mTORC1 (Figures 1 and 3G). As Akt/PKB is an insulin-stimulated kinase that phosphorylates PRAS40 on T246 (Kovacina et al., 2003), we hypothesized that Akt/PKB activates mTORC1 by phosphorylating and suppressing PRAS40. The in vitro addition of active T308D Akt/PKB and ATP to mTORC1 obtained from serum-deprived cells substantially increases mTORC1 kinase activity (Figure 7A). Consistent with a key role for PRAS40 in this activation, Akt/PKB does not activate mTORC1 that has been washed with buffers containing high salt concentrations, conditions that strip off PRAS40 (Figure 3B). To directly test the role of PRAS40 phosphorylation on mTORC1 activity, we generated phosphorylated PRAS40 by incubating it with active Akt/PKB and ATP. As a control we mock phosphorylated PRAS40 by incubating it with active Akt/PKB in the absence of ATP. As before (Figure 4A), the addition of 40 nM non-phosphorylated PRAS40 strongly inhibited the activity of mTORC1 washed with buffers containing high salt concentrations while, in contrast, the equivalent amount of phosphorylated PRAS40 had a much reduced inhibitory effect (Figure 7B). These results suggest that although 14-3-3 proteins interact with phosphorylated PRAS40 (Kovacina et al., 2003), the binding of 14-3-3 to PRAS40 is not necessary to repress the inhibitory function of PRAS40 on the in vitro kinase activity of mTORC1. Of course, it is possible that 14-3-3 does have a necessary role within cells that we do not detect in vitro.

To confirm the role of PRAS40 phosphorylation in regulating mTORC1 within cells, we co-expressed in HEK-293E cells an HA-GST-S6K1 reporter together with low

amounts of wild-type or T246A mutant PRAS40 and stimulated the cells with insulin (Figure 7C). As expected, insulin strongly boosted the T389 phosphorylation of the S6K1 reporter and the low amounts of co-expressed wild-type PRAS40 only weakly diminished this phosphorylation. On the other hand, the expression of the T246A mutant of PRAS40, completely blocked insulin stimulated phosphorylation of T389 of S6K1 (Figure 7C). We obtained equivalent results in a conceptually similar experiment in which we used the expression of constitutively active Akt/PKB (myr-Akt) instead of insulin to promote S6K1 phosphorylation (Figure 7D). As with insulin, the expression of low amounts of T246A, but not wild-type, PRAS40 mutant blocked the increase in T389 phosphorylation caused by the expression of constitutively active Akt/PKB. Thus, both in vitro and within cells, Akt/PKB mediated phosphorylation of PRAS40 represses its inhibitory function.

Discussion

A frustrating aspect of studying mTORC1 has been the difficulty of preserving its regulation in vitro. Even when isolated from cells with vastly different levels of mTORC1 signaling (like serum deprived and insulin stimulated cells), the mTORC1 obtained through most purification protocols exhibits at best modest differences in mTORC1 kinase activity. Using a newly developed purification protocol and mTORC1 kinase assay, we provide two findings that help explain this discrepancy. First, the in vitro addition of soluble rheb1-GTP to mTORC1 dramatically activates mTORC1 kinase activity, but even the gentlest purification schemes do not preserve the interaction between endogenous mTORC1 and rheb. Therefore, in cellular states where rheb plays a major role in activating the mTORC1 pathway—like in insulin stimulated cells—the in vitro kinase activity of mTORC1 will be artificially low and not reflect true mTORC1 activity within cells. Second, we identify PRAS40 as a raptor-binding protein that inhibits mTORC1 activity in vitro and mTORC1 signaling within cells. PRAS40 is largely lost from mTORC1 under purification conditions in which its core, evolutionarily-conserved components—mTOR, raptor, and mLST8/GβL—remain together. Thus, as PRAS40

negatively regulates the mTORC1 kinase, its loss artifactually increases the in vitro activity of mTORC1 from insulin-deprived cells.

The fact that both PRAS40 and rheb-GTP strongly regulate mTORC1 activity raises the question of which protein is the predominant regulator of mTORC1 in cells. Our overexpression and in vitro work shows that either regulator is capable of overcoming the effects of the other. However, in normal cells insulin-stimulated Akt/PKB signaling aligns the activities of PRAS40 and rheb so that both push mTORC1 activity in the same direction (Figure 7E). That is, in insulin treated cells, phosphorylated PRAS40 does not repress mTORC1 while GTP-loaded rheb activates it. In serum-deprived cells, dephosphorylated PRAS40 represses mTORC1 while GDP-loaded rheb does not activate it. So far, we know of only one signaling state, that of TSC2 null MEFs, in which PRAS40 and rheb push mTORC1 activity in opposite directions (Figure 7E). In these cells, rheb is constitutively loaded with GTP while PRAS40 is in its dephosphorylated inhibitory state because of suppressed insulin signaling. The high and constitutive activity of mTORC1 within TSC2 null MEFs indicates that hyperactive rheb can overcome PRAS40-mediated inhibition of mTORC1. Interestingly, the battle between PRAS40 and rheb1-GTP can be observed in vitro. The kinase activity of mTORC1 purified from TSC2 null MEFs under conditions that preserve the PRAS40 interaction is very low, but can be activated by the in vitro addition of rheb1-GTP or removal of PRAS40.

The relative strengths of the rheb and PRAS40 inputs to the mTORC1 pathway may vary in different cell types depending on the activity and abundance of each protein. In the serum-deprived cells we have examined, partial depletion of PRAS40 has modest activating effects on the mTORC1 pathway, presumably because in the absence of insulin the positive signal from rheb to mTORC1 is missing. Although our work has focused on the coordinated regulation of rheb and PRAS40 by insulin, it is likely that pathways exist that can signal to PRAS40 and rheb independently, allowing cells to fine tune mTORC1 activity to specific environmental conditions.

A recent parallel study also identifies PRAS40 as an mTORC1 interacting protein (Haar et al., 2007) and focuses on its role in modulating the feedback loop between mTORC1 and the IRS/PI3K pathway. While this study shows that PRAS40 suppresses mTORC1 signaling within cells, our in vitro data reveals that PRAS40 is a direct inhibitor of the mTORC1 kinase and that it antagonizes the activation of mTORC1 caused by rheb1-GTP. We also come to differing conclusions on a number of important points. First, a key question is whether PRAS40 or rheb1 plays the dominant role in establishing mTORC1 activity within cells. Haar et al. conclude from experiments based on tissue culture cells that PRAS40 has dominant effects over the TSC1/TSC2-rheb axis. However, our results with TSC2-null MEFs and in vitro kinase assays suggest that the situation is more complicated, and that either component can overcome the effects of the other when sufficiently activated. Second, our in vitro data suggests that 14-3-3 binding is not required for the Akt/PKB mediated repression of PRAS40, though we acknowledge that 14-3-3 may play a role in insulin stimulation of mTORC1 within cells. Third, Haar et al. describe PRAS40 as an mTOR-binding protein, but we find clear evidence for preferential binding to raptor, a result that is more consistent with the finding that PRAS40 binds only to mTORC1. Fourth, we have been unable to observe any effect of amino acid signaling on the mTORC1-PRAS40 interaction, and suspect that different cell lysis conditions between our two studies accounts for this discrepancy. In contrast to our previous work (Kim et al., 2002), we have now developed mild lysis and purification conditions under which the integrity of the mTOR-raptor interaction is not affected by changes in environmental leucine levels. Haar et al. do not examine the effects of leucine on PRAS40 phosphorylation and we believe that the leucine-induced decrease in the amount of PRAS40 recovered in mTOR immunoprecipitates correlates with a similar decrease in the amount of raptor bound to mTOR.

Given the complexity of the mTORC1 pathway and the presence of feedback loops between components, it is likely that a full understanding of the physiologic

consequences of altering the rheb-PRAS40 balance will require the development of animal models overexpressing or missing these proteins.

Experimental Procedures

Materials

Reagents were obtained from the following sources: antibodies to raptor, human PRAS40, and multi species phospho-T246 PRAS40, as well as GTPγS, GDP and T308D Akt/PKB from Upstate/Millipore; an antibody to mouse PRAS40 from Biosource; antibodies to mTOR, β-catenin, and S6K1 as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, phospho-T37/T46 4E-BP1, phospho-S473 Akt/PKB, phospho-T398-dS6K, phospho-S505 dAkt, Akt (pan), 4E-BP1, and the myc epitope from Cell Signaling Technology; an antibody to HA from Bethyl laboratories; FLAG M2 affinity gel, FLAG M2 antibody, ATP, and human recombinant insulin from Sigma Aldrich; protein G-sepharose and immobilized glutathione from Pierce; DMEM from SAFC Biosciences; LY294002 from Calbiochem; PreScission protease from Amersham Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; 4E-BP1 from A.G. Scientific; and SimplyBlue Coomassie G, Schneider's medium, Drosophila-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Cell Lines and Tissue Culture

The HEK-293E cell line was kindly provided by John Blenis (Harvard Medical School). $p53^{-/-}/TSC2^{-/-}$ as well as $p53^{-/-}/TSC2^{+/+}$ mouse embryonic fibroblasts (MEFs) were kindly provided by David Kwiatkowski (Harvard Medical School). Cell lines were cultured in the following media: HEK-293E, HEK-293T, HeLa, and HT-29 cells and MEFs in DMEM with 10% IFS. HEK-293E and HEK-293T cells express E1a and SV40 large T antigen, respectively. In HEK-293E, but not HEK-293T, cells the mTORC1

pathway is strongly regulated by serum and insulin.

Cell Lysis and Immunoprecipitations

Cells rinsed once with ice-cold PBS were lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS, and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 hours at 4°C. 60 μl of a 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hour. Immunoprecipitates were washed three times each with low salt wash buffer (40 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS). When specified, wash buffers contained the indicated increased concentrations of NaCl. Immunoprecipitated proteins were denatured by the addition of 20 μl of sample buffer and boiling for 5 minutes, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting as described (Kim et al., 2002).

In Vitro Kinase Assay for mTORC1 Activity

For kinases assays, immunoprecipitates were washed 3 times in low salt wash buffer, or once in low salt wash buffer followed by two additional washes in buffers containing the NaCl concentrations indicated in the figures. Immunoprecipates were then washed twice in 25 mM HEPES [pH 7.4], 20 mM potassium chloride. Kinase assays were performed for 20 min at 30°C in a final volume of 15 μl consisting of mTORC1 kinase buffer (25 mM Hepes [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$, 250 μM ATP) and 150 ng inactive S6K1 or 4E-BP1 as the substrate. Reactions were stopped by the addition of 30 μl of sample buffer and boiling for 5 min and analyzed by SDS-PAGE and immunoblotting. Note: the kinase assay buffer used in this work does not contain

manganese, which is present in buffers we have used previously (Kim et al., 2002). PRAS40, tubulin, rheb1, or rap2a were added to mTORC1 for 5-20 minutes before the addition of ATP to the kinase assay.

Preparation of S6K1, PRAS40, phosphorylated PRAS40, rheb1, rap2a, and α-tubulin for Use in mTORC1 Kinase Assays

Full-length rat p70 S6K1 was cloned into an HA-GST pRK5 vector modified so as to contain a PreScission protease site between the GST tag and the initiator codon of S6K1. The expression construct was transfected into HEK-293T cells using Fugene6 and, after 48 hours, the cells were treated with 20 μM LY294002 for 1 hour prior to cell harvesting and lysis. HA-GST-PreSciss-S6K1 was purified as described (Burnett et al., 1998), and the affinity tag removed with the PreScission protease. S6K1 was separated from free GST by gel filtration on a HiLoad 16/60 Superdex 200 column (Amersham) and the purified protein stored at -80°C in 20% glycerol.

Rheb1 or rap2a cDNAs in HA-GST-prk5 were transfected as above into HEK-293T cells. Cell were lysed with rheb lysis buffer (the lysis buffer used above but without EDTA and containing 5 mM $MgCl_2$) and cleared lysates were incubated with immobilized glutathione for 2 hours at 4°C. Beads were washed twice with rheb lysis buffer and once with rheb storage buffer (20 mM Hepes [pH 8.0], 200 mM NaCl, and 5 mM $MgCl_2$). GST-rheb1 and rap2a were eluted from the beads with 10 mM glutathione in rheb storage buffer. Eluted proteins were incubated with 10 mM EDTA and 1 mM GDP or 0.1 mM GTPγS at 30°C for 10 min. 20 mM $MgCl_2$ was then added and the proteins kept on ice until use. PRAS40 or α-tubulin cDNAs in flag-prk5 were transfected into HEK-293T cells, the cells treated with 20 μM LY294002 for 15 min prior to lysis, and the proteins purified using immobilized FLAG-antibody resin. Proteins were eluted from the resin with rheb storage buffer containing 50 μg/μl flag peptide, and stored on ice until use. In the experiments using PRAS40 phosphorylated by Akt/PKB, flag-PRAS40 still bound to the FLAG-antibody resin was incubated for 30 minutes at 30°C in mTORC1 kinase assay

buffer containing 400 ng of T308D Akt/PKB in the presence or absence of 500 μM ATP. The resin-bound flag-PRAS40 was then washed three times with mTORC1 kinase assay buffer and eluted as above and stored on ice until use.

Mass Spectrometric Analysis

mTOR and raptor immunoprecipitates prepared from 30 million HEK-293E cells were resolved by SDS-PAGE and the Coomassie stained 40 kDa band corresponding to PRAS40 was excised and digested with trypsin overnight. The resulting peptides were separated by liquid chromatography (NanoAcquity UPLC, Waters) using a self-packed Jupiter 3 micron C18 column. The eluting peptides were mass analyzed prior to collisionally induced dissociation (CID) using a ThermoFisher LTQ linear ion trap mass spectrometer equiped with a nanospray source. Selected mass values from the MS/MS spectra were used to search the human segment of the NCBI non-redundant protein database using Xcalibur Mass Spectrometry software (Thermo Fischer Scientific). Depending on the purification, 2-6 distinct PRAS40-derived peptides were identified.

Cell Size Determinations

To measure cell size, cells were grown to confluence in 6 cm culture dishes, harvested, diluted 1:10, and re-plated into fresh media. 12 hours later the cells were harvested by trypsinization in a 1 ml volume, diluted 1:20 with counting solution (Isoton II Diluent, Beckman Coulter), and cell diameters and volumes determined using a particle size counter (Coulter Z2, Beckman Coulter) with Coulter Z2 AccuComp software.

cDNA Manipulations, Mutagenesis, and Sequence Alignments as well as *Drosophila* and Mammalian RNAi and Analysis

See Supplemental Experimental Procedures.

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Figure legends

Figure 1. A salt-sensitive factor inhibits the kinase activity of mTORC1.

(A) When mTORC1 is washed with buffers containing high salt concentrations, there is no difference in the kinase activity of mTORC1 isolated from serum-deprived or insulin treated cells. HEK-293E cells were deprived of serum for 14 hours or serum-deprived and treated with 150 nM insulin for 15 minutes. mTOR immunoprecipitates were prepared from cell lysates (1 mg total protein), washed with buffers containing 500 mM NaCl, and analyzed for mTORC1 kinase activity towards S6K1 and levels of mTOR and raptor. Lysates were analyzed by immunoblotting for indicated proteins and phosphorylation states. (B) An insulin-stimulated difference in mTORC1 activity is detected only when mTORC1 is washed with low salt buffers. mTOR immunoprecipitates from HEK-293E cells treated as in (A) were washed with buffers containing 400, 300, or 150 mM NaCl and analyzed for mTORC1 kinase activity. (C) Raptor immunoprecipitates were prepared from indicated cell types that had been serum deprived and treated with insulin as in (A), washed with buffers containing 150 mM NaCl, and analyzed for mTORC1 activity. Cell lysates were analyzed by immunoblotting for levels of indicated proteins. (D) HA-immunoprecipitates from HEK-293E cells stably expressing HA-raptor were prepared and analyzed for mTORC1 activity as in (C). (E) Raptor-immunoprecipitates from $TSC2^{-/-}$ or $TSC2^{+/+}$ MEFs treated as in (A) were washed with buffers containing 150 mM or 500 mM NaCl and analyzed for mTORC1 activity. Cell lysates were analyzed by immunoblotting for levels of indicated proteins and phosphorylation states.

Figure 2. In vitro soluble rheb1-GTP strongly stimulates mTORC1 kinase activity towards S6K1 and 4E-BP1.

(A) Rheb1, rap2a, or material obtained in mock purifications were loaded with GDP or the non-hydrolyzable GTP-mimetic GTPgS and added to mTORC1 immunopurified from serum-deprived HEK-293E cells. mTORC1 kinase assays were performed and analyzed by immunoblotting for indicated proteins and phosphorylation states. (B) Experiment was performed as in (A) using mTORC1 from both serum-deprived and insulin-treated cells. (C) Experiment was performed as in (A) using mTORC1 containing HA-raptor. (D) Experiment was performed as in (A) using mTORC1 obtained from $TSC2^{-/-}$ MEFs. (E) Experiment was performed as in (B) using 4E-BP1 as a substrate.

Figure 3. PRAS40 is an mTORC1 interacting protein.

(A) Schematic of PRAS40 indicating the C-terminal domain conserved with the Drosophila Lobe protein (LCD) and the proline-rich regions (P). Below the PRAS40 schematic is an amino acid sequence alignment of the LCD of PRAS40 orthologues from indicated species showing that the T246 Akt/PKB phosphoryation site is conserved. (B) In HEK-293E cells the interaction of PRAS40 with mTORC1 is regulated by insulin and disrupted in vitro by high salt containing buffers. Raptor or p53 immunoprecipitates were prepared from HEK-293E cells deprived of serum for 14 hours or serum-deprived and treated with 150 nM insulin for 15 minutes. Immunoprecipitates were washed with buffers containing 400 or 150 mM NaCl, and analyzed by immunoblotting for indicated proteins. In cell lysates levels of PRAS40 do not change upon insulin stimulation. (C) PRAS40 binds to mTORC1 and not mTORC2. Raptor and rictor immunoprecipitates were prepared from HEK-293E cells treated with insulin as in (B), washed with buffers containing 150 mM NaCl, and analyzed by immunoblotting for the indicated proteins. (D) Endogenous PRAS40 binds to mTORC1 containing recombinant epitope-tagged raptor. Flag-immunoprecipitates from HEK-293E cells expressing flag-raptor or a control vector and treated as in (C) were prepared and washed with buffers containing 150 mM NaCl and analyzed by immunoblotting for the indicated proteins. (E) PRAS40 preferentially binds the raptor component of mTORC1. Indicated cDNAs in expression vectors were co-transfected in HEK-293E cells and cell lysates prepared. Half of each cell lysate was used to prepare HA-immunoprecipitates and the other half for myc-immunoprecipitates. Both were analyzed by immunoblotting for the indicated proteins. Cell lysates contain equal levels of flag-PRAS40. Samples in which cDNAs for raptor and mTOR were co-transfected were performed in duplicate. (F) PRAS40 association with mTORC1 requires raptor. HEK-293E cells were infected with the specified lentiviral shRNAs and mTOR and raptor immunoprecipitates were analyzed by immunoblotting for the levels of the indicated proteins. (G) In HEK-293E and HeLa cells and wild-type, but not TSC2 null MEFs, insulin stimulates the phosphorylation of the PRAS40 bound to mTORC1. Cells were treated with and without insulin as in (B). Raptor immunoprecipitates and cell lysates were analyzed for the levels of the indicated proteins and phosphorylation states.

Figure 4. In vitro PRAS40 potently inhibits mTORC1 kinase activity induced by insulin or GTP-loaded rheb1.

(A) PRAS40 inhibits mTORC1 activated by washing in high salt-containing buffers. HA-raptor immunoprecipitates were prepared from HEK-293E cells stably expressing HA-raptor deprived of serum for 14 hours or serum-deprived and treated with 150 nM insulin for 20 minutes. Immunoprecipitates were washed with buffers containing 150, or where indicated, 500 mM

NaCl. mTORC1 kinase assays containing the specified concentrations of PRAS40 or tubulin were performed and analyzed by immunoblotting for the indicated proteins and phosphorylation states. (B) PRAS40 inhibits active mTORC1 isolated from insulin-stimulated HEK-293E cells. Experiment was performed and analyzed as in (A) except that all immunoprecipitates were washed with buffers containing 150 mM NaCl. (C) PRAS40 blocks mTORC1 activation induced by GTP-loaded rheb1. Experiment was performed and analyzed as in (A) except that all immunoprecipitates were from serum-deprived cells and washed with buffers containing 150 mM NaCl. Kinase assays contained 100 nM rheb1 or rap2a loaded with GDP or GTP. (D) PRAS40 blocks, in a dose sensitive fashion, mTORC1 activation induced by GTP-loaded rheb1. Experiment was performed and analyzed as in (C) using indicated concentrations of rheb1-GTP and PRAS40. (E) At high ratios of rheb1-GTP to PRAS40, rheb1-GTP can overcome PRAS40-mediated suppression of mTORC1. Experiment was performed and analyzed as in (D). (F) PRAS40 inhibits mTORC1 activity towards 4E-BP1. Experiment was performed as in (B) using mTORC1 obtained from insulin-stimulated cells and 4E-BP1 as the substrate.

Figure 5. In mammalian cells PRAS40 inhibits mTORC1 signaling and cell growth.

(A) Overexpression of PRAS40 inhibits insulin-mediated phosphorylation of S6K1. HEK-293E cells were co-transfected with expression plasmids for HA-GST-S6K1 (500 ng) as well as myc-tubulin or myc-PRAS40 (2 mg), serum deprived for 14 hours or serum deprived and treated with 150 nM insulin for 15 minutes. Cell lysates were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. (B) Overexpression of PRAS40 inhibits the large increase in S6K1 phosphorylation caused by the overexpression of rheb1. HEK-293E cells were co-transfected with expression plasmids for HA-GST-S6K1 (500 ng) and 2 mg of the other specified plasmids. In HEK-293T cells 50 ng was used of the HA-GST-S6K1 expression plasmid and 1 mg of the other plasmids. Cell lysates were prepared and analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. (C) Stable overexpression of PRAS40 inhibits cell size. HEK-293E cells or TSC2 null MEFs were infected with retroviruses expressing PRAS40 or tubulin and, 48 hours later, cell size was measured using a Coulter counter. (D) Validation of shRNAs directed against human or mouse PRAS40. HT-29 cells or wild-type MEFs were infected with lentiviruses expressing shRNAs targeting mouse or human PRAS40, respectively, and cell lysates analyzed by western blotting for the indicated proteins. Control shRNAs targeted raptor or luciferase. (E) Knockdown of endogenous PRAS40 activates the mTORC1 pathway. Wild-type MEFs or HT-29 cells infected with lentiviruses expressing shRNAs targeting the indicated genes were incubated in the presence or absence of serum for 3 hours. Cell lysates were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states.

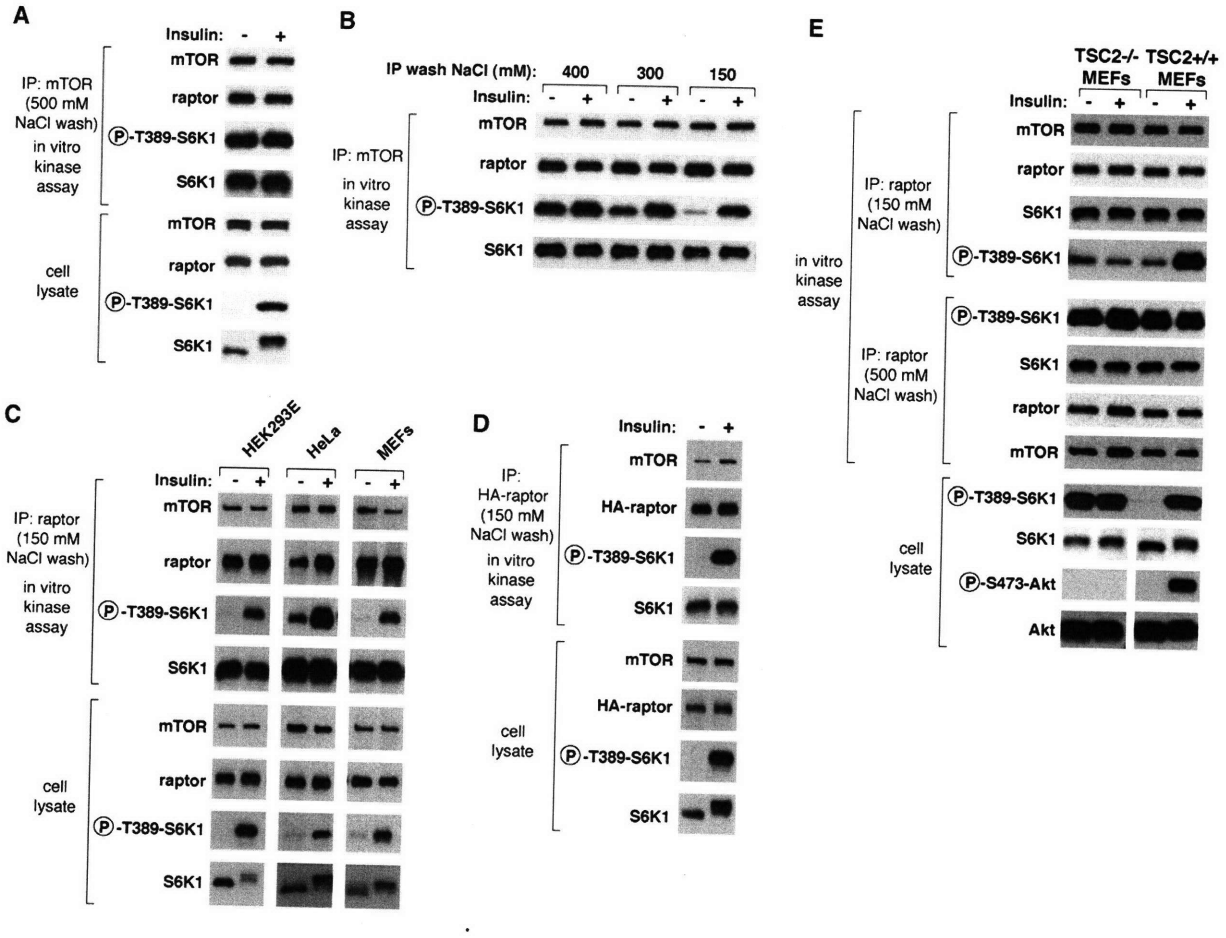
Figure 6. In *Drosophila* cells Lobe inhibits dTORC1 signaling and cell growth.

(A) A knockdown of Lobe using two distinct dsRNAs increases dS6K phosphorylation. S2 cells were transfected with the indicated dsRNAs as described in the Experimental Procedures. (B) A knockdown of Lobe increases the mean size of S2 cells. Cells were transfected as in (A) with the GFP, Lobe #1, Lobe #2, or dTSC2 dsRNAs and cell size measured with a Coulter counter. Mean cells diameters ± standard error for n = 3 are: GFP dsRNA, 10.71 ± 0.02 mm; Lobe #1 dsRNA, 10.98 ± 0.03 mm; Lobe #2 dsRNA, 10.95 ± 0.04 mm; dTSC2 dsRNA, 11.26 ± 0.02 mm. The differences in cell diameter between cells transfected with the GFP dsRNA and the Lobe or dTSC2 dsRNAs is significant to at least $p < 0.05$. For clarity, histogram shows data for only one of the Lobe dsRNAs. (C) A Lobe knockdown suppresses the decrease in dS6K-phosphorylation caused by a knockdown of dRheb but not of dTOR. Kc167 cells were transfected as described in the Experimental Procedures. Cell lysates were analyzed with immunoblotting for the levels and phosphorylation states of the indicated proteins. (D) A knockdown of Lobe increases S2 cell size in a dTOR-dependent but dRheb-independent fashion. Cells were transfected as in (C) with the indicated dsRNAs and cell sizes were measured 24 hours after the final transfection. Cell volumes were normalized within each group of three in order to compare the cell volume changes that occur when Lobe or dTSC2 is knocked down alone or together with dRheb or dTOR. Error bars indicate standard deviations for n = 3.

Figure 7. Akt/PKB-mediated phosphorylation of PRAS40 blocks its inhibition of mTORC1.

(A) The addition of active T308D Akt/PKB stimulates the kinase activity of mTORC1 washed with low but not high salt buffers. Raptor immunoprecipitates from serum-deprived cells were washed with buffers containing 150 or 500 mM NaCl as described in the Experimental Procedures. mTORC1 kinase activity in the immunoprecipitates was assayed in the presence or absence of 400 ng active Akt/PKB and levels and phosphorylation state of S6K1 was analyzed by immunoblotting. (B) PRAS40 phosphorylated by Akt/PKB has a reduced capacity to inhibit mTORC1. 40 nM of non-phosphorylated or phosphorylated PRAS40 was added to mTORC1 immunoprecipitates washed with high salt. Kinase assays were performed and analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. (C) Expression of T246A PRAS40 blocks insulin-stimulated phosphorylation of S6K1. HEK-293E were co-transfected with expression plasmids for HA-GST-S6K1 (500 ng) as well as low (25 ng) or high (250 ng) amounts of expression plasmids for wild-type or T246A myc-PRAS40. 250 ng was used of the plasmid encoding myc-tubulin. Cells were deprived of serum or serum-deprived and stimulated with 150 nM insulin for 20 minutes. Cell lysates were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. (D) Expression of T246A PRAS40 blocks phosphorylation of S6K1 induced by the expression of constitutively active myristoylated Akt/PKB (myr-Akt). HEK-293E cells were co-transfected and analyzed as above except that 2 mg of the expression plasmid for myr-Akt was included were indicated. The increasing amounts of PRAS40 expression vectors used were: 3, 10, 30, 100, and 300 ng. (E) Models depicting regulation of mTORC1 signaling by rheb and PRAS40 in insulin treated (+Insulin), serum deprived (-Insulin), and TSC2 null ($TSC2^{-/-}$) cells. Green and red colors indicate active and inactive components and signaling events, respectively. In insulin treated cells, GTP-loaded rheb stimulates mTORC1 while phosphorylated PRAS40 does not repress mTORC1. In serum-deprived cells, GDP-loaded rheb is inactive while dephosphorylated PRAS40 represses mTORC1. In TSC2 null cells GTP-loaded rheb overcomes the mTORC1 inhibition mediated by dephosphorylated PRAS40.

Figure 1


A
Insulin: - +
IP: mTOR (500 mM NaCl wash) in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
cell lysate
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
B
IP wash NaCl (mM): 400 300 150
Insulin: - + - + - +
IP: mTOR in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
C
HEK293E
HeLa
MEFs
Insulin: - + - + - +
IP: raptor (150 mM NaCl wash) in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
cell lysate
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
D
Insulin: - +
IP: HA-raptor (150 mM NaCl wash) in vitro kinase assay
mTOR
HA-raptor
Ⓟ-T389-S6K1
S6K1
cell lysate
mTOR
HA-raptor
Ⓟ-T389-S6K1
S6K1
E
TSC2-/- MEFs
TSC2+/+ MEFs
Insulin: - + - +
IP: raptor (150 mM NaCl wash) in vitro kinase assay
mTOR
raptor
S6K1
Ⓟ-T389-S6K1
IP: raptor (500 mM NaCl wash)
Ⓟ-T389-S6K1
S6K1
raptor
mTOR
cell lysate
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S6K1
Ⓟ-S473-Akt
Akt

Figure 2

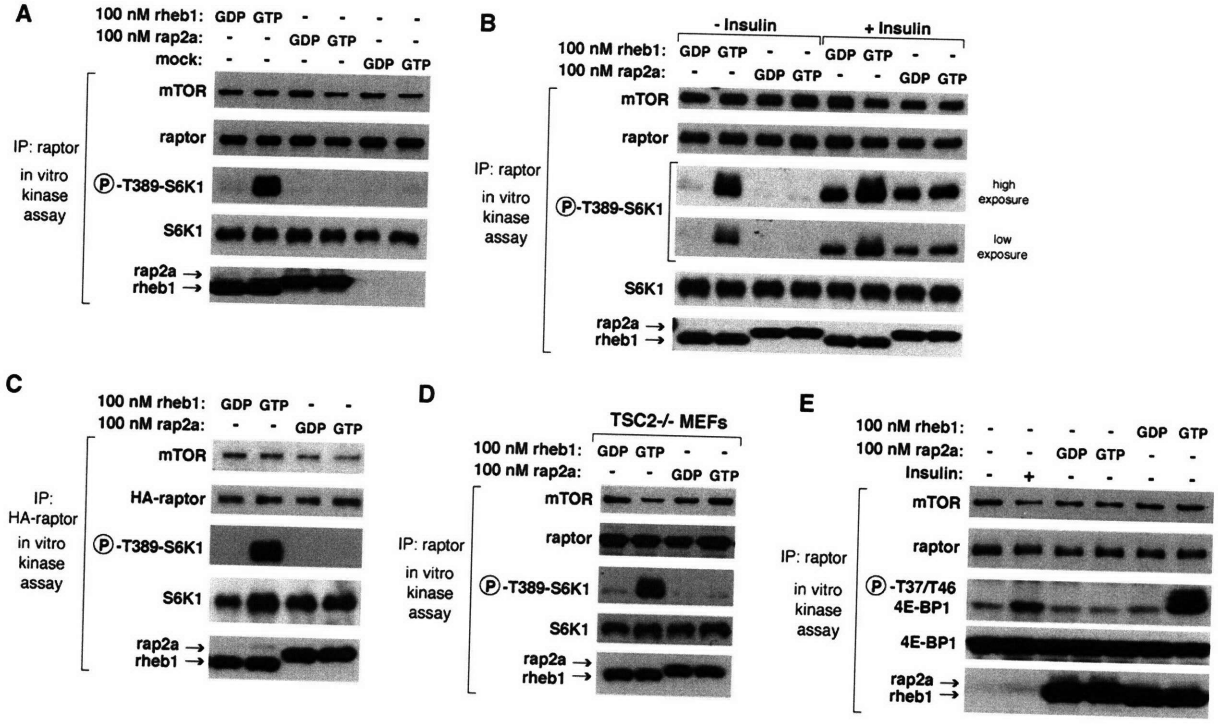

A
100 nM rheb1: GDP GTP - - - -
100 nM rap2a: - - GDP GTP - -
mock: - - - - GDP GTP
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
rap2a →
rheb1 →
B
- Insulin
+ Insulin
100 nM rheb1: GDP GTP - - GDP GTP - -
100 nM rap2a: - - GDP GTP - - GDP GTP
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
high exposure
low exposure
S6K1
rap2a →
rheb1 →
C
100 nM rheb1: GDP GTP - -
100 nM rap2a: - - GDP GTP
IP: HA-raptor
in vitro kinase assay
mTOR
HA-raptor
Ⓟ-T389-S6K1
S6K1
rap2a →
rheb1 →
D
TSC2-/- MEFs
100 nM rheb1: GDP GTP - -
100 nM rap2a: - - GDP GTP
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
rap2a →
rheb1 →
E
100 nM rheb1: - - - - GDP GTP
100 nM rap2a: - - GDP GTP - -
Insulin: - + - - - -
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T37/T46 4E-BP1
4E-BP1
rap2a →
rheb1 →

Figure 3

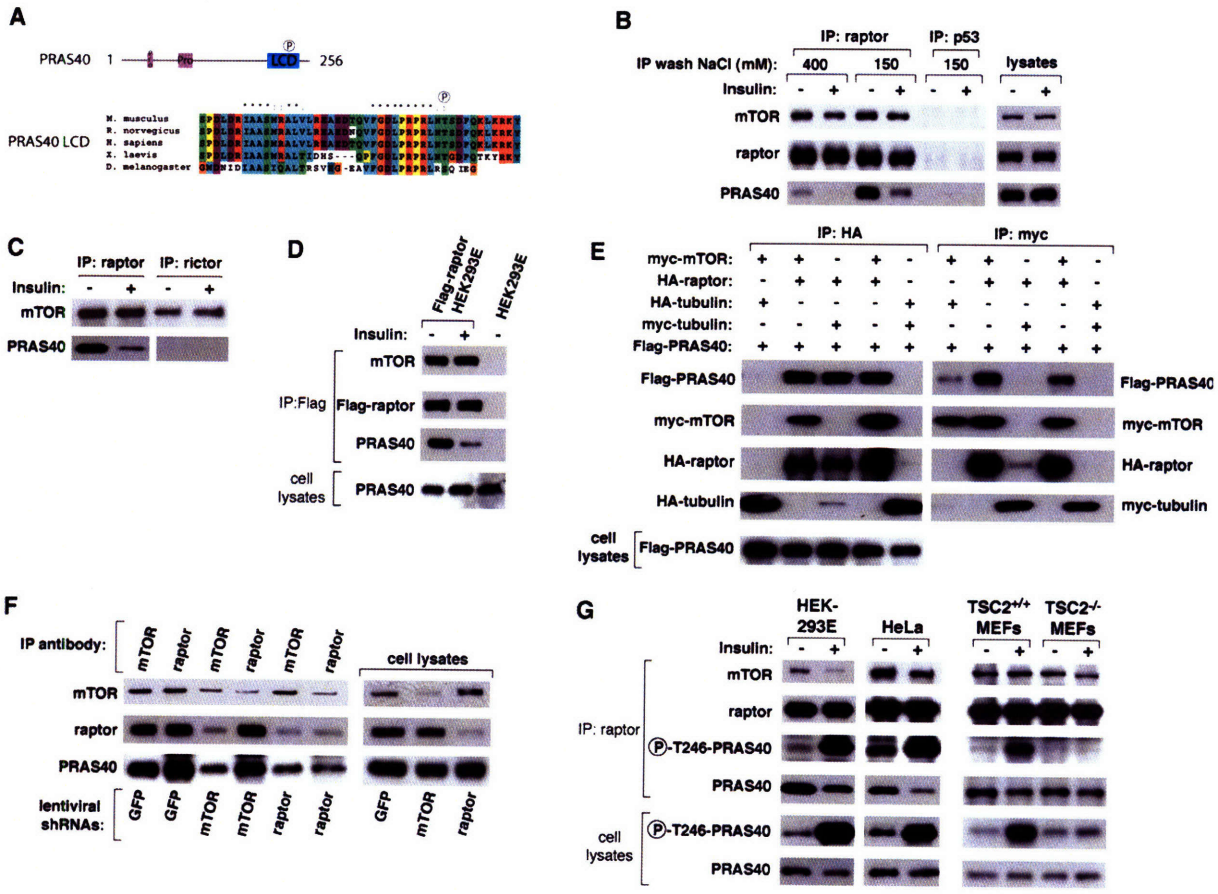
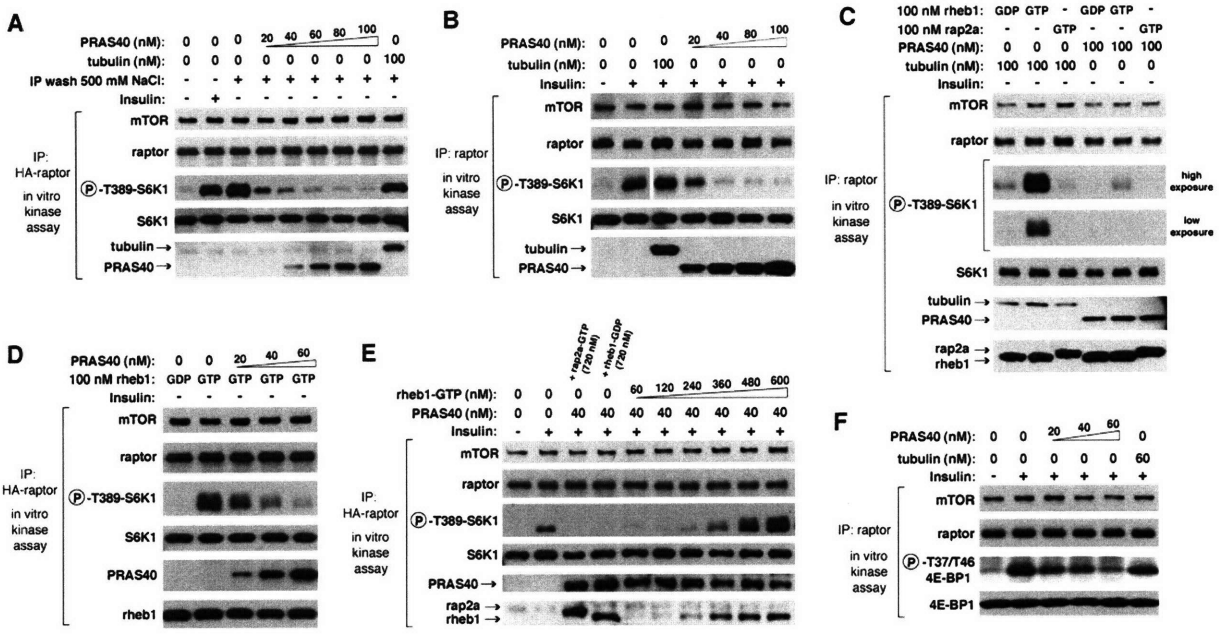

A
PRAS40
1
256
LCD
PRAS40 LCD
M. musculus
R. norvegicus
H. sapiens
X. laevis
D. melanogaster
B
IP: raptor
IP: p53
IP wash NaCl (mM):
400
150
150
lysates
Insulin:
mTOR
raptor
PRAS40
C
IP: raptor
IP: rictor
Insulin:
mTOR
PRAS40
D
Flag-raptor HEK293E
HEK293E
Insulin:
mTOR
IP:Flag
Flag-raptor
PRAS40
cell lysates
PRAS40
E
IP: HA
IP: myc
myc-mTOR:
HA-raptor:
HA-tubulin:
myc-tubulin:
Flag-PRAS40:
Flag-PRAS40
myc-mTOR
HA-raptor
HA-tubulin
cell lysates
Flag-PRAS40
Flag-PRAS40
myc-mTOR
HA-raptor
myc-tubulin
F
IP antibody:
mTOR
raptor
mTOR
raptor
mTOR
raptor
cell lysates
mTOR
raptor
PRAS40
lentiviral shRNAs:
GFP
GFP
mTOR
mTOR
raptor
raptor
GFP
mTOR
raptor
G
HEK-293E
HeLa
TSC2+/+ MEFs
TSC2-/- MEFs
Insulin:
mTOR
raptor
IP: raptor
P-T246-PRAS40
PRAS40
cell lysates
P-T246-PRAS40
PRAS40

Figure 4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tubulin (nM): 0 0 0 0 0 0 0 0 100
IP wash 500 mM NaCl: - - + + + + + + +
Insulin: - + - - - - - - -
IP: HA-raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
tubulin →
PRAS40 →
B
PRAS40 (nM): 0 0 0 20 40 80 100
tubulin (nM): 0 0 100 0 0 0 0
Insulin: - + + + + + +
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
tubulin →
PRAS40 →
C
100 nM rheb1: GDP GTP - GDP GTP -
100 nM rap2a: - - GTP - - GTP
PRAS40 (nM): 0 0 0 100 100 100
tubulin (nM): 100 100 100 0 0 0
Insulin: - - - - - -
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
high exposure
low exposure
S6K1
tubulin →
PRAS40 →
rap2a →
rheb1 →
D
PRAS40 (nM): 0 0 20 40 60
100 nM rheb1: GDP GTP GTP GTP GTP
Insulin: - - - - -
IP: HA-raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
PRAS40
rheb1
E
+ rap2a-GTP (720 nM)
+ rheb1-GDP (720 nM)
rheb1-GTP (nM): 0 0 0 0 60 120 240 360 480 600
PRAS40 (nM): 0 0 40 40 40 40 40 40 40 40
Insulin: - + + + + + + + + +
IP: HA-raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T389-S6K1
S6K1
PRAS40 →
rap2a →
rheb1 →
F
PRAS40 (nM): 0 0 20 40 60 0
tubulin (nM): 0 0 0 0 0 60
Insulin: - + + + + +
IP: raptor
in vitro kinase assay
mTOR
raptor
Ⓟ-T37/T46 4E-BP1
4E-BP1

Figure 5

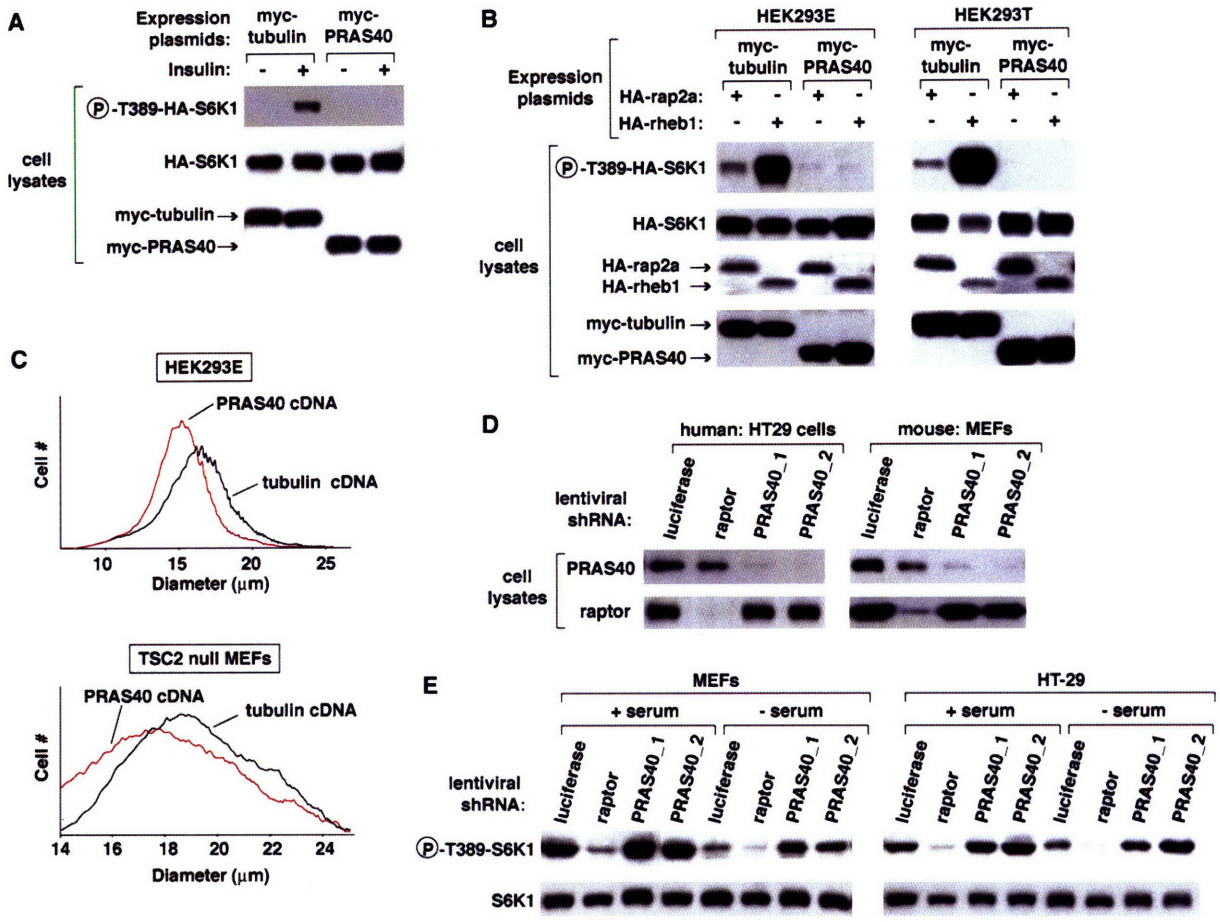

A
Expression plasmids:
myc-tubulin
myc-PRAS40
Insulin:
- + - +
Ⓟ-T389-HA-S6K1
HA-S6K1
cell lysates
myc-tubulin →
myc-PRAS40 →
B
HEK293E
HEK293T
myc-tubulin
myc-PRAS40
Expression plasmids
HA-rap2a:
HA-rheb1:
Ⓟ-T389-HA-S6K1
HA-S6K1
cell lysates
HA-rap2a →
HA-rheb1 →
myc-tubulin →
myc-PRAS40 →
C
HEK293E
PRAS40 cDNA
tubulin cDNA
Cell #
10 15 20 25
Diameter (μm)
TSC2 null MEFs
PRAS40 cDNA
tubulin cDNA
Cell #
14 16 18 20 22 24
Diameter (μm)
D
human: HT29 cells
mouse: MEFs
lentiviral shRNA:
luciferase
raptor
PRAS40_1
PRAS40_2
cell lysates
PRAS40
raptor
E
MEFs
HT-29
+ serum
- serum
lentiviral shRNA:
luciferase
raptor
PRAS40_1
PRAS40_2
Ⓟ-T389-S6K1
S6K1

Figure 6

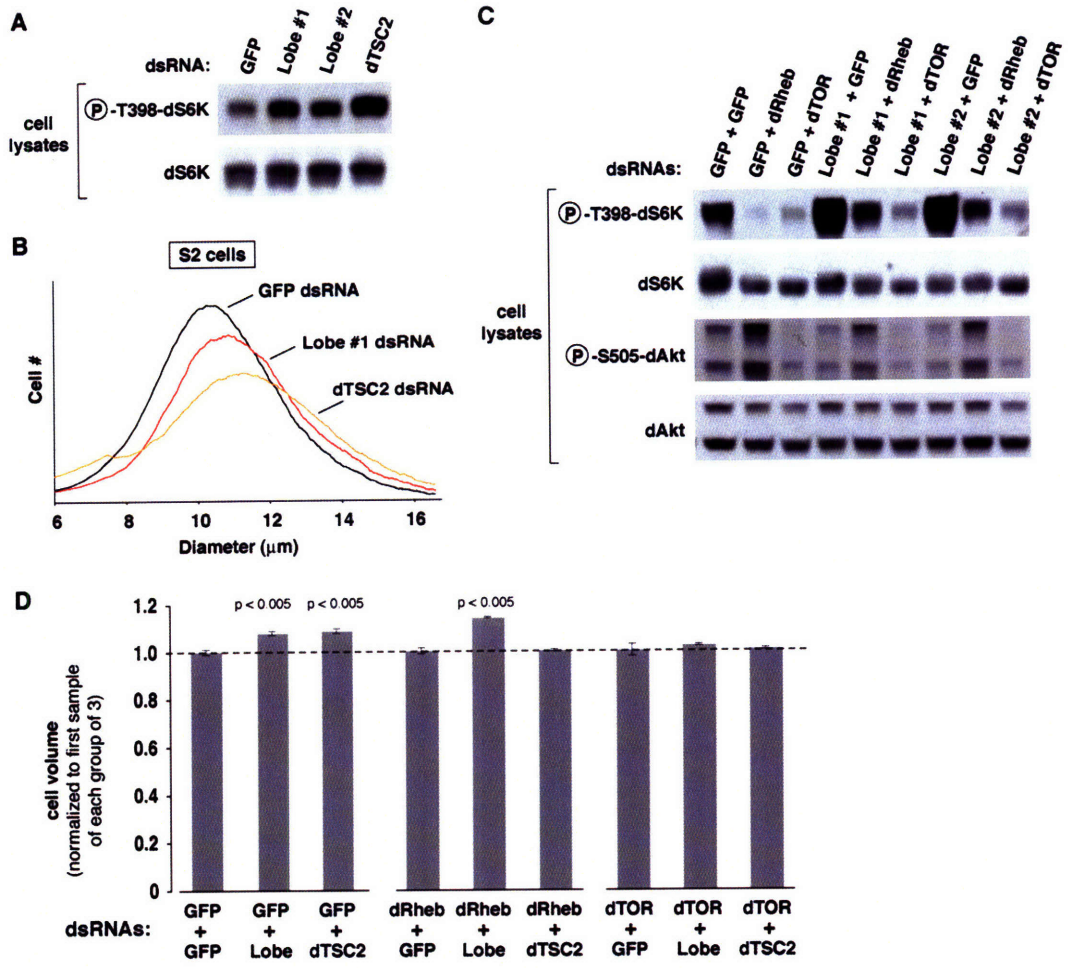
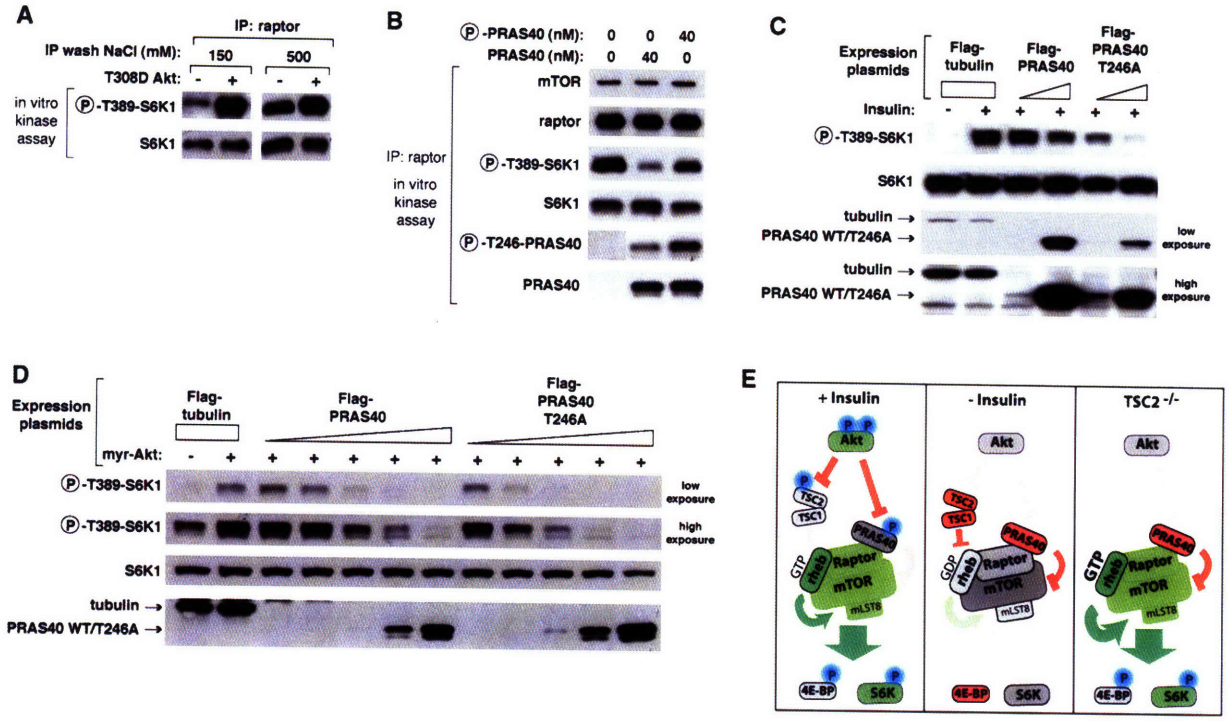

A
dsRNA:
GFP
Lobe #1
Lobe #2
dTSC2
cell lysates
Ⓟ-T398-dS6K
dS6K
B
S2 cells
GFP dsRNA
Lobe #1 dsRNA
dTSC2 dsRNA
Cell #
6
8
10
12
14
16
Diameter (μm)
C
dsRNAs:
GFP + GFP
GFP + dRheb
GFP + dTOR
Lobe #1 + GFP
Lobe #1 + dRheb
Lobe #1 + dTOR
Lobe #2 + GFP
Lobe #2 + dRheb
Lobe #2 + dTOR
cell lysates
Ⓟ-T398-dS6K
dS6K
Ⓟ-S505-dAkt
dAkt
D
cell volume
(normalized to first sample of each group of 3)
1.2
1.0
0.8
0.6
0.4
0.2
0
p < 0.005
p < 0.005
p < 0.005
dsRNAs:
GFP + GFP
GFP + Lobe
GFP + dTSC2
dRheb + GFP
dRheb + Lobe
dRheb + dTSC2
dTOR + GFP
dTOR + Lobe
dTOR + dTSC2

Figure 7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500
T308D Akt:
in vitro kinase assay
Ⓟ-T389-S6K1
S6K1
B
Ⓟ-PRAS40 (nM):
PRAS40 (nM):
mTOR
raptor
IP: raptor
in vitro kinase assay
Ⓟ-T389-S6K1
S6K1
Ⓟ-T246-PRAS40
PRAS40
C
Expression plasmids
Flag-tubulin
Flag-PRAS40
Flag-PRAS40 T246A
Insulin:
Ⓟ-T389-S6K1
S6K1
tubulin →
PRAS40 WT/T246A →
low exposure
high exposure
D
Expression plasmids
Flag-tubulin
Flag-PRAS40
Flag-PRAS40 T246A
myr-Akt:
Ⓟ-T389-S6K1
low exposure
high exposure
S6K1
tubulin →
PRAS40 WT/T246A →
E
+ Insulin
- Insulin
TSC2 -/-
Akt
TSC2
TSC1
PRAS40
GTP
GDP
Rheb
Raptor
mTOR
mLST8
4E-BP
S6K

Chapter 3

Pharmacological inhibition of mTORC1 and mTORC2 reveals redundant roles for PI3K and RAS pathways in tumorigenesis

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[2]MIT Center for Cancer Research, 77 Massachusetts Avenue, Cambridge, MA 02139.

[3]Broad Institute, Seven Cambridge Center, Cambridge, MA 02142.

[4]Dana–Farber Cancer Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Experiments in Figure 1 were performed by C.C.T.
Experiments in Figure 2 were performed by C.C.T.
Experiments in Figure 3 and Table 2 were performed by C.C.T. and S.A.K.

Summary

Mutations in the PI3K/PTEN signaling pathway are implicated in many cancers and drive tumorigenesis by promoting proliferation, cell survival and increased protein synthesis. The mTORC1 and mTORC2 complexes, which contain the mTOR kinase, are important effectors of PI3K signaling and control many of these processes by regulating the AGC kinases S6K and AKT, respectively. In the laboratory, inhibition of mTORC1 with the FDA-approved drug rapamycin has shown remarkable efficacy against tumors with hyper-activated PI3K signaling. Unfortunately, rapamycin has had only limited success in the clinic. Recent work has shown that, under certain circumstances, rapamycin inhibits both complexes and this dual inhibition is more strongly anti-proliferative than inhibition of mTORC1 alone. This variable effect may account for cases where rapamycin is effective, and argues that inhibitors of both complexes may have more consistent anti-cancer efficacy. Towards this end, we developed a small molecule that potently and specifically inhibits both complexes by directly targeting mTOR. This molecule exhibits strong anti-proliferative effects against many tumor cell lines, but, surprisingly, not against those carrying mutations in the RAS pathway. These results suggest that PI3K/mTOR and RAS signaling engage a common mechanism to drive tumorigenesis, and that combined inhibition might be a particularly effective therapy for a broad range of cancers.

Introduction

Mutations in PI3K occur in approximately 30% of all solid tumors, earning it the distinction, along with K-RAS, as one of the two most frequently activated oncogenes. PI3K drives growth and proliferation by catalyzing the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphoate (PIP3). The accumulation of PIP3 leads to the activation of 3-phosphoinositide-dependent kinase (PDK1), the mTORC1/2 complexes, and the proto-oncogene AKT/PKB kinases, AKT1, AKT2 and AKT3(Engelman et al., 2006). Other members of the PI3K signaling pathway are also frequently mutated in cancer. Monoallelic mutations in the tumor suppressor PTEN (phosphatase and tensin homolog), which counters the activity of PI3K by converting PIP3 to PIP2, are found in 50-80% of sporadic tumors and 30-50% of breast, colon and lung tumors (Salmena et al., 2008). Moreover, germline PTEN heterozygosity is the cause of the familial Cowden Syndrome, which is characterized by benign tumors and a high risk of cancer. Overexpression of AKT2 has also been implicated in tumorigenesis, confirming its role as a key downstream effector (Engelman et al., 2006). The transforming ability of PTEN loss, PI3K activation and overexpression of AKT have all been established in both cell culture and mouse tumor models (Engelman et al., 2006).

The large multi-protein complex mTORC2 is integral in transmitting signals from PI3K to AKT and may mediate many of PI3Ks downstream effects. AKT activity is regulated by a two-step mechanism where full activation requires phosphorylation at a site located in the activation loop (T308) and a site at the C-terminus known as the hydrophobic motif (S473) (Alessi et al., 1996). Accumulation of PIP3 targets AKT to the plasma membrane where PDK1 phosphorylates T308 and mTORC2 phosphorlyates S473 (Alessi et al., 1996). Although phosphorylation of both sites generally occurs in a

coordinated fashion, there is no absolute dependency between them (Collins et al., 2003). Deletion of mTORC2 components ablates phosphorylation at S473 and blunts the effects of PTEN loss, but, importantly, does not completely inhibit AKT activity, suggesting that S473 might primarily be required for transmitting spikes in PI3K activity (Guertin et al., 2006; Hietakangas and Cohen, 2007).

The connection between deranged PI3K signaling and cancer has driven the search for pharmacological inhibitors of various pathway components. Several AKT inhibitors exist, though the therapeutic value of these is complicated by the existence of the three highly similar, partially redundant but also physiologically distinct isoforms (LoPiccolo et al., 2007). Furthermore, the similarity between AKT and other AGC kinases, such as serum-glucocorticoid kinase (SGK) and the S6-ribosomal kinase (S6K), has made target specificity a challenge. The most potent inhibitors to date are reasonably efficacious against mouse xenograft tumor models, but also cause acute metabolic toxicity, which has dampened enthusiasm for their prospects in humans (Luo et al., 2005). A second strategy has been to develop molecules that inhibit PI3K directly, though these are also complicated by the existence of multiple p110 isoforms and similar toxicity concerns because of the broad range of downstream processes (Fan et al., 2006).

The mTORC1 complex, which acts downstream of AKT, is also considered a therapeutic target. AKT activates mTORC1 by phosphorylating and inactivating TSC2, a component of the heterodimeric tuberous sclerosis complex (TSC) that negatively regulates mTORC1(Inoki et al., 2002; Potter et al., 2002). Upon activation, mTORC1 phosphorylates and regulates the S6-ribosomal kinase (S6K) and the eIF4E inhibitor 4E-BP1, and contributes to tumorigenesis by promoting cap-dependent translation through eIF4E (Guertin and Sabatini, 2007). mTORC1 is potently and specifically inhibited by the FDA-approved drug rapamycin, and several studies have demonstrated that rapamycin is, experimentally, highly effective against neoplastic phenotypes induced by PTEN deletion or transgenic activation of AKT (Majumder et al., 2004; Neshat et al., 2001; Podsypanina

et al., 2001; Wendel et al., 2004).

Unfortunately, rapamycin has had far less clinical success as an anti-cancer treatment than had been hoped for. One hypothesis is that selective inhibition of mTORC1 paradoxically activates PI3K signaling. mTORC1 controls a powerful negative feedback loop that normally suppresses insulin signaling by targeting the adaptor protein IRS1 for degradation(Guertin and Sabatini, 2007). Rapamycin treatment disables this feedback loop, thereby hyper-activating PI3K and AKT to an extent that might overcome the growth-inhibitory effects of mTORC1 inhibition.

A second hypothesis is based on the observation that rapamycin, despite its historical classification as a specific mTORC1 inhibitor, can also inhibit mTORC2 during prolonged treatment (Sarbassov et al., 2006). Strangely, this effect only occurs in certain cell types, and we have proposed that the variation in tumor response might reflect whether or not both mTOR complexes are inhibited (Sarbassov et al., 2006). Shor et al recently showed that high but clinically relevant concentrations of rapamycin are also to inhibit both mTOR complexes(Shor et al., 2008). In support of a key role for mTORC2 in tumor growth, they further showed that high doses of rapamycin dramatically inhibited proliferation of many tumor cell lines that were unaffected by low doses, and caused a severe repression of global protein synthesis. Together, these observations suggest that mTORC2 inhibition may account for rapamycin's successes and provide rationale for developing a small molecule capable of inhibiting both mTORC1 and mTORC2. To this end, we developed a screening platform to identify promising small molecule candidates and then refined these to produce a highly potent and specific mTORC1/mTORC2 inhibitor.

Results

Automated screen for mTOR-specific inhibitors

To identify candidate mTOR inhibitors, we developed an in vitro kinase assay that was compatible with automated screening methods. We chose this approach over a cell-based one because mTORC1 and mTORC2 are downstream of many signaling pathways, and we believed that off-target effects in cells would generate an unacceptably high false-positive rate. Although we were interested in molecules that inhibit mTOR directly, we based our assay on mTORC1 with the reasoning that most hits would also be active against mTORC2 because mTOR is the catalytic domain for both complexes.

We designed our assay to maintain as many regulatory features as possible with the belief that some molecules might act by engaging these mechanisms. To this end, we developed a method for purifying intact soluble mTORC1, which contains other mTORC1 components raptor, mLST8 and PRAS40 that are important for regulation of kinase activity(Guertin and Sabatini, 2007). In the absence of raptor, purified monomeric mTOR is no longer regulated in vitro by insulin and cannot be activated in vitro by rheb (data not shown). For similar reasons, we chose to use full-length p70S6K as a substrate. We have shown previously that mTORC2 can phosphorylate a C-terminal truncation of S6K1, but not the full-length protein, suggesting that full-length substrates are subject to regulatory mechanisms that don't affect shorter fragments (Ali and Sabatini, 2005).

To purify intact mTORC1, we stably infected HEK293T cells with an MSCV vector expressing N-terminal FLAG-tagged raptor. The mTORC1 complex is sensitive to the stoichiometry of components, and transiently expressing high levels of a single component can disrupt complex integrity (data not shown). By generating cell lines that stably expressed low levels of tagged raptors, we were able to purify large amounts of complex that maintained all of its constituent pieces (data not shown). After purification by FLAG antibody, mTORC1 was eluted with FLAG peptide and stored at –80C. We then used this kinase in combination with full-length p70S6K to perform kinase assays in

96-well plates.

Our initial screening panel contained 200 compounds, many of which were ATP-competitive inhibitors of PI3K. mTOR belongs to the PI3K-like kinase (PIKK) family of kinases, which is defined by sequence similarity between the PIKK and PI3K kinase domains(Abraham, 2004). Because many structurally unrelated PI3K inhibitors, such as wortmannin, LY294002, and PI-103, also inhibit mTOR and other PIKKs, we hoped to find a compound with similar cross-selectivity that was more amenable to further modification. To this end, we determined mTORC1 IC50s for each compound by measuring their effect on mTOR activity at 8 different concentrations. As a positive control, we included PI-103, which yielded the expected IC50 of approximately 100nM(Knight et al., 2006). We also determined mTOR IC50s in cells for top hits by treating mouse embryonic fibroblasts (MEFs) with varying concentrations for 30 min or 1h and monitoring phosphorylation of p70S6K T389 and pAkt S473, which are established targets of mTORC1 and mTORC2, respectively (Figure 1A) (Burnett et al., 1998; Sarbassov et al., 2005).

Increasing selectivity for mTOR over PI3K and other PIKK kinases

Our next step was to determine which of our top hits was more selective for mTOR versus PI3K and other PIKK kinases. Assessing PI3K activity in cells is difficult, and generally involves measuring incorporation of 32P into PIP3 or indirect FRET-based detection systems. Instead, we made use of the observation that phosphorylation of AKT at T308 depends on two processes that directly reflect PI3K activity: PIP3-dependent targeting of AKT to the plasma membrane and activation of PDK1. In wild-type cells, phosphorylation of T308 is also influenced by phosphorylation at S473 (Sarbassov et al., 2005). To remove this variable, we tested compounds in MEFs where mLST8, an essential mTORC2 component, is deleted and AKT S473 is constitutively

dephosphorylated, or in PC3 cells that express an S473D AKT phospho-mimetic mutant. Because S473 is fixed in a single state in these cell lines, phosphorylation at T308 only reflects PI3K activity. Using these two systems, we determined cellular PI3K IC50s for each compound (Figure 1B). We also determined in vitro IC50s for PI3Kalpha using well-established methods that are described elsewhere (Figure 2) (Knight et al., 2006).

Compounds that inhibit PI3K and mTOR are also likely to inhibit other PIKK kinases, including the DNA-damage response kinases ATM, ATR and DNA-PK. Like mTOR, these kinases are unusually large and difficult to screen in a high-throughput format, and so we relied on in vitro and in vivo assays to determine selectivity. For DNA-PK, ATM and ATR, we measured IC50s using in vitro assays (Figure 2). We also measured inhibition of ATM in cells by monitoring phosphorylation of Chk2 T68 following a 2h treatment with the DNA-damage inducing compound doxorubicin. We also measured inhibition of the Class III PI3K hVps34. Some reports have proposed that hVps34 acts upstream of mTORC1, and we wanted to be sure that cross-reactivity with this kinase was not influencing mTORC1 activity in cells (Nobukuni et al., 2005). Finally, we screened top candidates at 10uM against a panel of 353 serine/threonine kinases using Ambit Biosciences scanMAX screening platform (Table 1).

Guided by selectivity information from the assays described above, we generated a series of derivatives of top candidates from our original screen that eventually produced JW-7-52-1, a potent and selective inhibitor of mTORC1 and mTORC2. The IC50 is 2nM for mTORC1 and 8nM for mTORC2 (Figure 1A). This compound is nearly 1000-fold selective for mTOR over PI3K (IC50 = 1.8uM), and at least 400-fold selective over other PIKK kinases and hVps34 (Figure 2). Of the 353 serine/threonine kinases screened using the Ambit panel, only the poorly characterized myotonic dystrophy kinase-related cdc42 binding kinase alpha (MRCKa) showed any substantial inhibition (Table 1). The slight difference in IC50 between mTORC1 and mTORC2 was puzzling at first, as both complexes share the same catalytic domain. However, inhibition of mTORC1 should

disengage the negative feedback loop that normally dampens insulin signaling in growing cells (Manning, 2004). This could sensitize PI3K and thus increase mTORC2 activation, countering the inhibitory effect of JW-7-52-1.

Effects of mTOR inhibition on tumor cell proliferation

Using JW-7-52-1, we next asked whether some tumor cell lines were more dependent on mTOR activity that others. To test this, we assembled a panel of 30 cell lines that represented a broad range of cancer types and mutational backgrounds (Table 2). Previous studies have established a relationship between activation of PI3K/PTEN signaling and sensitivity to rapamycin, and so we included cell lines that carry known lesions in PI3K (MDA-MB-435, MCF7, BT-20) and PTEN (U-87-MG, PC3, 786-O). We also included cell lines derived from tumors that are frequently clinically responsive to rapamycin, such as mantle cell lymphoma (MCLs) and NF1 malignant peripheral nerve sheath tumors (MPNST), which are characterized by loss of the NF1 tumor suppressor (Drakos et al., 2008,Johannessen, 2008 #189). Additional tumor cell lines were chosen to cover a broad range of cancers.

To measure inhibition, we seeded cell lines in 96-well plates, treated them with vehicle, 50nM rapamycin or increasing concentrations of JW-7-52-1 (Table 2). Cell proliferation was measured using a luminescent reagent that reflects the ATP content of the well, and measurements were taken at the time of drug addition on Day 1 and again at Day 6. The seeding density for each cell line was chosen to ensure exponential growth throughout the duration of the experiment. To measure the degree of growth inhibition, we calculated the number of cell doublings per day under each condition. Many groups measure inhibition by comparing the final difference in cell number between treated and untreated wells (McDermott et al., 2007). However, we found that this measurement is confounded by differences in growth rates between cell lines such that, for a given degree of inhibition, cells that divide more rapidly will seem more affected than cells that divide

slowly.

Overall, JW-7-52-1 treatment substantially slowed the growth of some cell lines and had little effect on others (Figure 3A). For instance, Hela cells continued to proliferate under even the highest concentrations of JW-7-52-1, while the breast cancer cell line MCF7 declined below what was initially seeded. In cell lines where mTOR inhibition reduced cell numbers, there was no evidence of substantial apoptosis, suggesting that the anti-proliferative effects are primarily due to cell-cycle arrest (data not shown). This is consistent with a recent report showing that inhibition of both complexes using high concentrations of rapamycin caused a similar arrest (Shor et al., 2008). Low doses of rapamycin are also known to cause a G1/S cell-cycle arrest in tumors where it is an effective anti-proliferative agent (Johannessen et al., 2008). However, our finding that JW-7-52-1 is more potently anti-proliferative than rapamycin in more cell lines argues that its effects cannot be explained by mTORC1 inhibition alone (Figure 3B).

Previous studies have described a relationship between increased sensitivity to rapamycin and activation of PI3K signaling, either through mutations in PI3K or loss of PTEN (Neshat et al., 2001; Wendel et al., 2004). There is good theoretical rationale for this expectation as both mTOR complexes are downstream of PI3K and may by its primary effectors. Accordingly, we had expected tumor cell lines with PI3K/PTEN mutations to be more sensitive to mTOR inhibition with JW-7-52-1. However, we failed to detect a clear relationship between the two (Table 2). Although some PTEN-null cell lines were particularly sensitive, such as U87-MG and MCF7, others, such as HCT-116 and HT-29 cell lines were much less affected. Moreover, some of the most sensitive cell lines lacked obvious activating mutations in the PI3K/PTEN signaling pathway. MCL lines (ie. Rec-1, JVM-2) and MPNST lines (90-8T, S462) are all strongly inhibited by JW-7-52-1 but are mostly characterized by amplified Cyclin D1 expression (Table 2).

Conversely, we found a strong and unexpected relationship between mutations in the RAS/MAPK signaling pathway and resistance to JW-7-52-1 (Table 2). The RAS

proteins are family of small GTPases that are activated by growth factor signaling through RTKs such as the EGF-receptor (EGFR). Oncogenic mutations have been identified in three isoforms, KRAS, NRAS and HRAS, and are implicated in a wide variety of cancers (Rosen and She, 2006). Upon activation, RAS signals through a complicated cascade of downstream effectors that involves activation of the RAF kinases, the MEK1/2 kinases and the ERK1/2 kinases (Rosen and She, 2006). RAS can also activate PI3K directly (Rodriguez-Viciana et al., 1994). Our panel included 10 cell lines with canonical mutations in RAS pathway components, including KRAS, BRAF, and EGFR. Under 250nM JW-7-52-1 treatment, none of these cell lines declined in number and all were among the 14 least-inhibited cancer cell lines tested (excluding both MEF cell lines, which are non-transformed). Interestingly, some of these cell lines also carried PI3K or PTEN mutations, suggesting that RAS mutations can overcome whatever sensitivity to mTOR inhibition that PI3K or PTEN mutations might confer. The only exceptions to this pattern were the MPNST cell lines, which are defined by mutations in the complicated tumor suppressor NF1. NF1 contains a RAS-GAP domain that normally suppresses RAS signaling (Dasgupta and Gutmann, 2003). However, re-addition of the NF1 RAS-GAP domain to NF1-null cells does not suppress their transformed phenotype, indicating that loss of NF1 might drive tumor growth through other mechanisms (Dasgupta and Gutmann, 2003).

We also included two non-transformed MEF cell lines in our panel (Table 2). Both contained mutations in p53, which is necessary for immortalization, and one carried a mutation in the mTORC2 component mLST8 (listed as MEF and mLST8-null MEF). Treatment with JW-7-52-1 only mildly affected both cell lines. Rapamycin treatment, which should mimic general mTOR inhibition in mLST8-null cells because they already lack a functional mTORC2, also had little effect in both lines (Table 2). It is tempting to conclude that non-transformed cells are less sensitive to mTOR inhibition, an ideal therapeutic situation, though this will require further investigation.

Discussion

We have developed an mTOR inhibitor, JW-7-52-1, which potently suppresses the mTORC1 and mTORC2 complexes and thereby inhibits two key effectors of PI3K signaling. This inhibitor is strongly anti-proliferative against tumor cell lines representing a variety of cancers and mutational defects. However, we have made the surprising discovery that cell lines carrying mutations in RAS pathway components, including KRAS and BRAF, are largely resistant to mTOR inhibition. Interestingly, a similar phenomenon exists in yeast, where activation of RAS signaling can cause resistance to rapamycin (Schmelzle et al., 2004). These findings suggest that deregulated RAS and PI3K pathways converge on a common mechanism to drive tumor growth and fulfill partly redundant functions. Clinical evidence that PI3K and RAS pathway mutations are mutually exclusive in some cancers supports this theory by suggesting that there is little selective advantage for mutations in both pathways (Hollestelle et al., 2007; Velasco et al., 2006).

There is already known to be extensive cross talk between these two pathways in both normal and transformed cells. Activation of RAS promotes activation of PI3K signaling, both by direct interaction as well as through downstream components. Erk1/2, a downstream RAS effector, activates mTORC1 by phosphorylating and inhibiting the TSC complex(Ma et al., 2005). Rsk, which is a direct substrate of Erk1/2, can inhibit TSC through a similar mechanism, and also phosphorlyates a subset of sites on S6 that are targets of mTORC1 effector p70S6K (Roux et al., 2007). Conversely, activation of PI3K signaling suppresses RAS/MAPK pathway components. Both AKT and SGK can phosphorylate and inactivate the RAF kinases (Zhang et al., 2001; Zimmermann and Moelling, 1999). There are also indications that rheb can interact with and inhibit C-Raf (Karbowniczek et al., 2006). The sum of these observations suggest a negative feedback relationship between these two pathways, where RAS activates PI3K signaling, and PI3K

dampens RAS signaling by inhibiting downstream effectors.

Our results suggest that hyper-activation of RAS signaling can overcome the anti-proliferative effects of mTOR inhibition. Although hyperactive RAS signaling may accomplish this through mechanisms that are independent of mTOR status, several lines of evidence suggest that these two pathways converge on many common targets and may redundantly drive key growth- and proliferation-promoting processes. One example of this convergence is the eIF4F translation initiation complex, which promotes translation by coordinating the eIF4E mRNA cap binding -protein, the eIF3 translation factor, and the S6 ribosomal subunit. eIF4E is the limiting component, and preferentially promotes the translation of mRNAs with extensive secondary structure in the 5'-UTR, including the proto-oncogenes c-myc and cyclin D1 and the antiapoptotic gene MCL1 (Silva and Wendel, 2008). Partly through this relationship, eIF4E can drive tumorigenesis and transgenic overexpression in mice leads to the development of multiple cancers (Ruggero et al., 2004; Wendel et al., 2004). Effectors of both the RAS and mTOR pathways impinge upon eIF4F activity by phosphorylating several key regulatory factors. The mTORC1 complex phosphorylates and inactivates 4E-BP1, which otherwise binds to and suppresses eIF4E, and activates S6K, which phosphorylates a series of sites on S6 that promote its assembly into the pre-initiation complex (Guertin and Sabatini, 2007). The RAS/ERK regulated kinase Rsk phosphorylates overlapping sites on S6 to similar effect (Roux et al., 2007). Both Rsk and S6K have also been shown to regulate eIF4B, which mediates the association between S6 and eIF4F (Shahbazian et al., 2006). Additionally, ERK1/2 is the primary activator of the MNK1/2 kinases, which associate with and phosphorylate a critical regulatory site on eIF4E that is required for its oncogenic activity (Waskiewicz et al., 1999; Wendel et al., 2007). Through these mechanisms, activation of RAS or PI3K/mTOR pathways leads to increased translation of eIF4E-dependent proteins (Aktas et al., 1997; Albers et al., 1993).

Several studies have further demonstrated that the transforming ability of

PI3K/mTOR and RAS pathways depends on eIF4E activity. For instance, a mouse lymphoma model driven by overexpression of AKT1 is normally sensitive to rapamycin treatment(Wendel et al., 2004). Overexpression of eIF4E recapitulates the effects of AKT1, but generates tumors that are no longer sensitive to rapamycin, implying that AKT1 drives tumor growth through mTORC1-dependent control of eIF4E (Wendel et al., 2004). RAS-induced transformation has also been shown to depend on cyclin D1 expression, implying a similar requirement for eIF4E activity (Robles et al., 1998; Yu et al., 2001). Thus, it may be that activation of PI3K/mTOR signaling drives tumorigenesis in part by activating eIF4E-dependent processes, and that a hyperactive RAS pathway can overcome mTOR inhibition by activating some of the same processes (Figure 4). This model predicts that combined inhibition of RAS and mTOR pathways may be a particularly effective therapeutic strategy. Current evidence demonstrating the increased efficacy of combined EGFR and mTOR inhibition in some lung cancers supports this hypotheses, though there is clearly more work to be done (Li et al., 2007).

There are some discrepancies between our work and previous reports. In particular, Skeen et al showed that transformation by HRAS in mice is dependent on functional AKT1 signaling and is inhibited by rapamycin, contradicting our hypothesis that RAS activation can compensate for mTORC1 inhibition(Skeen et al., 2006). However, these differences might reflect differential requirements for RAS and PI3K/ mTOR signaling at early stages of tumor development. Another explanation is that Skeen et al used HRAS, while KRAS, NRAS and BRAF are more common defects in human cancers. Mutations in these components might have different dependencies on PI3K/ mTOR signaling for transformation.

Mutations in the PI3K/mTOR and RAS/MAPK pathways are implicated in many human cancers and are sufficient to initiate and maintain tumorigenesis in experimental systems. A key goal in cancer research is to understand how these pathways promote

unregulated cell growth and proliferation while evading the many cellular safeguards that normally prevent it. Both pathways are connected to many growth related processes, such as metabolism, survival, proliferation, protein synthesis, though it is unclear which of these is relevant to tumor growth. Our finding that mutational activation of RAS signaling correlates with insensitivity to mTORC1/2 inhibition in many tumor cell lines indicates that a common fundamental oncogenic mechanism may be under redundant control of both pathways. We have proposed that there is good rationale to suspect that this mechanism is eIF4E-dependent translation, though others, such as the NF-kappaB survival pathway and eEF2 translation control, are also downstream of both pathways (Ghosh et al., 2006; Wang et al., 2001). Answering this question should help to identify the most effective therapeutic targets.

Experimental procedures

Materials

Reagents were obtained from the following sources: purified active PI3K p110/ p85 alpha, P81 phosphocellulose, and antibodies to raptor from Upstate/Millipore; antibodies to mTOR and S6K1 as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, phospho-S473 Akt/PKB, phospho-T308 Akt/PKB, Akt (pan), phospho-T68 Chk2 from Cell Signaling Technology; FLAG M2 affinity gel, FLAG M2 antibody, ATP, and human recombinant insulin from Sigma Aldrich; protein G-sepharose and immobilized glutathione from Pierce; DMEM from SAFC Biosciences; LY294002, wortmannin and PI-103 from Calbiochem; PreScission protease from Amersham Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; Inactivated Fetal Calf Serum (IFS) and fetal bovine serum (FBS) from Invitrogen; CellTiter Glo, DNA-PK, and DNA-PK peptide from Promega; phosphatidylinositol and phosphatidylserine from Avanti Polar Lipids;

EasyTide 32P-gamma-ATP from PerkinElmer.

Cell Lines and Tissue Culture

$p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by David Kwiatkowski (Harvard Medical School). $p53^{-/-}$/$mLST8^{-/-}$ MEFs and AKT473D PC3 cells have been described elsewhere (Guertin et al., 2006; Sarbassov et al., 2006). 90-8T and S462 MPNST cells were provided by Karen Cichowski (Harvard Medical School). MCL cell lines (Rec-1, JVM-2, JVM-13, Jeko-11, NCEB-1, and Mino) were provided by Mark Raffeld (NCI). Other cell lines were obtained from ATCC. HEK-293T, HeLa, and MEFs were cultured in DMEM with 10% IFS. All others were cultured in DMEM with 10% FBS.

mTORC1 purification

To produce soluble mTORC1 we generated HEK-293T cell lines that stably express N-terminally FLAG-tagged Raptor using VSVG-pseudotyped MSCV retrovirus. mTORC1 was purified by lysing cells in 50mM Hepes pH 7.4, 10mM NaPyrophosphate, 10mM NaBetaglycerophosphate, 100mM NaCl, 2mM EDTA, 0.3% CHAPS. Cells were lysed at 4C for 30min, and the insoluble fraction was removed by microcentrifugation at 13,000RPM for 10min. Supernatants were incubated with FLAG-M2 mAb agarose (Sigma) for 1h, and then washed 3 times with lysis buffer and once with lysis buffer containing a final concentration of 0.5M NaCl. Purified mTORC1 was eluted with 100ug/ml 3X FLAG peptide (Sigma) in 50mM Hepes pH 7.4, 100mM NaCl. Eluate can be aliquoted and stored at -80C. Substrate p70S6K was purified as described previously (Sancak et al., 2007).

High-throughput mTORC1 kinase assay

Purified soluble mTORC1 kinase activity was assayed in standard 96-well format as follows: purified mTORC1 was combined with purified p70S6K in reaction buffer (25mM Hepes pH 7.4, 50mM KCl, 10mM MgCl2) and aliquoted into individual wells. 100uM ATP and test compound are added to each well, and reactions were allowed to proceed at 25C for 30min. Reactions were stopped by the addition of cold PBS and EDTA to a final concentration of 15mM. Reaction mixture is then transferred to MaxiSorp (NUNC) 96-well high-protein binding plates and incubated for 1h at 25C with gentle shaking. Wells are then aspirated and blocked with 5%BSA/PBST for 1h at 25C. Block is then aspirated and wells are incubated with primary antibody specific for phospho-S6K T389 in 5% BSA/PBST for 1h at 25C. Antibody mix is aspirated, and plates are washed 4X with PBST. Goat anti-rabbit IgG HRP-conjugated antibody (Pierce) in 5% BSA/PBST is added and plates are incubated for 1h at 25C. Antibody mix is aspirated again and wells are washed 4X PBST and 2X PBS. Chemiluminescense reagent (Perkin Elmer, Western Lightning) is added and luminescence is read using a standard luminescence reader.

Cellular and in vitro kinase assays

mTOR IC50s for top hits were validated in cell culture and in vitro according to previously published protocols (Sancak et al., 2007). Briefly, p53-/- MEFs were incubated with vehicle, 2, 10, 50 or 250nM compound for 1h and then lysed. Phosphorylation of p70S6K at T389 and phosphorylation of AKT at S473 were assessed by western blot and used as indicators for mTORC1 and mTORC2, respectively. For in vitro measurements of mTOR IC50s, mTORC1 or mTORC2 were immunoprecipitated from HEK-293T cells using antibody specific for raptor or rictor, respectively. Immunoprecipitates were washed and then combined with vehicle, 10, 50 or 250nM compound, full-length p70S6K or Akt1, and 500uM ATP in reaction buffer and allowed to proceed for 20min. Phosphorylation of substrates was assessed by western blot using phospho-specific

antibodies.

PI3K and hVps34

Cellular IC50s for PI3K were determined using mLST8-/- MEFs and PC3 that were engineered to express a phospho-mimetic 473D Akt1 mutant. Cells were treated with vehicle or increasing concentrations of compound for 1h and then lysed. Phosphorylation of Akt T308 was monitored by western blot using phospho-specific antibody. In vitro PI3K IC50s were determined as described previously (Knight et al., 2006). Briefly, chloroform stocks of phosphatidylinositol (PI) and phosphatidylserine (PS) were combined in equimolar ratios, dried under nitrogen gas, resuspended in 50mM Hepes pH 7.4, 100mM KCl, sonicated to clarity using a bath sonicator, and aliquoted and stored at –80C. For kinase assays, purified PI3Kalpha was combined with 100uM PS/PI, compound and 10uCi 32P-gamma-ATP (100uM final concentration) in kinase buffer, and incubated at 37C for 20min. Reactions were stopped with 1N HCl. Lipid was extracted with a 1:1 mixture of chloroform:methanol and separated on silica TLC plates. 32-P labeled PIP was quantitated by phosphorimager. hVps34 was GST-purified from HEK293T cells and assayed using the same procedure.

ATM, ATR and DNA-PK

For DNA-PK kinase assays, purified DNA-PK (Promega) was combined with DNA-PK peptide substrate (derived from the N-terminal sequence of p53), compound and 10uCi/rxn 32P-gamma-ATP (100uM final concentration) in kinase buffer, and incubated for 10min at 37C. Reactions were stopped with 1N HCl and spotted onto P81 phosphocellulose squares. P81 squares were washed 3X5min in 0.75% phosphoric acid, and 1X5min in acetone, dried and measured by scintillation counter. ATM and ATR in vitro kinase assays were performed according to previously published protocols (Knight

et al., 2006).

ATM activity was also measured in cells. Hela cells were treated with vehicle or compound for 30min, and then treated with 1uM doxorubicin for an additional 2h. Cells were lysed and phospho-T68 of Chk2 was assessed by western blot.

Cell proliferation

To determine optimal cell density, cell lines were seeded in 96-well plates at 500, 1000, 2000, 4000 and 8000 cells/well on Day 0. Cell density was quantitated at Day 1, Day 3 and Day 6 using CellTiter Glo (Promega). Seeding densities that permitted exponential growth through Day 6 were used for later experiments. For growth curves, cells were seeded in duplicate at Day 0 and treated with vehicle, 50nM rapamycin, or 2, 10, 50, or 1250nM JW-7-52-1 on Day 1. Cell density was measured using CellTiter Glo (CTG) on Day 1, Day 3, and Day 6. For measurements, CTG and cells were allowed to equilibrate at room temperature for at least 2h. 50ul of CTG was added to each well, plates were incubated on a shaker at RT for 12min, and then incubated at RT for an addition 8min. Luminescence was measured using a standard luminometer. Growth was measured as doublings/day and was calculated as $5/\log_2(\text{Day6/Day1})$.

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Figures

Figure 1. JW-7-52-1 potently inhibits mTOR but not PI3K

A. In wild-type (p53-/-) MEFs, JW-52-1 inhibits the phosphorylation of mTOR targets S6K and Akt. MEFs were treated with vehicle, JW-7-52-1 (2, 10, 50 and 250nM) or PI-103 (500nM) for 30min, lysed and analyzed by immunoblot using phospho-specific antibodies to detect phosphorylation of Akt on S473 and S6K on T389. S6K and pan-Akt antibodies were used to detect total S6K and total Akt, respectively.

B. JW-7-52-1 does not inhibit PI3K in mLST8-/-, p53-/- MEFs. MEFs were treated with vehicle, JW-7-52-1 (2, 10, 50 and 250nM) or PI-103 (500nM) for 30min, lysed and analyzed by immunoblot using phospho-specific antibodies to detect phosphorylation of Akt on T308. JW-7-52-1 has no effect on T308 phosphorylation up to 250nM, indicating that it does not inhibit PI3K activity in cells at concentrations where mTOR is potently inhibited.

Figure 2. JW-7-52-1 is highly selective for mTOR over related kinases

JW-7-52-1 IC50s were determined for mTORC1, PI3Kalpha, DNA-PK, hVps34, ATM and ATR using in vitro kinase assays. 500uM ATP was used in mTORC1 assays, while 100uM ATP was used for all other assays. Some IC50 values for PI-103 have been reported earlier (Knight et al., 2006).

Figure 3. JW-7-52-1 selectively inhibits growth of some tumor cells but not others

A. Response to 250nM JW-7-52-1 defines a sensitive and insensitive group of tumor cell lines. Cells were seeded in 96-well plates at Day 0 at densities that ensure exponential growth for the duration of the experiment. At Day 1, cells were treated with vehicle (DMSO), 50nM rapamycin or JW-7-52-1 (50, 250 or 1250nM). At Day 6 cells were lysed and wells were analyzed for ATP content, which is a proxy for cell number. Number of cell divisions/day was calculated as described in Experimental Procedures and used to determine relative rates of growth for each condition compared to vehicle control. Relative growth rates for cells treated with 250nM JW-7-52-1 are shown as a histogram.

B. JW-7-52-1 has greater anti-proliferative effects than rapamycin. Relative growth rates were calculated for cells treated with rapamycin or JW-7-52-1 at approximately 50-fold IC50s (50nM and 250nM, respectively) as in A. Growth rates were than plotted as points where the X-axis value is the growth rate under 50nM rapamycin and the Y-axis is the growth rate under 250nM

JW-7-52-1. The diagonal line delineates where the response to 50nM rapamycin is equal to 250nM JW-7-52-1. Points above this line identify cell lines that were inhibited more by JW-7-52-1 than by rapamycin.

C. Both sensitive and insensitive cell lines show incomplete inhibition of known Akt substrates. HeLa cells (insensitive) and MCF7 cells (sensitive) were treated for 30 or 60 minutes with 50nM JW-7-52-1. Lysates were analyzed by immunoblot using phospho-specific antibodies to detect phosphorylation of Akt on S473, PRAS40 on T246 and FoxO1 on T24.

Figure 4. RAS/ERK and PI3K/mTOR pathways converge on the translational apparatus to drive tumorigenesis

RAS/ERK and PI3K/mTOR converge on the eIF4F, eIF3, and S6 translational complex. The RAS pathway drives cap-dependent translation by activating eIF4E (via MNK1/2, not shown) and S6 (through Rsk). Furthermore, RAS/ERK signaling can activate the mTORC1 pathway by ERK-dependent inactivation of TSC. PI3K/mTOR signaling similarly drives cap-dependent translation by phosphorylation of S6 (through S6K), inactivation of the translational inhibitor (4E-BP1) and phosphorylation of other sites within the eFI4E complex. Activation of eIF4E is sufficient to drive tumorigenesis in some systems, and may be a mechanism that both RAS and PI3K/mTOR-driven cancers engage. Because of the overlapping functionality, inhibition of only one or the other pathway in established tumors may be insufficient to halt tumor growth.

Table 1. JW-7-52-1 shows very few off-target effects in a panel of 353 kinases

JW-7-52-1 was tested at 10uM against a panel of 353 kinases by Ambit Biosystems. The relative binding score reflects the affinity of the indicated kinase for JW-7-52-1 and is proportional though not equivalent to the Kd. For kinases where no number is listed there was no measurable binding. As an indication for how these numbers translate to IC50s, the binding score for PI3Kalpha is 1.5 and the IC50 is 1.8uM.

Table 2. mTOR inhibition selectively inhibits the growth of a subset of tumor cell lines

Cells were seeded in 96-well plates at Day 0 at densities that ensure exponential growth for the duration of the experiment. At Day 1, cells were treated with vehicle (DMSO), 50nM rapamycin or 250nM JW-7-52-1 (approximately 50-fold greater than mTOR IC50s for each compound). At Day 6 cells were lysed and wells were analyzed for ATP content, which is a proxy for cell number. Number of cell divisions/day was calculated as described in Experimental Procedures and used to determine relative rates of growth for each condition compared to vehicle control. Cell lines are listed in ascending order by relative growth rate under 250nM JW-7-52-1. For

each cell line, the table lists: doubling time (days), relative growth under each condition, mutational background according to the Sanger Centre Cancer Cell Genome project (http://www.sanger.ac.uk/genetics/CGP/), and tissue origin.

Figure 1

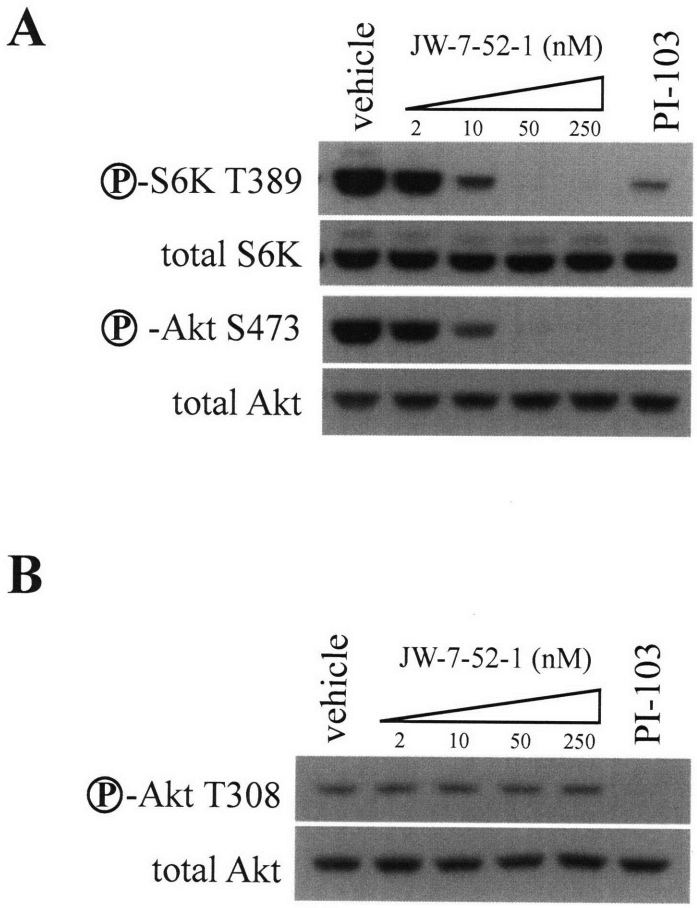

A
vehicle
JW-7-52-1 (nM)
2
10
50
250
PI-103
Ⓟ-S6K T389
total S6K
Ⓟ -Akt S473
total Akt
B
vehicle
JW-7-52-1 (nM)
2
10
50
250
PI-103
Ⓟ-Akt T308
total Akt

Figure 2

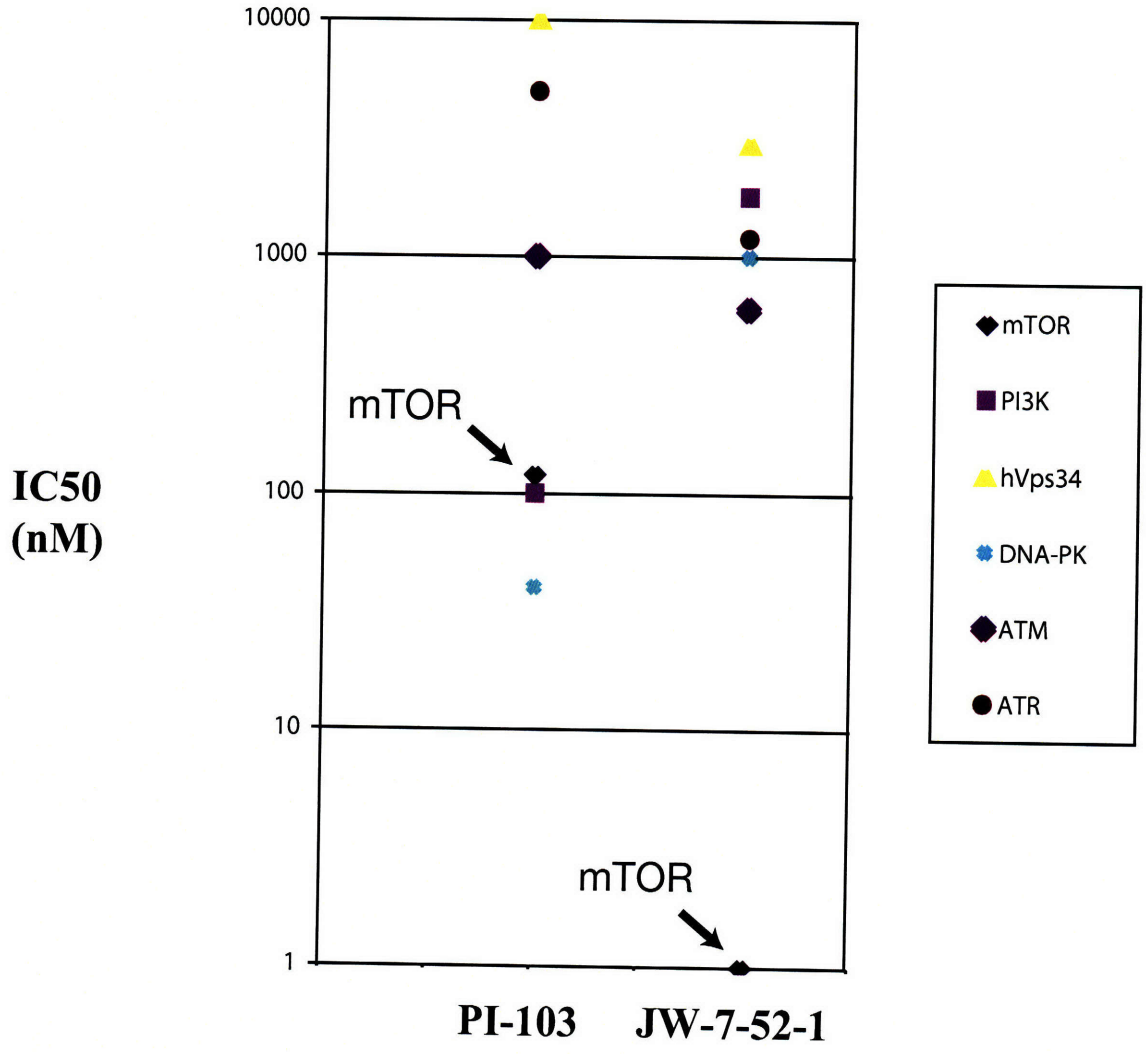
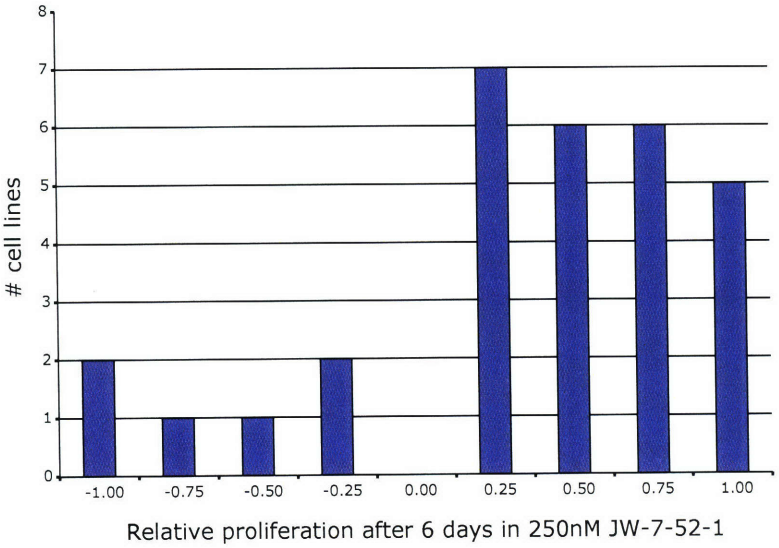
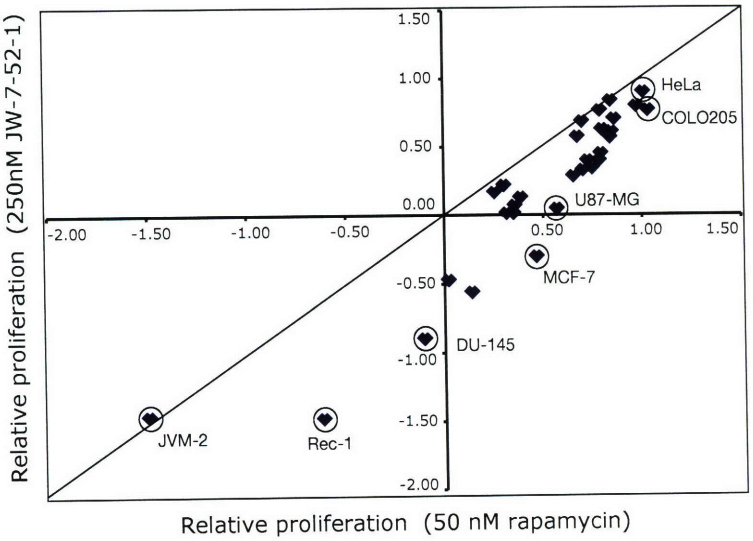

IC50
(nM)
10000
1000
100
10
1
mTOR
mTOR
PI-103
JW-7-52-1
mTOR
PI3K
hVps34
DNA-PK
ATM
ATR

Figure 3

A


cell lines
0
1
2
3
4
5
6
7
8
-1.00
-0.75
-0.50
-0.25
0.00
0.25
0.50
0.75
1.00
Relative proliferation after 6 days in 250nM JW-7-52-1

B


Relative proliferation (250nM JW-7-52-1)
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.00
-1.50
-1.00
-0.50
0.50
1.00
1.50
HeLa
COLO205
U87-MG
MCF-7
DU-145
JVM-2
Rec-1
Relative proliferation (50 nM rapamycin)

C

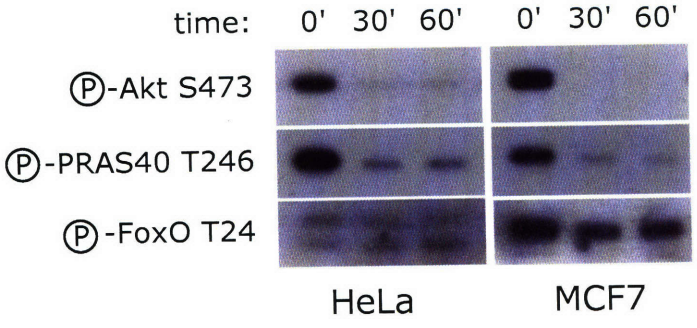

time: 0' 30' 60' 0' 30' 60'
Ⓟ-Akt S473
Ⓟ-PRAS40 T246
Ⓟ-FoxO T24
HeLa
MCF7

Figure 4

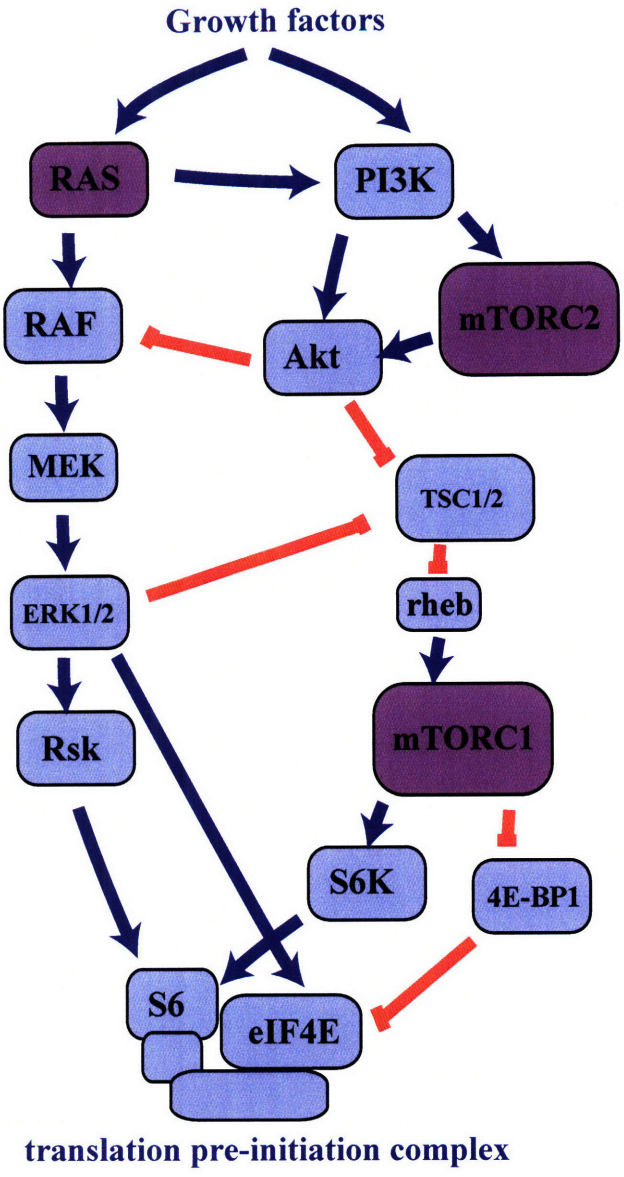

Growth factors
RAS
PI3K
RAF
Akt
mTORC2
MEK
TSC1/2
ERK1/2
rheb
Rsk
mTORC1
S6K
4E-BP1
S6
eIF4E
translation pre-initiation complex

Table 1

Kinase ID	Relative binding	Kinase ID	Relative binding	Kinase ID	Relative binding	Kinase ID	Relative binding
AAK1		EGFR(L861Q)		MLCK		RPS6KA6(Kin.Dom.2)	
ABL1		EGFR(S752-I759del)		MLK1		SgK085	
ABL1(E255K)		EPHA1		MLK2		SgK110	
ABL1(F317I)		EPHA2		MLK3		SLK	
ABL1(F317L)		EPHA3		MRCKA		SNARK	
ABL1(H396P)		EPHA4		MRCKB		SNF1LK	
ABL1(M351T)		EPHA5		MST1		SNF1LK2	25
ABL1(Q252H)		EPHA6		MST1R		SRC	
ABL1(T315I)	33	EPHA7		MST2		SRMS	
ABL1(Y253F)		EPHA8		MST3		SRPK1	
ABL2		EPHB1		MST4		SRPK2	
ACVR1		EPHB2		MUSK		SRPK3	
ACVR1B		EPHB3		MYLK		STK16	25
ACVR2A		EPHB4		MYLK2		STK33	
ACVR2B		ERBB2		MYO3A		STK35	28
ACVRL1		ERBB4		MYO3B		STK36	18
ADCK3	15	ERK1		NDR2		SYK	
ADCK4	23	ERK2		NEK1		TAK1	
AKT1		ERK3		NEK2		TAOK1	
AKT2		ERK4		NEK5		TAOK3	
AKT3		ERK5		NEK6	6.4	TEC	
ALK		ERK8		NEK7		TESK1	
AMPK-alpha1		FER		NEK9		TGFBR1	
AMPK-alpha2		FES		NLK		TGFBR2	
ANKK1		FGFR1		p38-alpha		TIE1	
ARK5		FGFR2		p38-beta	25	TIE2	25
AURKA		FGFR3		p38-delta		TLK1	
AURKB		FGFR3(G697C)		p38-gamma		TLK2	
AURKC		FGFR4		PAK1		TNIK	
AXL		FGR		PAK2		TNK1	15
BIKE		FLT1		PAK3	20	TNK2	
BLK		FLT3		PAK4		TNNI3K	
BMPR1A		FLT3(D835H)		PAK6		TRKA	
BMPR1B		FLT3(D835Y)		PAK7/PAK5		TRKB	
BMPR2		FLT3(ITD)		PCTK1		TRKC	
BMX		FLT3(K663Q)		PCTK2	29	TSSK1	
BRAF		FLT3(N841I)		PCTK3		TTK	30
BRAF(V600E)		FLT4		PDGFRA		TXK	
BRSK1		FRK		PDGFRB	26	TYK2(Kin.Dom.1)	
BRSK2		FYN		PDPK1		TYK2(Kin.Dom.2)	
BTK		GAK		PFTAIRE2		TYRO3	
CAMK1		GCN2(Kin.Dom.2,S808G)		PFTK1		ULK1	
CAMK1D		GSK3A		PHKG1		ULK2	
CAMK1G		GSK3B		PHKG2		ULK3	
CAMK2A		HCK		PIK3C2B		VEGFR2	26
CAMK2B		HIPK1		PIK3CA		WEE1	
CAMK2D		IGF1R		PIK3CA(E545K)		WEE2	
CAMK2G		IKK-alpha		PIK3CB		YANK2	28
CAMK4		IKK-beta		PIK3CD		YANK3	
CAMKK1		IKK-epsilon		PIK3CG		YES	34
CAMKK2		INSR		PIM1		YSK1	
CDC2L1		INSRR		PIM2		ZAK	
CDC2L2		IRAK3		PIM3		ZAP70	23
CDK11	2	ITK		PIP5K1A			
CDK2		JAK1(Kin.Dom.1)		PIP5K2B	27		
CDK3		JAK1(Kin.Dom.2)		PKAC-alpha			
CDK5		JAK2(Kin.Dom.2)		PKAC-beta			
CDK7		JAK3(Kin.Dom.2)		PKMYT1			
CDK8	13	JNK1		PKN1	33		
CDK9		JNK2		PKN2	33		
CDKL2		JNK3		PLK1			
CHEK1		KIT		PLK3			
CHEK2		KIT(D816V)		PLK4			
CIT		KIT(V559D)		PRKCD			
CLK1		KIT(V559D,T670I)		PRKCE	19		
CLK2		KIT(V559D,V654A)		PRKCH			
CLK3		LATS1		PRKCQ			
CLK4		LATS2		PRKD1			
CSF1R		LCK		PRKD2			
CSK		LIMK1		PRKD3			
CSNK1A1L		LIMK2		PRKG1			
CSNK1D		LKB1		PRKG2	15		
CSNK1E		LOK		PRKR	6.8		
CSNK1G1		LTK		PRKX			
CSNK1G2		LYN		PTK2			
CSNK1G3		MAP3K3		PTK2B			
CSNK2A1		MAP3K4		PTK6			
CSNK2A2		MAP3K5		RAF1	33		
DAPK1		MAP4K1		RET			
DAPK2		MAP4K2		RET(M918T)			
DAPK3		MAP4K3		RET(V804L)	25		
DCAMKL1		MAP4K4		RET(V804M)	15		
DCAMKL2		MAP4K5		RIOK1	18		
DCAMKL3		MAPKAPK2		RIOK2	10		
DDR1		MAPKAPK5		RIOK3	16		
DDR2		MARK1		RIPK1			
DLK		MARK2		RIPK2	3		
DMPK		MARK3		RIPK4			
DMPK2	3	MARK4		ROCK2			
DRAK1		MEK1		ROS1			
DRAK2		MEK2		RPS6KA1(Kin.Dom.1)			
DYRK1B		MEK3		RPS6KA1(Kin.Dom.2)			
EGFR		MEK4		RPS6KA2(Kin.Dom.1)			
EGFR(E746-A750del)		MEK6		RPS6KA2(Kin.Dom.2)			
EGFR(G719C)		MELK		RPS6KA3(Kin.Dom.1)			
EGFR(G719S)		MERTK		RPS6KA4(Kin.Dom.1)			
EGFR(L747-E749del, [illegible]		MET		RPS6KA4(Kin.Dom.2)			
EGFR(L747-S752del, [illegible]		MINK		RPS6KA5(Kin.Dom.1)			
EGFR(L747-T751del,Sins)		MKNK1		RPS6KA5(Kin.Dom.2)			
EGFR(L858R)		MKNK2		RPS6KA6(Kin.Dom.1)			

Table 2

Cell line	Rel. Proliferation (50nM Rapa)	Rel. Proliferation (250nM JW)	Mutations	Tissue Origin
Rec-1	-0.62	-1.47	CYLD	MCL
JVM-2	-1.50	-1.45	CYLD	MCL
DU 145	-0.10	-0.89	TP53, STK11, RB1, CDKN2A	Prostate
90-8T	0.14	-0.55	NF1	Peripheral nerve
MDA-MB-453	0.02	-0.46	PI3K, CDH1	Breast
MCF7	0.47	-0.29	PI3K, CDKN2A	Breast
BT-20	0.35	0.02	TP53, PI3K, CDKN2A	Breast
S462	0.32	0.02	NF1	Peripheral nerve
U-87-MG	0.57	0.05	PTEN	Brain
A431	0.36	0.08	TP53	Skin
JVM-13	0.39	0.14	CYLD	MCL
Jeko-1	0.26	0.17	CYLD	MCL
NCEB-1	0.30	0.23	CYLD	MCL
Mino	0.66	0.29	CYLD	MCL
SK-MEL-28	0.71	0.33	TP53, EGFR, BRAF	Skin
A549	0.75	0.35	KRAS, STK11, CDKN2A	Lung
DLD-1	0.73	0.40	PI3K, KRAS	Colorectal
A2058	0.79	0.40	TP53, PTEN, BRAF	Skin
786-O	0.80	0.46	TP53, PTEN, VHL, CDKN2A	Kidney
MDA-MB-231	0.84	0.57	TP53, NF2, KRAS, CDKN2A, BRAF	Breast
PC-3	0.68	0.58	TP53, PTEN	Prostate
HT-29	0.85	0.62	TP53, PI3K, APC, SMAD4, BRAF	Colorectal
293E	0.80	0.63	E1A	Kidney, embryonic
mLST8-null MEF	0.70	0.69	TP53, mLST8	Embryonic
SW620	0.86	0.71	TP53, KRAS, APC	Colorectal

Chapter 4

mTORC2 is necessary for Akt/PKB turn motif phosphorylation

Carson C. Thoreen, David A. Guertin, Andrew L. Markhard, and David M. Sabatini

Experiments in Figure 1 were performed by D.A.G.
Experiments in Figure 2 and Figure 3 were performed by C.C.T.

Summary

The Akt/PKB kinase is hyperactive in a large fraction of human cancers and its regulation is an area of intense study. Growth factor-induced stimulation of PI3K leads to the phosphorylation of Akt/PKB at two sites, T308 and S473, which are necessary for its activity. PDK1 is the kinase for T308 while mTORC2, a multi-component protein kinase containing mTOR as its catalytic subunit, appears to be the principal kinase for S473. In addition to these regulated phosphorylation sites, Akt/PKB is phosphorylated at T450, also known as the turn motif site. This phosphorylation is largely constitutive and is not stimulated by growth factors. Here, we demonstrate that two core mTORC2 components, rictor and mLST8/GβL, are required for the phosphorylation of T450 within cells. However, while in vitro mTORC2 readily phosphorylates wild-type or kinase-dead Akt/PKB on S473, it does not phosphorylate Akt/PKB on T450. In addition, treatment of cells with a small molecule inhibitor of mTORC2 that directly inhibits the mTOR kinase domain eliminates S473 phosphorylation of Akt/PKB but has no acute effect on T450 phosphorylation. However, longer durations of mTOR inhibition decrease total levels of Akt/PKB as well as T450 phosphorylation. We conclude that mTORC2 likely regulates an unidentified kinase that phosphorylates the T450 site on newly translated Akt/PKB, thereby stabilizing the active conformation of the protein.

Introduction

The Akt/PKB kinase becomes hyperactive in tumors with deregulated PI3K signaling (reviewed in (Manning and Cantley, 2007)). Because of the connection of Akt/PKB to human disease there is much interest in understanding how it is regulated. In response to the production of phosphatidylinositol 3,4,5-trisphosphate, Akt/PKB is recruited to the plasma membrane through its pleckstrin homology (PH) domain. At the membrane it is phosphorylated on two sites, T308 in the activation loop, and S473 in the hydrophobic motif of the C-terminal tail (Alessi et al., 1996). Phosphorylation at both sites is needed for full Akt/PKB activation and is greatly stimulated by many growth factors, such as insulin. Substantial evidence suggests that the kinases that phosphorylate these sites are PDK1 and mTORC2, respectively (reviewed in (Guertin and Sabatini, 2007)).

In addition to phosphorylation at T308 and S473, Akt/PKB is also phosphorylated on T450 (Alessi et al., 1996), although in this case the phosphorylation is constitutive and not regulated by growth factors (Alessi et al., 1996; Bellacosa et al., 1998; Hauge et al., 2007). T450 is within a motif present in other members of the AGC kinase that has been called the turn motif (Toker and Newton, 2000), and, more recently the zipper motif (Hauge et al., 2007). Elegant recent work (Hauge et al., 2007) suggests that the function of the phosphorylated turn motif is to bind to the catalytic domain and stabilize an active conformation. This interaction may also help protect against dephosphorylation of the hydrophobic motif in some AGC kinases, such as S6K1 (Hauge et al., 2007), although this is unlikely to be the case for Akt/PKB because mutants of Akt/PKB that cannot be phosphorylated on T450 show normal levels of S473 phosphorylation. The identity of the Akt/PKB turn motif kinase remains unknown. We show that mTORC2 serves an essential role in the phosphorylation of the Akt/PKB turn motif site, but that it is unlikely to be the

direct T450 kinase.

Experimental procedures

Materials

Reagents were obtained from the following sources: protein G-sepharose and glutathione sepharose from Pierce; HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; Rictor antibodies from Bethyl Laboratories; phospho-473 Akt, phospho-T308 Akt, and phospho-T450 Akt, and mTOR antibodies from Cell Signaling Technologies; HA monoclonal antibody from Covance; DMEM, from Life Technologies; and rapamycin and PI-103 from Calbiochem.

Cell Lines

Murine embryonic fibroblasts (MEFs) null for rictor or mLST8/GβL were obtained from mice, cultured, and analyzed by immunoblotting as described (Guertin et al., 2006). PC3, HeLa, and HEK-293T cells were cultured as described (Sarbassov et al., 2006).

In Vitro Kinase Substrates

cDNAs for full-length wild-type and K179M kinase dead Akt1/PKB1 (from D. Kaplan) were sub-cloned into HA-GST PRK5 (Burnett et al., 1998). Plasmids were transfected into HEK-293T cells and HA-GST fusion proteins were purified as described (Burnett et al., 1998). Prior to purification cells were treated with 1 μM PI-103 for 1h to eliminate S473 phosphorylation. To dephosphorylate Akt1/PKB1 in vitro, HA-GST-Akt still bound to glutathione agarose was treated with 200 U lambda phosphatase (New England Biolabs) for 15 min at 30° C. After washing 3 x 1 ml with lysis buffer and 1 x 1 ml with wash buffer as described (Burnett et al., 1998), proteins were eluted as described (Sancak et al., 2007).

Immunoblots, Immunoprecipitations, and Kinase Assays

mTORC2 immunopurifications and assays of its kinase activity were performed as described (Sarbassov et al., 2005) as were all immunoblots (Sarbassov et al., 2004).

Lentiviral shRNAs

Lentiviral vectors expressing shRNAs targeting mouse mTOR or rictor were obtained from the TRC (Moffat et al., 2006) and used as described (Sarbassov et al., 2005).

Results and Discussion

To ask if mTORC2 is necessary for the phosphorylation of T450 of Akt/PKB, we analyzed by immunoblotting murine embryonic fibroblasts (MEFs) isolated from wild-type and rictor-null mouse embryos. Rictor is a component of mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004) and, as previously reported (Guertin et al., 2006; Shiota et al., 2006), is required for S473 but not T308 phosphorylation of Akt/PKB in these cells. Consistent with previous reports showing that Akt/PKB turn motif phosphorylation is constitutive (Bellacosa et al., 1998; Hauge et al., 2007), T450 phosphorylation was high in both serum-deprived and insulin-stimulated wild-type MEFs, while phosphorylation of S473 and T308 was dependent on insulin (Fig. 1A). In contrast, T450 phosphorylation was absent in MEFs lacking rictor, indicating that mTORC2 is necessary for the phosphorylation of Akt/PKB on the turn motif site (Fig. 1B). To expand upon these results we analyzed MEFs lacking mLST8/GβL, a component of mTORC2 that, like rictor, is essential for it to phosphorylate S473 within cells and in vitro (Guertin et al., 2006). As with the rictor-null MEFs, loss of mLST8/GβL eliminates phosphorylation of both S473 and T450 of Akt/PKB (Fig. 1C). Thus, these data indicate that mTORC2 components are necessary within cells for the phosphorylation of Akt/PKB on both its hydrophobic (S473) and turn motif (T450) sites.

Because mTORC2 is known to directly phosphorylate Akt/PKB on S473 (Sarbassov et al., 2005), we hypothesized that it may also be the direct kinase for T450. To test this hypothesis we used a rictor antibody to immunopurify mTORC2 and incubated it in a kinase reaction with recombinant wild-type (WT) or kinase-dead (KD) Akt1/PKB1 purified from HEK-293T cells under conditions where S473 is dephosphorylated. As expected, mTORC2 readily phosphorylated both forms of Akt/PKB on S473 (Fig. 2). It is worth noting that this finding is consistent with the proposal that mTORC2 directly phosphorylates Akt/PKB (Sarbassov et al., 2005) and inconsistent with a potential alternative model in which mTORC2 binds Akt/PKB and stimulates it to autophosphorylate itself on S473. In contrast, mTORC2 failed to increase the phosphorylation of T450 of either wild-type or kinase-dead Akt/PKB (Fig. 2). We reasoned that T450 might already be fully phosphorylated in our purified recombinant Akt/PKB, precluding the possibility of additional phosphorylation in vitro. To test this, we dephosphorylated Akt/PKB in vitro with lambda phosphatase to eliminate all phosphorylation and repeated the experiment. As before, mTORC2 readily phosphorylated S473 but had no effect on T450 (Fig. 2). Accordingly, we conclude that mTORC2 is unable to phosphosphorylate T450 under in vitro conditions in which it robustly phosphorylates S473, and is therefore unlikely to be the direct kinase in cell. It remains formally possible that mTORC2 directly phosphorylates T450 of newly synthesized and incompletely folded Akt in cells, but not the mature version of the protein used in vitro, though we consider this unlikely.

A second hypothesis to explain the absence of T450 phosphorylation in rictor or mLST8/GβL null MEFs is that a downstream target of the mTORC2 signaling pathway, but not mTORC2 itself, is responsible for phosphorylating the Akt/PKB turn motif site. To test this model, we used PI-103, a recently described dual mTORC2 and PI3K ATP-competitive inhibitor (Knight et al., 2006), to pharmacologically inhibit mTORC2

in MEFs. As expected, PI-103 eliminated the phosphorylation of Akt/PKB at S473. Surprisingly, it had no effect on the phosphorylation of T450, even after two days of treatment (Fig. 3A). We confirmed these results in two cancer cell lines, HeLa and PC3 cells. In both cell types, PI-103 had no effect on T450 phosphorylation even after 48 hours of treatment despite eliminating phosphorylation on T308 and S473 (Fig. 3B). However, recent work has suggested that T450 phosphorylation serves a structural role and might be inaccessible to mTOR in folded protein {Hauge, 2007 #2971}. To ask whether mTORC2 activity was required for T450 phosphorylation of newly synthesized protein, we transiently expressed Akt/PKB in cells that were already treated with PI-103. Under these conditions, the T450 site of recombinant Akt/PKB was completely dephosphorylated (Fig. 3C). Total levels of Akt/PKB were also suppressed, supporting a structural role for T450 phosphorylation (Fig. 3C).

mTORC2 is a large protein complex with many components, including mTOR, rictor, mSin1, mLST8/GβL, and the recently identified protor (Pearce et al., 2007; Thedieck et al., 2007; Woo et al., 2007). Given its apparent structural complexity, it is reasonable to believe that it senses many diverse signals and is capable of performing multiple functions. So far, the only molecular function ascribed to it is the direct phosphorylation of Akt/PKB on S473. Here we present evidence that mTORC2 has an indirect role in the phosphorylation of T450 of the turn motif of Akt/PKB as well. In support of this we find that loss of essential mTORC2 components inhibits T450 phosphorylation, but that purified mTORC2 is unable to phosphorylate this site in vitro. Moreover, pharmacological inhibition of mTORC2 kinase activity in cells only suppresses T450 phosphorylation of newly synthesized protein. Although it remains possible that mTORC2 phosphorylates this site directly in cells but not in vitro, we consider it more likely that mTORC2 regulates an unidentified kinase that phosphorylates T450 of Akt/PKB as it is translated, potentially reflecting a mechanism for adjusting total

levels of competent Akt/PKB.

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Competing financial interests

The authors declare that they have no competing financial interests.

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Figures

FIG. 1. mTORC2 is required for phosphorylation of Akt/PKB at T450.

A. Insulin stimulates the phosphorylation of the hydrophobic motif (S473) and activation loop (T308), but not the turn motif (T450) of Akt/PKB. Wild-type MEFs were serum-starved for 8 hours or serum-starved and then stimulated with 500 nM insulin for 15 minutes, and analyzed by immunoblotting for the indicated proteins.

B. Rictor is required for phosphorylation of Akt/PKB at S473 and T450. Lysates from wild-type and rictor-null MEFs under normal growth conditions were analyzed by immunoblotting for the indicated proteins.

C. mLST8/GβL is also required for phosphorylation of Akt/PKB at T450. Lysates from wild-type and mLST8-null MEFs under normal growth conditions were analyzed by immunoblotting for the indicated proteins.

D. RNAi-mediated knockdown of mTOR shows that it too is required to maintain normal levels of T450 phosphorylation of Akt/PKB. Lysates from wild-type MEFs infected with the indicated shRNAs and growing under normal conditions were analyzed by immunoblotting for the specified proteins.

FIG. 2. In vitro mTORC2 phosphorylates wild-type and kinase-dead Akt/PKB on S473 but not T450.

Immunoblot analysis of in vitro kinase assays using rictor immunoprecipitates from HEK-293T cells as the source of mTORC2 and recombinant wild-type (WT) or kinase-dead (KD) HA-GST-Akt1/PKB1 as the substrates. Kinase assays were performed as described in the presence or absence of 1 μM PI-103. Where indicated Akt1/PKB1 that had been de-phosphorylated with lambda protein phosphatase was used. Kinase assays were analyzed by immunoblotting for the phosphorylation of Akt/PKB at T450 and S473 using phospho-specific antibodies and for total levels of Akt/PKB using an HA-specific antibody.

FIG. 3. Short and long-term pharmacological inhibition of mTOR kinase activity does not affect the phosphorylation of Akt/PKB on T450.

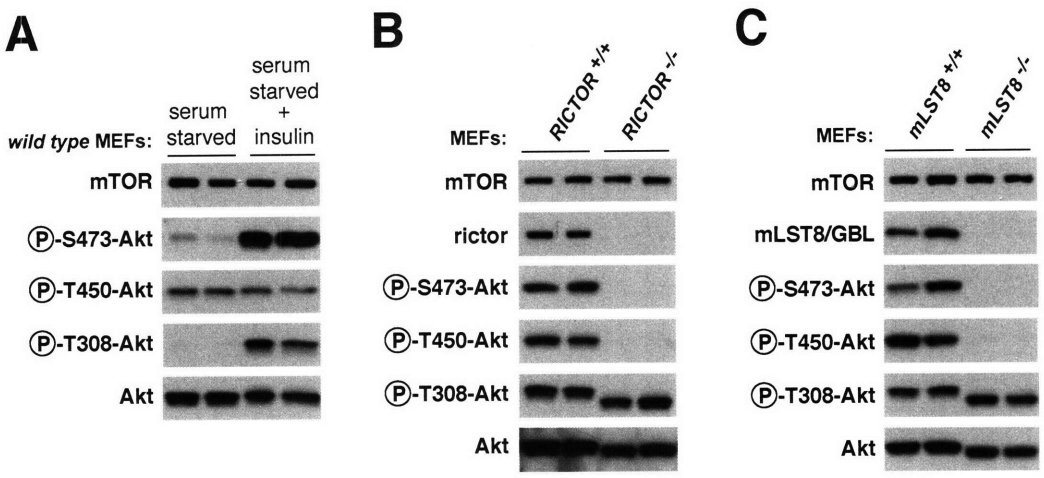
A. In wild-type MEFs inhibition of mTORC2 with PI-103 prevents the phosphorylation of Akt/PKB on S473, but not T450. Wild-type MEFs were treated with 250 nM PI-103 or DMSO vehicle for 1 or 48 hours as indicated. Lysates were analyzed by immunoblotting using phospho-specific antibodies to detect the phosphorylation of Akt/PKB S473 and T450 and pan-Akt antibodies to detect total Akt/PKB.

B. In HeLa and PC3 cells inhibition of mTORC2 with PI-103 prevents the phosphorylation of

Akt/PKB on S473, but not T450. HeLa and PC3 cells were treated and analyzed as described in *A* and in addition samples were analyzed for total levels of mTOR and rictor.

C. In wild-type MEFs inhibition of mTORC2 with PI-103 prevents phosphorylation of newly translated Akt/PKB on T450. Wild-type MEFs were treated with 500nM PI-103, 50nM rapamycin or DMSO vehicle for 1h. Cells were then transfected with 500ng HA-GST-tagged Akt/PKB and incubated for 20h. Lysates were analyzed for phosphorylation of Akt/PKB T450 as described in *A*.

Figure 1


A
wild type MEFs:
serum starved
serum starved + insulin
mTOR
Ⓟ-S473-Akt
Ⓟ-T450-Akt
Ⓟ-T308-Akt
Akt
B
MEFs:
RICTOR +/+
RICTOR -/-
mTOR
rictor
Ⓟ-S473-Akt
Ⓟ-T450-Akt
Ⓟ-T308-Akt
Akt
C
MEFs:
mLST8 +/+
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Ⓟ-T450-Akt
Ⓟ-T308-Akt
Akt

Figure 2

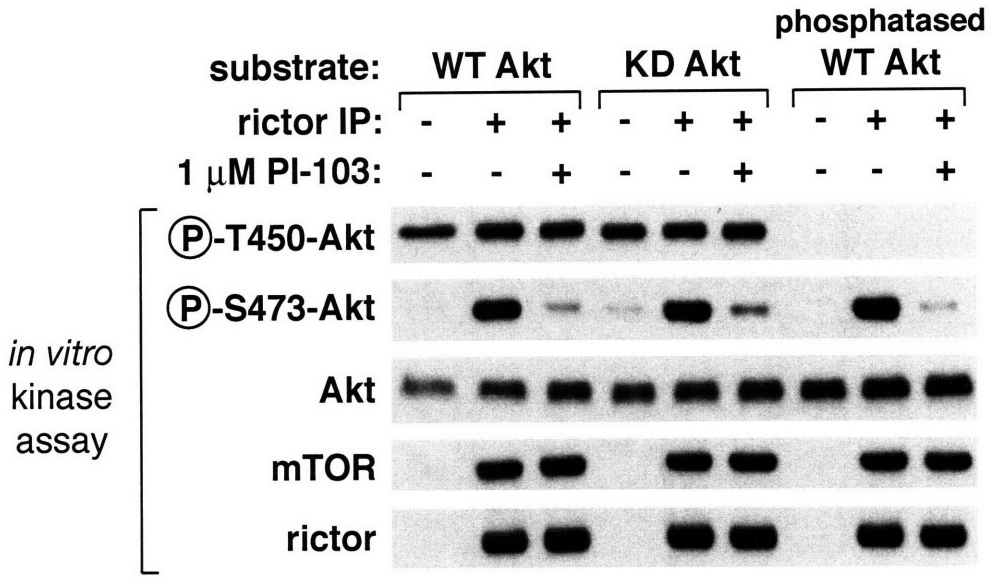
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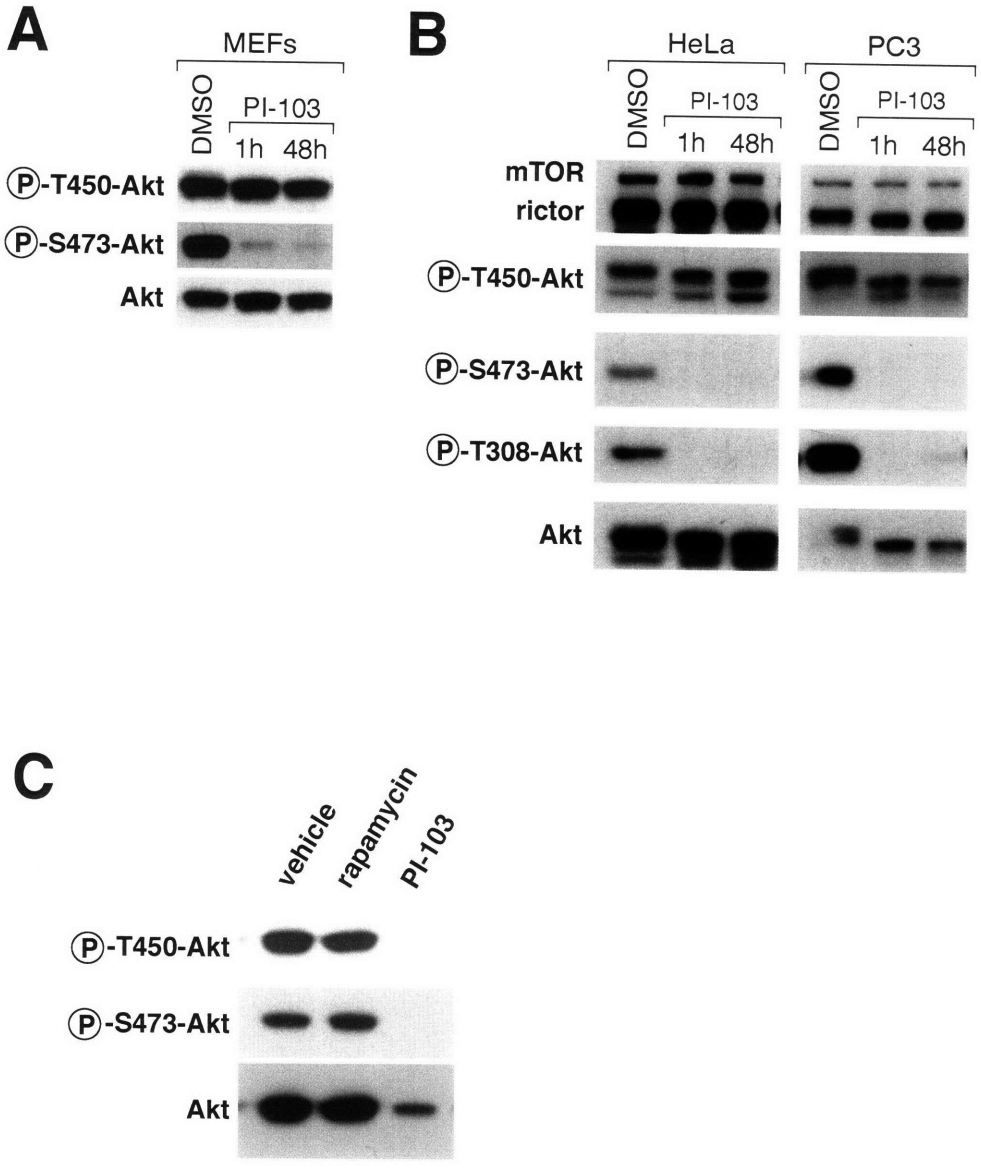
Figure 3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Ⓟ-S473-Akt
Ⓟ-T308-Akt
Akt
C
vehicle
rapamycin
PI-103
Ⓟ-T450-Akt
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Chapter 5

Future Directions

Summary

The mTORC1 and mTORC2 complexes are conserved central regulators that coordinate protein translation, nutrient uptake, metabolism and cell growth in response to growth factor and nutrient cues. In the work described here, we have explored the biochemical mechanisms that connect the mTOR complexes to upstream signals and developed novel tools to probe their function in normal and diseased physiology. In particular, the discovery of negative regulator PRAS40 has clarified how insulin regulates mTORC1 activity. In the absence of insulin, PRAS40 ensures that mTORC1 is inactivated, and thereby limits the energy expensive process of protein synthesis, and maintains cell's sensitivity to insulin signaling. We have also developed a novel, specific and potent inhibitor of mTOR, JW-7-52-1. Against a panel of tumor cell lines, JW-7-52-1 treatment elicited remarkable anti-proliferative effects that were far greater than those caused by inhibition of mTORC1 alone. We also made the surprising discovery that tumor cell lines with mutationally activated RAS signaling were largely resistant to mTOR inhibition, suggesting that the RAS and PI3K signaling pathways might converge on a common mechanism to drive tumorigenesis. We hope that this compound will eventually be therapeutically useful, but also believe that it provides a unique research tool. The following section explores some of the new questions that have emerged from this work.

What mTORC2 substrates are important for tumorigenesis?

Inhibition of mTOR with JW-7-52-1 prevents the phosphorylation of AKT at Ser473 and suppresses the proliferation of many tumor cell lines. Ser473 phosphorylation is required for full AKT activity, and so we were surprised to find that many established

substrates of AKT, such as GSK3, TSC2 and FoxO remained phosphorylated even when mTORC2 is inhibited. A similar phenomenon occurs in MEFs and Drosophila where TORC2 has been genetically inactivated by deletion of rictor (Guertin et al., 2006; Hietakangas and Cohen, 2007). Both observations suggest that AKT maintains a basal activity that doesn't depend on Ser473 phosphorylation or that a TORC2-independent kinase can compensate for AKT inhibition. The former hypothesis seems more likely because deletion of AKT in drosophila is lethal, while deletion of mTORC2 components is not(Hietakangas and Cohen, 2007). Moreover, unlike many AGC kinases, AKT can be phosphorylated at its activation loop site T308 in the absence of phosphorylation at Ser473, allowing it to bypass the mTORC2 input under certain circumstances. The mystery then becomes how does mTORC2 inhibition suppress the proliferation of so many tumor cell lines without fully inhibiting its only known downstream target?

One hypothesis is that mTORC2 activity is dispensable for normal cell growth and only required to mediate the kind of hyperactive PI3K signaling that occurs during tumor cell proliferation or tissue development (Guertin et al., 2006; Hietakangas and Cohen, 2007). This would imply that tumor cells in culture are adapted, or "addicted," to hyperactive AKT, and arrest when the kinase is forced to function at basal levels. To test this, we are generating cell lines that are normally sensitive to JW-7-52-1, but express the phospho-mimetic AKT1 or AKT2 S473D mutant (AKT3 is only expressed in a few tissues). If expression of these mutants renders cells insensitive to JW-7-52-1, or at least equally sensitive to mTORC1 inhibition with rapamycin, it indicates that mTORC2 drives tumor cell growth predominantly by activating AKT (at least in cell culture).

If these cells remain equally sensitive to mTOR inhibition, the interpretation is more complicated. One possibility is that a second, unidentified mTORC2 target that is *completely* inhibited by mTOR inhibition plays a more important role in tumor cell proliferation. An obvious candidate is the serum-glucocorticoid induced kinase (SGK). SGK, which exists as three isoforms, is also a member of the AGC kinase family and

shares many structural similarities to AKT, including phosphorylation sites that are equivalent to Thr308 and Ser473 (Biondi et al., 2001). Expression of SGK1, but not SGK2 or SGK3, is induced by glucocorticoids and all isoforms are thought to play a role in osmotic homeostasis (Lang and Cohen, 2001). Unlike AKT, activation of SGK requires phosphorylation at its hydrophobic site before it can be phosphorylated at the activation loop site (Biondi et al., 2001). Interestingly, there is increasing evidence that SGK can phosphorylate many of the substrates that were previously thought to be exclusive AKT targets, such as FOXO and GSK3, indicating that these two kinases may have overlapping functions in driving cell proliferation and survival (Brunet et al., 2001; Dai et al., 2002).

There is currently no indication of whether or not mTORC2 regulates SGK, partly because SGK reagents are stuck in the dark ages of forgotten kinases. Whether mTORC2 phosphorylates SGK is easy to answer using standard radio-labeling techniques. The stickier question is whether mTORC2 inhibition leads to inhibition of SGK activity in cells. The lack of well-established SGK substrates makes this difficult to test, but can probably be accomplished by immuno-precipitating recombinant SGK from treated cells, and convincing it to phosphorylate a FoxO-derived peptide. A more functional question, as with AKT, is to ask whether expression of the phospho-mimetic SGK mutant can overcome the growth suppressive effects of mTOR inhibition. Given that these are purely speculative predictions, it may be that SGK has no role at all, and that mTORC2 mediates its effects through other downstream targets. Identifying these, either through phospho-proteomic analysis or substrate motif mapping, will bring many years of research excitement to the field.

Do RAS and mTOR cooperate to drive tumorigenesis?

Another one of our more surprising findings is that RAS pathway mutations in

tumor cell lines predict resistance to mTOR inhibition. If a causal relationship exists, it would suggest that RAS and PI3K pathways redundantly converge on one or several mechanisms that drive tumor cell growth. Currently, our work only establishes a correlation, but provides a hypothesis that can be tested in several ways. Our first goal is to ask whether introducing an activated RAS mutant can convert a sensitive cell line into an insensitive one. We plan to do this by introducing the canonical KRasV12 GTPase-deficient mutant into MCF7 and U87MG cells, which carry PI3K and PTEN mutations, respectively, and asking whether they retain their sensitivity to JW-7-52-1 treatment. Ectopic expression of RAS mutants is sometimes problematic, and so we may also try expression of the BRAFV600E mutant, which is also represented in our screening panel.

The converse experiment is to convert insensitive cell line into sensitive ones by inactivating RAS pathway components. One approach is to knock-down RAS or BRAF using RNAi and another is to use small-molecule inhibitors. There are currently no inhibitors available for any of the RAS isoforms, but inhibitors for MEK (U0126) and BRAF (SB590885) are both readily available and reasonably specific (King et al., 2006). Our prediction is that combinations of these inhibitors with JW-7-52-1 will have an additive effect on tumor cell growth inhibition. There are currently many reports of synergism between PI3K/mTOR pathway and EGFR inhibition, which would support this theory, as EGFR is a potent RAS activator (Fan et al., 2007; Wang et al., 2006). Moreover, in glioblastoma with EGFR mutations, mutations in PTEN cause resistance to the EGFR-inhibitor erlotinib, suggesting that activation of the PI3K pathway can compensate for EGFR/Ras inhibition as well (Wang et al., 2006).

What process downstream of mTORC1 and mTORC2 drives tumorigenesis?

Another take on understanding how mTOR drives tumorigenesis is to identify the downstream processes that are most responsible. Both mTOR complexes are linked

to many fundamental cell programs, including protein translation, proliferation, nutrient uptake and cell survival, but it is unclear which of these is the most important in normal and tumor cell physiology. Many clues point to translational control. mTORC1 has had a long relationship with the translational machinery through its substrates, S6K and 4E-BP1, both of which participate in assembling the translational pre-initiation complex (Holz et al., 2005). Recent work has also indicated a role for mTORC2 (Shor et al., 2008). Shor et al found that high concentrations of rapamycin, like JW-7-52-1, inhibit both mTORC1 and mTORC2 and that this causes a profound suppression of global protein synthesis that greatly exceeds the effects of mTORC1 inhibition. They also provide some evidence that these effects are mediated through inactivation of two translational components, eIF2 and eEF2.

eEF2, which participates in translational elongation, has been connected to mTOR before (Wang et al., 2001). eEF2 is phosphorylated and inhibited by the eEF2-kinase (eEF2K), and Wang et al showed that eEF2K is phosphorylated and inhibited by S6K and the Erk-regulated kinase Rsk (Wang et al., 2001). Clearly this is at odds with a claim for mTORC2 involvement, as neither S6K nor Rsk is directly affected by mTORC2. Additionally, rictor-null MEFs have no obvious defect in protein synthesis and proliferate at rates comparable to wild-type cells, though mTORC1, Erk/Rsk or other pathways might compensate and maintain eEF2 activity. Regardless, Shor et al convincingly show that combined mTORC1/2 inhibition causes a dramatic increase in eEF2 phosphorylation, warranting further investigation. Furthermore, eEF2 is a target of both mTOR and RAS pathways, supporting its role as a common mechanism in both kinds of cancers.

mTOR and the MNK kinases

Another possible convergence point that was discussed in an earlier chapter is the translational pre-initiation complex and, in particular, the mRNA cap-binding

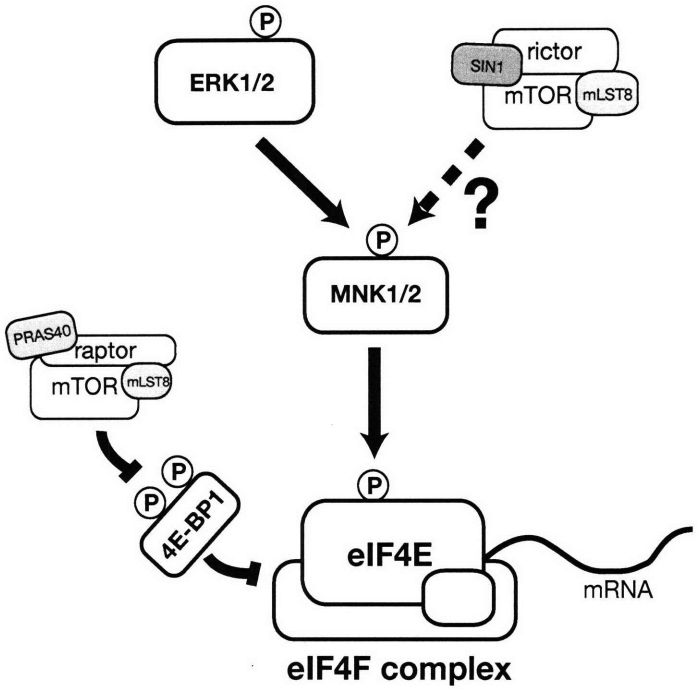

Figure 1. RAS/ERK and PI3K/mTOR pathways may converge on MNK kinases

The MNK1/2 kinases promote 5'-cap-dependent translation by phosphorylating a key site (Ser209) on the 5'-cap-binding protein eIF4E. MNK1/2 are known substrates of the ERK1/2 RAS pathway kinases. Indirect evidence suggests they may also be downstream of PI3K/ mTORC2 signaling, indicating a possible convergence point for these two pathways and suggesting a potential mechanism for their coordinate regulation of cell growth and proliferation.

protein eIF4E. eIF4E is itself oncogenic and overexpression drives tumorigenesis in a similar fashion to AKT1 overexpression (Wendel et al., 2004). eIF4E is inhibited by the small protein 4E-BP1, which is a substrate of mTORC1 and possibly other kinases (Bjornsti and Houghton, 2004). However, 4E-BP1 isn't directly affected by mTORC2, and therefore can't explain why dual mTORC1/2 inhibition would have a greater anti-proliferative effect than mTORC1 inhibition alone.

Instead, mTORC2 may act through the MNK1/2 kinases (Figure 1). These

kinases are not well understood, but can phosphorylate eIF4E at a site (Ser209) that is important for its activity and is its only known post-translational modification (Ueda et al., 2004). Surprisingly, deletion of both MNK kinases, and consequently loss of eIF4E phosphorylation, has no overt effect on mice (Ueda et al., 2004). However, eIF4E loses its transforming and tumorigenic ability in this context (Wendel et al., 2007). MNK activity is associated with the TOR pathway in two non-obvious ways. The first is that rapamycin treatment causes an increase in eIF4E phosphorylation that is MNK-dependent and sensitive to PI3K inhibitors, suggesting involvement of feedback activation of PI3K and possibly mTORC2 (Sun et al., 2005; Wang et al., 2007). Erk can also phosphorylate MNK, but has no role in this rapamycin-mediated activation (Wang et al., 2007).

A second observation connecting mTOR to MNK comes from Drosophila. Reiling et al showed that deletion of the Drosophila MNK homologue, Lk6, has no effect under normal growth conditions, but causes a severe growth defect when flies are fed a low protein diet (Reiling et al., 2005). These results suggest that mTORC1, which is suppressed by starvation conditions, and MNK might cooperate to control protein synthesis. It is possible that mTORC2 maintains basal eIF4E activity through MNK in wild-type cells, and loss of this input sensitizes flies to starvation. It would be interesting to see whether rictor-null flies are similarly sensitive. In summary, both studies indicate that eIF4E and MNK are, to some degree, under the joint regulation of the PI3K/mTOR and RAS/ERK signaling pathways. The mechanistic details are far from clear, but suggest many testable hypotheses.

mTOR inhibition and apoptosis

A reason that is often cited for the limited success of clinical rapamycin treatment is the failure to cause apoptosis in cancer cells. In many situations rapamycin only causes

a cell cycle arrest (Bjornsti and Houghton, 2004). The most common molecular rationale is that rapamycin-mediated mTORC1 inhibition suppresses cap-dependent translation by inhibiting eIF4E activity, and this prevents translation of transcripts such as cyclin D1 that are required for proliferation. The fact that many cancers that are highly responsive to rapamycin treatment show elevated cyclin D1 expression, such as NF1-null malignant peripheral nerve sheath tumors (MPNST) and mantle cell lymphomas (MCL) is often used to support this theory. From a therapeutic perspective, cell cycle arrest is less than ideal because it leaves open the opportunity for cells to acquire mutations that circumvent mTORC1 inhibition and continue growing. This is what appears to happen in the clinic, as well as in some laboratory cancer models (Yilmaz et al., 2006). There are some tumor cell lines where rapamycin does induce apoptosis, especially when PTEN is lost and in combination with other chemotherapy, but the underlying mechanism that differentiate these from other PTEN cancers are not clear (Guertin and Sabatini, 2007).

We had hoped that dual mTORC1/2 inhibition would further sensitize cells towards apoptosis because of a greater effect on AKT. AKT is often characterized as a survival-promoting kinase because it can phosphorylate and inhibit the proapoptotic factors BAD and FoxO, and is also thought to promote the activity of the prosurvival transcription factor NF-kappaB (Manning and Cantley, 2007). However, this does not seem to occur as early indications suggest that cells treated with JW-7-52-1 are more likely to arrest that become apoptotic. Nonetheless, it seems likely that mTOR inhibition, and consequently AKT inhibition, should increase sensitivity to other apoptotic stimuli, just as rapamycin treatment sensitizes some cancers to other therapeutic interventions. Identifying other signaling pathways, using both small molecules and RNAi, that synergize with mTOR inhibition to cause apoptosis is a future priority.

Does mTOR inhibition prevent tumor growth in animals?

We are currently preparing to test the effectiveness of mTOR inhibition in preventing tumor growth in animals. Our first step will be to ask whether daily treatment with JW-7-52-1 can stop the growth of MCF7, U87MG, or PC3 xenografts. We have already shown that reasonable does of JW-7-52-1 can inhibit S6 and AKT phosphorylation in tissue, and so we are hopeful that these tumor cell lines will respond in vivo as well as they do in culture. A better test of the therapeutic value of mTOR inhibition is to ask whether it can prevent or cause remission of an endogenous genetic cancer model. As has been mentioned, there are many examples showing that cancers with activated PI3K/PTEN signaling are more responsive to mTOR inhibition, particularly with rapamycin (Guertin and Sabatini, 2007). A good model is PTEN-driven prostate cancer, which can be initiated in mice by conditional deletion of PTEN in the prostate (Wang et al., 2003). The tumors that result progress through stereotypical stages that closely mimic the progression of the disease in humans, and are thus considered particularly realistic.

Another useful study would be to compare the effectiveness of mTOR inhibition against cancers caused by PTEN deletion to those caused by KRAS activation. Expression of the KRAS V12 mutant in hematopoetic stem cells initiates an aggressive myeloproliferative disorder (MPD) that causes significant disease within a few weeks (Braun et al., 2004). Both NRAS and KRAS mutations are frequently found in leukemias, including MPDs, supporting the belief that these cancers develop through representative mechanisms (Bos, 1989). PTEN mutations are much less common in human leukemia, but can nonetheless initiate these types of cancers in laboratory settings (Salmena et al., 2008; Yilmaz et al., 2006). This would be a useful test of our prediction that KRAS-driven tumors, but not those caused by PTEN, are refractory to mTOR inhibition, and that combination therapy with RAS pathway inhibitors should be more effective.

Strategies for identifying mTORC2-specific inhibitors

There are obvious drawbacks of dual-mTORC1/mTORC2 inhibition as an anti-cancer therapy. Inhibition of mTORC1 with either JW-7-52-1 or rapamycin causes PI3K activation by disengaging the negative feedback loop. Concurrent inhibition of mTORC2 can blunt some of these effects, but PI3K has mTOR-independent functions that might promote tumor growth through other means. Dual PI3K/mTOR inhibition is another possible strategy, though potent suppression of PI3K may cause an unacceptable degree of toxicity. We have shown that genetic deletion of mTORC2 components doesn't cause feedback activation of PI3K, indicating that specific inhibition of mTORC2 might be a more promising therapeutic strategy (Guertin et al., 2006). However, identifying mTORC2-specific inhibitors is a challenging task. Small-molecule inhibitors that are specific for mTORC2 will need to act through an allosteric mechanism – ATP-competitive molecules, which constitute the overwhelming majority of kinase inhibitors, are guaranteed to inhibit mTORC1 as well.

To identify molecules that act outside of the kinase domain, we will need screen a much larger library, which is not easily compatible with our current setup. Currently, our biggest limitation is the quality of our detection system, as it is currently too slow and lacks the dynamic range required for testing millions of molecules. A more attractive strategy is to adapt it to use a lanthanide-based reporter system (Robers et al., 2008). LanthaScreen was developed by Invitrogen and leverages several useful fluorescent properties of terbium (Tb) and EGFP to compose a robust reporter system. Terbium is an element belonging to the "rare earth" lanthanide series, and absorbs light at 335nM and, after a short time delay, emits at peaks centered at 490nM, 550nM, 580nM and 630nM. EGFP absorbs at 488nM and emits at 509, making it an ideal partner for fluorescence resonance energy transfer (FRET) with Tb. Thus, when a Tb-labeling a phospho-

specific antibody is mixed with an EGFP-labeled substrate, binding of the antibody to the phosphorylated residue brings the Tb and EGFP close enough for FRET to occur, permitting efficient detection of phosphorylation in a single-well system. We are currently working on adapting this system to S6K and Akt substrates.

A second strategy is to develop a cell-based assay, which are routinely used to screen large compound libraries. We have avoided cell-based assays in the past because an enormous number of signaling pathways influence mTORC1/2 activity, leading such screens to generate an unacceptably high false-positive rate. However, many secondary pathways signal through PI3K, and conducting the screen in cell lines with a constitutively active PI3K pathway might limit their effect on mTORC2. Additionally, we could independently measure PI3K activity by expressing a GFP tagged with a pleckstrin-homology (PH) domain. The PH domain specifically binds PIP3, and would localize PIP3 to the plasma membrane in response to PI3K activation. By combining this assay with an immunofluorescent readout of AKT S473 phosphorylation, we could identify small molecules that inhibit mTORC2 without affecting PI3K signaling. These kinds of assays would have the unique advantage over their in vitro counterparts by additionally identifying molecules that disrupt mTORC2 assembly or thwart associations with other cellular proteins or structures that promote activation.

Conclusions

Many of the original questions in the mTOR field have been answered over the last few years. We know that mTOR acts in two functionally distinct but complementary complexes, and that only one is acutely inhibited by rapamycin. We have a reasonable understanding of how both nutrients and growth factors regulate mTORC1. We have identified many of the key substrates for both complexes that mediate their downstream effects. There are, of course, important regulatory features that we don't understand.

For instance, what metabolite does mTORC1 actually sense, and what mechanism connects PI3K to mTORC2? There are also many more questions about the cellular and organismal physiologic role of mTOR signaling. Rapamycin treatment has limited effects on humans and mice, and mTORC2 components are dispensable in adult flies. Is mTOR signaling only required for processes that demand rapid proliferation and growth such as development, immune response and tumorigenesis? Hyperactive mTORC1 undoubtedly suppresses insulin signaling, but is this the mechanism of insulin insensitivity caused by a high-fat diet? How does fat activate mTORC1? Do cancer cells some cancers have a unique dependency on mTOR signaling, and, if so, what genetic markers determine this sensitivity? Does the mTOR pathway cooperate with other signaling pathways in cancer?

Our next step should be to apply our biochemical understanding to answering these kinds of questions. Small molecule inhibitors are a powerful way to probe the role of mTOR signaling because they allow acute and reversible inhibition that isn't possible with any other approach. Rapamycin and JW-7-52-1 have already opened many new windows into mTOR physiology, and mTORC2-specific inhibitors, if they can be developed, will surely do the same. Conditional-knockouts for many mTORC-related genes are also now available and offer a complimentary genetic approach. Together, these tools will help to answer the questions described above and clarify the role that this fundamental cellular signaling pathway plays in normal and disease physiology.

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Appendix A

mTOR in vitro kinase assays

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Experiments in Figure 1A were performed by Y.S.
Experiments in Figure 1B were performed by C.C.T.
Experiments in Figure 2 were performed by C.C.T.

Introduction

In vitro kinase assays are a powerful approach to studying the function and regulation of complex kinases. Their most common use is to corroborate genetic data and prove that kinase X phosphorylates Y, but this is only the most basic application. We have used in vitro kinase assays extensively to understand the mechanisms that regulate the mTORC1 complex. Both mTOR complexes are fragile machines, and certain amount of care. The following summarizes our technical knowledge about using them in vitro. Most of the information was developed for mTORC1 kinase assays, though the findings are likely applicable to mTORC2 as well. In general, our primary goal has been to find purification conditions that maintain whatever regulatory features exist in cells.

Detergents for mTOR purification

One of the earliest technical observations in the mammalian TOR field was that the stability of the complex was highly sensitive to the choice detergents (Hara et al., 2002; Kim et al., 2002). Original purifications used Triton-X100 or the related NP-40 to lyse cells, both of which are common "gentle" non-ionic detergents. Indeed, mTOR can be immuno-purified using these detergents and is highly active towards endogenous substrates S6K and 4E-BP1 (Burnett et al., 1998). However, under these conditions, the complex fails to reflect its cellular regulatory state. For instance, in cells, nutrient availability and growth factor signaling are potent mTOR regulators, but none of these changes in activity were preserved once mTOR was purified from lysates. Soon after, our lab showed that mTOR partners with another protein, raptor, and that this interaction can only be maintained by using CHAPS, another non-ionic detergent (Hara et al., 2002; Kim et al., 2002). Use of CHAPS is also necessary to maintain the interaction between mTOR

and its mTORC2 partner Rictor, which is required for its ability to phosphorylate AKT. Another approach that avoids these troubles is to lyse cells using sonication rather than detergent. The drawback of sonication is that it fails to solubilize membranes as well as detergents do, and so proteins that are strongly associated with membranes are often lost in the insoluble fraction of the lysate. See the section below for more information about the lysis buffer we currently use.

Effects of pyrophosphate

An observation following the identification of Raptor was that nutrient availability altered the stability of the mTOR/Raptor interaction (Kim et al., 2002). However, another group found that the integrity of the complex was unchanged(Hara et al., 2002). In the end, it was realized that the different results were due to the presence or absence of sodium pyrophosphate in the buffer. We had included it because it acts as a general phosphatase inhibitor, while Hara et al did not. It isn't clear why pyrophosphate causes this effect, but we have speculated in the past that the Raptor/mTOR interaction might involve binding to phosphorylated residues and that pyrophosphate can compete for these binding sites. There is, of course, no evidence to support this theory. A final note on pyrophosphate is that, although it has no effect on kinase activity when present in the lysis buffer, it is inhibitory when added to kinase reactions (data not shown). This may be because pyrophosphate can bind metal ions, which are required for kinase activity.

Ionic strength of the lysis buffer

It is common practice to wash immuno-purified proteins with high-salt buffers to remove impurities. We found that washing mTOR immuno-purifications with 0.5M NaCl caused a dramatic increase kinase activity. Although high-salt washes partly disrupted the binding between mTOR and Raptor, we found that similarly washing raptor immuno-

purifications caused the same increase in kinase activity, indicating that this phenomenon was not caused by the loss of raptor. Instead, we suspected that high-salt caused the dissociation of a normally inhibitory component of the mTORC1 complex, which led to the identification of PRAS40 (Sancak et al., 2007). We also believed that other important regulatory components of mTORC1 might be affected by the ionic strength of the lysis buffer. To this end, we lysed cells that had been starved or stimulated with leucine in a buffer containing a minimal concentration of salt, and then immuno-purified mTOR or Raptor to ask if the in vitro activity remained regulated (Figure 2A). It was, and we further showed that increasing salt concentrations in the lysis buffer obscured this regulation (Figure 2B). We have recently proposed that amino acids regulate mTORC1 activity by causing it to translocate to membranous structures that contain rheb, so it is unclear what part of that activating mechanism remains intact when the complex is purified(Sancak et al., 2008). Although a small amount of activating rheb may remain bound to raptor, we were never able to detect it in mTOR immunoprecipitates. However, other groups have made the claim that the interaction is maintained (Long et al., 2005a; Long et al., 2005b).

Kinase reaction conditions

The previous discussions have all referred to the lysis buffer composition. The contents of the kinase reaction buffer also affect mTOR regulation. Divalent metal ions are required in kinase reactions to help coordinate ATP in the catalytic pocket. Mg++ is the physiologic ion, but Mn++ is commonly used in the laboratory because it results in greater kinase activity. We found that Mn++ was indeed activating for mTORC1, but that it overcame whatever mechanisms normally regulate the kinase activity in response to leucine (Figure 2). Thus, it is important to use Mg++ in the kinase reaction buffer.

Substrates for in vitro kinase assays

An interesting story about mTORC1 substrates concerns the "rapamycin-insensitive" S6K. This protein, which differed from wild-type S6K by the removal of a C-terminal fragment known as the auto-inhibitory domain, was phosphorylated by mTOR in a rapamycin-insensitive fashion. A popular theory was that the C-terminal domain in wild-type S6K coordinated a rapamycin-activated phosphatase, and that loss of this domain rendered the kinase insensitive to rapamycin. Despite years of searching, no one has found a rapamycin-activated phosphatase. Instead, Ali et al found that the "rapamycin-insensitive" S6K was phosphorylated by the rapamycin-insensitive mTOR complex, which we now call mTORC2 and which normally phosphorylates AKT (Ali and Sabatini, 2005). S6K and AKT share a very similar structure, and we now know that the S6K C-terminal domain normally restricts it to mTORC1. Partly because of this story, we try to use full-length substrates for our kinase assays, because the structure of the substrate is often important for the kinase's regulatory mechanism. To purify p70 S6K for use in kinase reactions, we express it with an N-terminal GST tag and a PreScission protease cleavage site in HEK293T cells, precipitate with glutathione-coated agarose, cleave with PreScission protease, and separate the protein on a HiLoad 16/60 Superdex 200 column (Amersham). The substrate can be stored at –80C.

Rheb purification and use in kinase assays

Rheb is a small GTPase that is a key activator of mTORC1 in response to insulin stimulation. We showed that purified rheb activates mTORC1 directly in in vitro kinase assays(Sancak et al., 2007). The following describes the protocol for purification. Rheb1 in HA-GST-pRK5 is transfected into HEK293T cells. Cells are then lysed with rheb lysis buffer (the lysis buffer used for mTOR immunoprecipitations, but without EDTA and containing 5 mM $MgCl_2$), and cleared lysates are incubated with immobilized glutathione for 2 hr at 4°C. Beads are then washed twice with rheb lysis buffer and once with rheb storage buffer (20 mM HEPES [pH 8.0], 200 mM NaCl, and 5 mM $MgCl_2$). GST-rheb1

can be eluted from the beads with 10 mM glutathione in rheb storage buffer. Eluted rheb can be GTP loaded by incubating it with 10 mM EDTA and 1 mM GDP or 0.1 mM GTP S at 30°C for 10 min, though this step is not necessary. $MgCl_2$ (20 mM) is then added to permit stable GTP binding and the proteins kept on ice until use.

Procedure for mTORC1 kinase assays

Cells are lysed in 50mM Hepes pH 7.4, 10mM NaPyrophosphate, 10mM NaBetaglycerophosphate, 2mM EDTA, 0.3% CHAPS. Complex is immunoprecipitated with raptor, rictor or mTOR-specific antibody for 1h, followed by incubation with protein-G agarose beads for 1h. Immunoprecipitates are then washed once in lysis buffer followed by two additional washes in lysis buffer containing a final concentration of 150mM NaCl. Immunoprecipates are then washed twice in 25 mM HEPES (pH 7.4), 20 mM potassium chloride. Kinase assays are performed for 20 min at 30°C in a final volume of 15 ul consisting of mTORC1 kinase buffer (25 mM HEPES [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$, 250 uM ATP) and 150 ng inactive S6K1 or 4E-BP1 as the substrate. Reactions are stopped by the addition of 30 ul of sample buffer and boiling for 5 min and analyzed by SDS-PAGE and immunoblotting.

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Figures

Figure 1. Leucine stimulation of cells causes an activation of mTORC1 that is preserved in vitro.

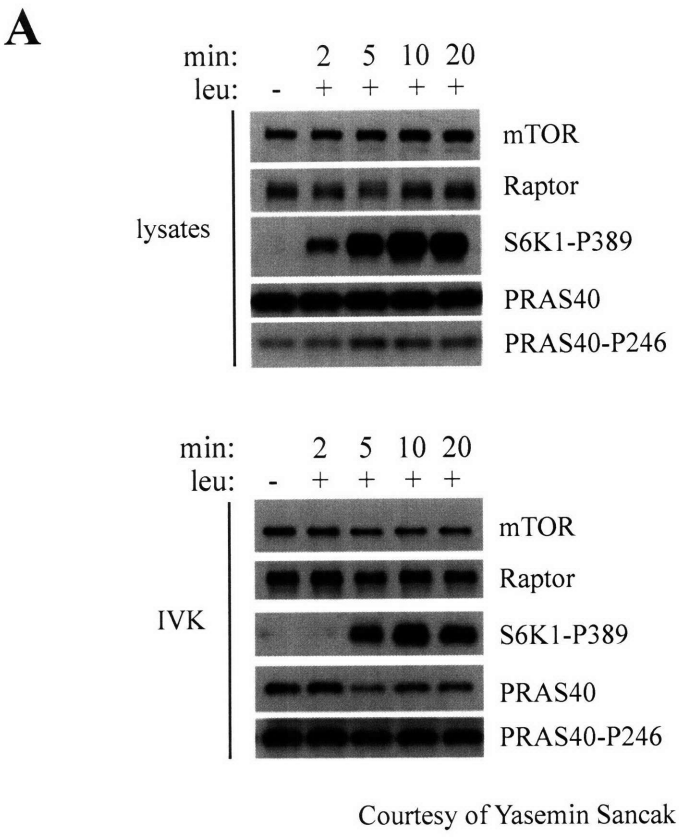
A. mTORC1 immunopreciptated from leucine stimulated cells remains active in vitro. HEK293T cells we grown in leucine-free RPMI for 1h and then stimulated with 52ug/ml leucine for the indicated times. Cells were lysed and mTORC1 was immunoprecipitated using raptor-specific antibody and analyzed in in vitro kinase assays as described in Experimental Procedures. Cell lysates and kinase reactions were both analyzed by immunoblot using phospho-specific antibodies to detect phosphorylation of S6K at T389 and phosphorylation of PRAS40 at T246. Antibodies specific for mTOR and raptor were used to determine total levels of those proteins. B. In vitro leucine regulation of mTORC1 is sensitive to high-salt. mTORC1 was immunoprecipitated from leucine starved and stimulated cells as described in A. However, immunoprecipitates were washed three times in buffer containing 50mM Hepes pH 7.4 and indicated amounts of NaCl. Immunoprecipitates were then subjected to kinase assays and analyzed as in A.

Figure 2. Use of manganese instead of magnesium in kinase reactions disables mTORC1 regulatory mechanisms.

HEK293T cells were grown in leucine-free RPMI for 1h, and then stimulated as indicated with 52ug/ml leucine for 10min. mTORC1 was immunoprecipitated from cell lysates using raptor-specific antibody. Immunoprecipitates were prepared as described in Experimental Procedures and washed one additional time with 0.5M NaCl where indicated. Immunoprecipitates were then

subjected to in vitro kinase assays, except that 5mM $MnCl_2$ was used in place of $MgCl_2$ where indicated.

Figure 1



Courtesy of Yasemin Sancak

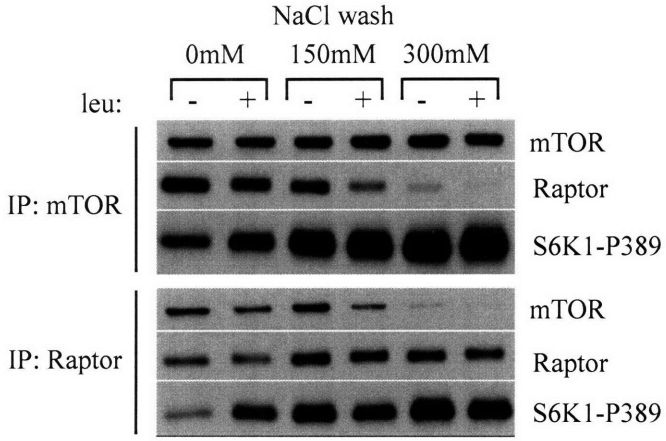
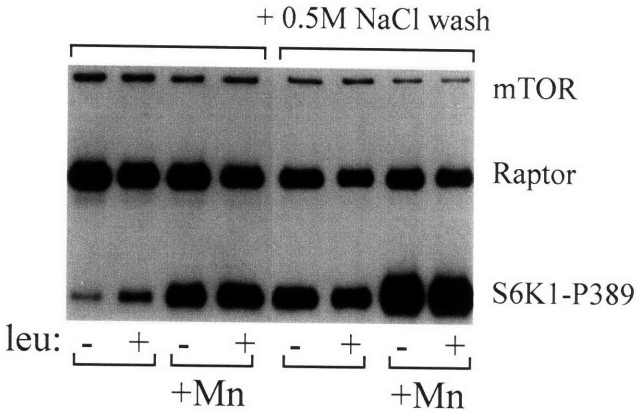


Figure 2



Appendix B

Lipid activation of mTORC1

Carson C. Thoreen and David M. Sabatini

All experiments were performed by C.C.T.

Introduction

The mTORC1 serine/threonine kinase complex is the founding member of a signaling pathway that senses nutrients and growth factors, and exerts powerful influence over the cell's translational machinery. Through one of its substrates, S6K1, mTORC1 can also affect insulin sensitivity (Shah et al., 2004). S6K1 phosphorylates the insulin receptor substrate (IRS1), which couples the insulin receptor to the lipid kinase PI3K, marking it for degradation and dampening the effects of insulin stimulation (Shah et al., 2004). Because of its sensitivity to nutrients and feedback effect on insulin signaling, several groups have proposed that excessive nutrients, a situation called "nutrient overload," might constitutively activate mTORC1 and lead to insulin insensitivity (Um et al., 2006).

Despite fervent interest, controversy remains over the nature of the nutrient input to mTORC1. Many signals upstream of mTORC1 are channeled through the tuberous sclerosis complex (TSC), which is a heterodimeric complex composed of tuberin (TSC1) and hamartin (TSC2) (Kwiatkowski and Manning, 2005). TSC negatively regulates mTORC1 through its role as a GTPase-activating protein for the small GTPase rheb (ras homolog enriched in brain), which directly stimulates mTORC1 (Kwiatkowski and Manning, 2005). Many signaling pathways are thought to activate mTORC1 by altering the activity of TSC. For instance, AKT and Erk phosphorylate and inactive TSC in response to growth factor signals (Kwiatkowski and Manning, 2005). Alternatively, the AMP-activated kinase (AMPK) phosphorylates and stabilizes TSC in response to rising cellular AMP and other forms of energetic stress. The gene Redd1 activates TSC in response to hypoxia (Brugarolas et al., 2004; Reiling and Hafen, 2004). Amino acid availability regulates mTORC1 through a separate mechanism that involves the Rag family of small GTPases (Sancak et al., 2008). Glucose availability may be signaled

through AMPK and TSC or through another route.

Within cells, nutrients are rapidly broken down into more basic molecules that can be utilized for energy or other biosynthetic pathways, such nucleotide or fatty acid synthesis. We wanted to ask whether the mTORC1 pathway senses glucose or amino acids directly, or whether it responds to one of these downstream intermediates. Identifying this molecule will shed light on the relationship between nutrient metabolism and mTORC1 activity.

Results and Discussion

RNAi directed screen of metabolic genes in Drosophila cells

To identify metabolic pathways that are required for TORC1 activity, we conducted a broad RNAi-based silencing screen in Drosophila KC 167 cells of metabolic genes that are rate-limiting and/or cannot be bypassed. The advantage of doing this work in a Drosophila system is that RNAi-mediated gene silencing is particularly efficient (Clemens et al., 2000). Although a significant evolutionary gap separates insect and mammalian lineages, the considerable conservation of TORC1 pathway components, such as TOR, raptor, rheb, TSC1/2 and S6K, leads us to believe that products of this screen will be good candidates for further characterization in mammalian systems.

Our initial screen targeted components of the following metabolic pathways: glycolysis, glucosamine synthesis, pentose phosphate shunt, branched chain amino acid catabolism, and fatty acid synthesis and oxidation. Surprisingly, dTOR activity was affected most significantly by silencing of genes involved in fatty acid synthesis (Figure 1A). These included acetyl-CoA carboxylase (ACC), the rate limiting and regulated step in fatty acid synthesis, and fatty acid synthase (FAS), the primary endogenous producer of intracellular fatty acids (Figure 1A). ACC causes the irreversible carboxylation of acetyl-CoA, a TCA cycle intermediate that is exported from the mitochondira, to

malonyl-CoA. FAS then uses malonyl-CoA as the building block for free-fatty acid synthesis (FFAs).

Unsaturated fatty acids are required for TOR activity

Fatty acids are further modified either by elongation or desaturation of their acyl chain. We were unable to identify homologues of human fatty acid elongation enzymes, but did identify several fatty acids desaturases. Of these, knock-down of several enzymes with significant homology to the human stearoyl-CoA desaturases (SCD) caused suppression of S6K phosphorylation (Figure 1B). SCD catalyzes the synthesis of monounsaturated fatty acids, such as oleate, from the saturated products of FAS and is the primary source of these molecules in cells (Ntambi and Miyazaki, 2003). We also tested the effect of inhibiting fatty acid synthesis more broadly by knocking down the transcription factor and lipid sensor SREBP (Seegmiller et al., 2002). Unfortunately, depletion of SREBP was lethal to cells, though phosphorylation of S6K was decreased (data not shown). Despite many attempts, using this system, we were unable to identify a more specific lipid species that was required for TORC1 activity.

Fatty acids are also required for mTORC1 signaling

Mammalian cells synthesize fatty acids through a similar mechanism. To test whether fatty acid synthesis was required for mTOR signaling, we treated cells with the fatty acid synthase inhibitor C75. C75 is a derivative of the small molecule cerulenin, which has been shown to inhibit FAS and has attracted interest as an anti-cancer drug (Kuhajda, 2000). Like knockdown of FAS, ACC and SCD in Drosophila cells, C75 treatment rapidly caused dephosphorylation of S6K in HEK293T cells (Figure 1C).

mTORC1 may sense lipids directly

Cellular ipids are incorporated into many molecules, including triglycerides, proteins, phospholipids and many other lipid species. We thought that mTORC1 might sense a particular lipid species directly. In fact, earlier reports had indicated that mTOR binds phosphatidic acid (PA), and suggested that this increases kinase activity (Fang et al., 2001). We first ask whether certain phospholipids can stimulate mTOR activity when added exogenously, and found that phosphatidic acid and phosphatidylserine (PS) were both stimulatory (Figure 2A). We tested this hypothesis directly by adding PA liposomes to immunoprecipitated mTOR and showed substantial activation (Figure 2B). However, this effect is highly variable, and frequently PA does not change mTOR kinase activity, possibly indicating a requirement for a component that does not always co-immunoprecipitate with mTOR. Moreover, it is unclear what concentrations of PA exist in cells, and so we are unsure if our in vitro conditions are within a reasonably physiologic range.

Conclusions

We have provided evidence that mTORC1 kinase activity depends on fatty acid synthesis in both Drosophila and mammalian systems. We have further suggested that mTORC1 might be directly activated by certain phospholipids, though this remains to be confirmed more rigorously. The finding that mTORC1 signaling is sensitive to lipid biosynthesis identifies a new class of nutrients that can influence this pathway, and also adds a new twist on the idea that "nutrient overload" can lead to insulin insensitivity. "Nutrient overload" generally refers to the consequences of consuming a high-fat diet. We show here that high levels of certain intracellular lipids might activate mTORC1 directly, thus bypassing the normal requirement for other signals such as growth factors and amino acids and potentially desensitizing cells to insulin.

Figures

Figure 1. Fatty acid synthesis is required for mTORC1 activity

A. RNAi-mediated knock-down of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) in Drosophila cells inhibits mTORC1. Kc 167 cells were transfected with dsRNA specific for either FAS or ACC and grown for 4 days. Cell lysates were then analyzed by immunoblot using phospho-specific antibody to detect phosphorylation of S6K on T389.

B. RNAi-mediated inhibition of monounsaturated fatty acid synthesis inhibits mTORC1. Kc 167 cells were transfected with dsRNA specific for putative Drosophila homologues of the mammalian stearoyl-CoA desaturases and analyzed as in A. Knockdown of SCD B strongly affected cell viability, and sufficient protein could not be extracted from cell lysates. Thus, this lane cannot be directly compared with other lanes in the gel.

C. Pharmacological inhibition of FAS causes inhibition of mTORC1 in mammalian cells. HEK293T cells were treated with 50ug/ml C75 for increasing amount of time, lysed, and analyzed as in A.

Figure 2. Phospholids can activate mTORC1

A. Exogenous phospholipids can activate mTORC1. Hela cells were serum-starved in DMEM for 4h and then stimulated with 100uM of the indicated phospholipids for 30 minutes. Phospholipids had been dried from chloroform stocks under nitrogen gas and resuspended in DMSO. Cell lystates were analyzed by immunoblot using phospho-specific antibodies to detect phosphorylation of S6K at T389.

B. Phosphatidic acid can activate mTORC1 directly. Phosphatidic acid (PA) was dried from chloroform stocks under nitrogen gas, resuspended in 50mM Hepes pH 7.4, 150mM KCl, and then sonicated to clarity in a bath sonicator. PA liposomes were than added to mTOR immunoprecipitates at the indicated concentrations, and in vitro kinase assays were conducted as described in the Experimental Procedures. Kinase assays were analyzed by immunoblot, using phospho-specific antibody to detect phosphorylation of S6K at T389.

Figure 1

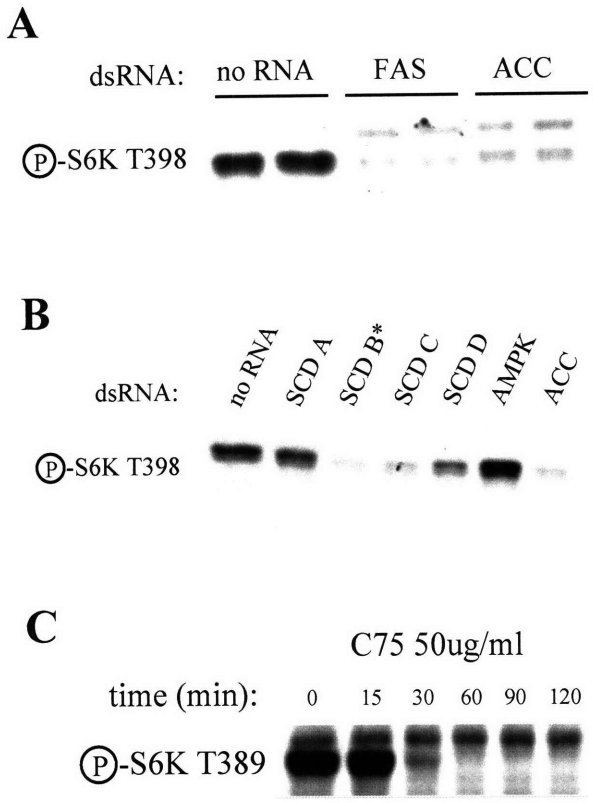
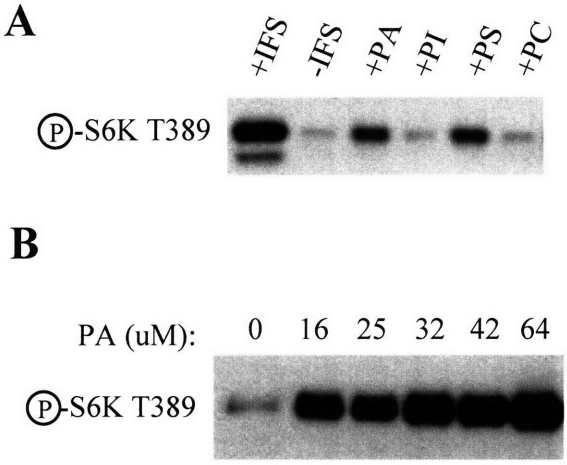


Figure 2



Experimental procedures

Materials

Reagents were obtained from the following sources: antibodies to raptor from Upstate/Millipore; antibodies to mTOR and S6K1 as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1 from Cell Signaling Technology; ATP from Sigma Aldrich; protein G-sepharose from Pierce; DMEM from SAFC Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; Schneider's insect cell media, Inactivated Fetal Calf Serum (IFS) and fetal bovine serum (FBS) from Invitrogen; phospholipids from Avanti Polar Lipids; C75 from Calbiochem.

In vitro mTORC1 kinase assays

mTORC1 in vitro kinase assays were conducted as described earlier (Sancak et al., 2007). Briefly, mTORC1 was immunoprecipitated from HEK-293T cells using antibody specific for raptor or mTOR. Immunoprecipitates were washed and then combined with full-length p70S6K, 500uM ATP in reaction buffer and allowed to proceed for 20min. Phosphorylation of substrates was assessed by western blot using phospho-specific antibodies. Phospholipids were dried from chloroform stocks under nitrogen gas and resuspended in 50mM Hepes pH 7.4, 150mM KCl. Lipid mixtures were then sonicated to clarity using a bath sonicator.

Drosophila RNAi

Drosophila RNAi experiments were conducted as earlier described (Guertin et al., 2006). Briefly, dsRNAs targeting *Drosophila* metabolic enzymes were synthesized by in vitro transcription in 20 ul reactions using a T7 MEGAscriptTM kit (Ambion).

DNA templates for IVT were generated by RT-PCR from total *Drosophila* cellular RNA using the OneStep RT-PCR kit (Qiagen). Drosophila KC167 cells were seeded in 6-well culture dishes. dsRNAs were administered to cells using FuGENE 6 transfection reagent (Roche). After 4 days total of incubation to allow turnover of the target mRNAs, cell lysates were prepared as described (Guertin et al., 2006). Cellular protein was loaded onto 8% SDS-PAGE gels, separated, transferred to nitrocellulose membranes and analyzed by immunoblotting.

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