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GβL, a Positive Regulator of the Rapamycin-Sensitive Pathway Required for the Nutrient-Sensitive Interaction between Raptor and mTOR

Do-Hyung Kim,[1] Dos D. Sarbassov,[1] Siraj M. Ali,[1] Robert R. Latek,[1] Kalyani V.P. Guntur,[1] Hediye Erdjument-Bromage,[2] Paul Tempst,[2] and David M. Sabatini[1,*]

[1]Whitehead Institute for Biomedical Research and Department of Biology
Massachusetts Institute of Technology
Nine Cambridge Center
Cambridge, Massachusetts 02142

[2]Molecular Biology Program
Memorial Sloan-Kettering Cancer Center
New York, New York 10021

Summary

mTOR and raptor are components of a signaling pathway that regulates mammalian cell growth in response to nutrients and growth factors. Here, we identify a member of this pathway, a protein named GβL that binds to the kinase domain of mTOR and stabilizes the interaction of raptor with mTOR. Like mTOR and raptor, GβL participates in nutrient- and growth factor-mediated signaling to S6K1, a downstream effector of mTOR, and in the control of cell size. The binding of GβL to mTOR strongly stimulates the kinase activity of mTOR toward S6K1 and 4E-BP1, an effect reversed by the stable interaction of raptor with mTOR. Interestingly, nutrients and rapamycin regulate the association between mTOR and raptor only in complexes that also contain GβL. Thus, we propose that the opposing effects on mTOR activity of the GβL- and raptor-mediated interactions regulate the mTOR pathway.

Introduction

Cell growth is the fundamental biological process whereby cells accumulate mass and is an important determinant of the sizes of cells, organs, and organisms (Conlon and Raff, 1999; Dixon and Fordham-Skelton, 1998; Gomer, 2001; Johnston and Gallant, 2002; Stocker and Hafen, 2000). The mTOR pathway, along with the PI 3-Kinase/PKB/PTEN axis, is emerging as a critical regulator of growth in mammals in response to nutrients, hormones, and growth factors (Gingras et al., 2001; Kozma and Thomas, 2002; Schmelzle and Hall, 2000). The central component of the pathway, mTOR (also known as RAFT1 or FRAP), was discovered during studies into the mechanism of action of rapamycin (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995), an antiproliferative drug with valuable immunosuppressive and anticancer clinical applications (Saunders et al., 2001; Vogt, 2001). mTOR is a member of the PIK-related family of large protein kinases (Keith and Schreiber, 1995) and controls the phosphorylation of at least two regulators of protein synthesis and cell growth: S6 kinase 1 (S6K1) and an inhibitor of translation initiation, the eIF-4E binding protein 1 (4E-BP1) (Brunn et al., 1997; Burnett et al., 1998; Isotani et al., 1999). Recent work suggests that deregulation of the mTOR pathway plays a role in the pathogenesis of human disease, as the pathway is constitutively active in tuberous sclerosis complex (Goncharova et al., 2002; Kwiatkowski et al., 2002), a tumor-prone syndrome caused by mutations in the *TSC1* (van Slegtenhorst et al., 1997) or *TSC2* (Consortium, 1993) genes. Exactly how the mTOR, TSC1/2, and PI-3K/PKB/PTEN pathways interconnect is unknown, but it is likely that these systems integrate growth factor- and nutrient-derived signals to determine overall growth rates. The mTOR pathway is particularly sensitive to the levels of nutrients, such as amino acids (Hara et al., 1998) and glucose (Kim et al., 2002), and of ATP (Dennis et al., 2001), but the molecular mechanisms by which nutrients regulate mTOR are just starting to be understood.

Recent biochemical approaches have led to the identification of a subunit of an mTOR-containing complex, a protein we named raptor (*r*egulatory *a*ssociated *p*rotein of *TOR*), whose association with mTOR is modulated by nutrients and which regulates the mTOR kinase activity (Kim et al., 2002). Hara et al. (2002) independently identified the same interacting protein and adopted the raptor name. Raptor is a large 149 kDa protein and contains an N-terminal RNC (*r*aptor *N*-terminal *c*onserved) domain found in all its eukaryotic homologs, followed by three HEAT repeats and seven WD-40 repeats in the C-terminal third of the protein (Kim et al., 2002). The mTOR binding site on raptor is not easily defined and, based on mutagenesis and truncation studies, may require the overall conformation of raptor and/or multiple contacts between the proteins (Kim et al., 2002). Previously, we proposed that raptor has at least two functions in the mTOR pathway. It clearly has a positive role within cells in maintaining an active mTOR pathway, as revealed by the inhibitory effects of reducing raptor expression on S6K1 activity and cell size (Kim et al., 2002). Raptor overexpression also stimulates the kinase activity of recombinant mTOR toward S6K1 and 4E-BP1, suggesting that raptor may be an adaptor protein that recruits mTOR substrates to the mTOR kinase domain (Hara et al., 2002). In addition to a positive function for raptor, we proposed that raptor also negatively regulates the mTOR kinase. Conditions that inhibit the pathway, such as nutrient deprivation, stabilize the raptor-mTOR association and inhibit the kinase activity of endogenous mTOR, and raptor overexpression decreases the phosphorylation state of S6K1 within cells and the mTOR kinase activity in vitro (Kim et al., 2002). These results imply that regulation of mTOR kinase activity is likely complex and may be difficult to understand without knowledge of how mTOR and raptor interact with each other or with other unidentified components of the complex that may affect the regulatory function(s) of raptor.

In this study, we identify GβL as an additional subunit of the mTOR-signaling complex, a protein that stimulates the mTOR kinase activity and is critical for main-

*Correspondence: sabatini@wi.mit.edu

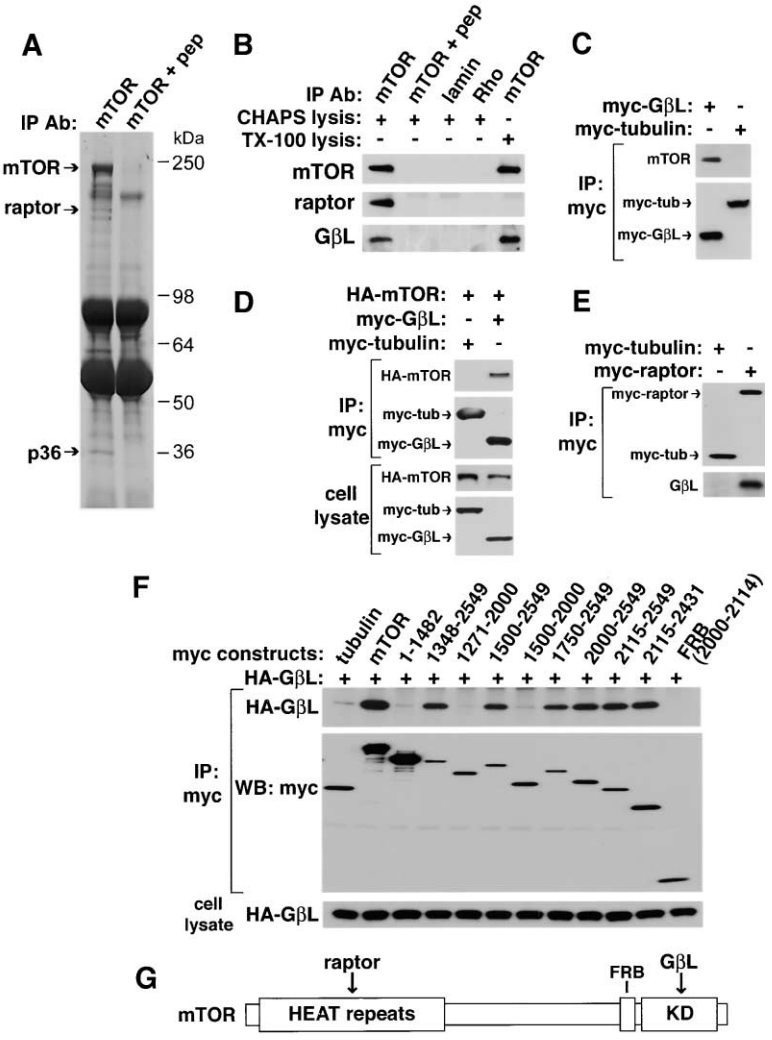

Figure 1. Identification of GβL as an mTOR-Associated Protein

(A) Coomassie blue-stained SDS-PAGE analysis of mTOR immunoprecipitates prepared from HEK-293T cell extracts in the absence or presence (+pep) of the blocking peptide for the anti-mTOR antibody.

(B) Specific in vivo interaction between mTOR and GβL. A polyclonal anti-GβL antibody recognizes GβL in mTOR immunoprecipitates prepared from cells lysed in buffers containing either 0.3% CHAPS or 1% Triton X-100, but not in immunoprecipitates prepared with the indicated control antibodies or in the presence of the blocking peptide for the mTOR antibody.

(C) Endogenous mTOR interacts with recombinant GβL but not γ-tubulin. Immunoblotting for mTOR and myc-tagged proteins was performed on anti-myc immunoprecipitates prepared from HEK-293T cells expressing myc-GβL or myc-γ-tubulin.

(D) Recombinant versions of mTOR and GβL interact with each other. Immunoblotting for HA-mTOR and myc-tagged proteins was performed on myc immunoprecipitates prepared from HEK-293T cells coexpressing HA-mTOR with either myc-GβL or myc-γ-tubulin.

(E) Immunoprecipitates of recombinant myc-raptor but not myc-γ-tubulin contain endogenous GβL.

(F) GβL interacts with the mTOR kinase domain. Myc-tagged full-length mTOR, its indicated fragments, or γ-tubulin were coexpressed with HA-GβL, and anti-myc immunoprecipitates were analyzed by anti-HA and anti-myc immunoblotting.

(G) Schematic diagram of mTOR showing the HEAT repeats, the FKBP12-rapamycin binding (FRB) and kinase (KD) domains, and the raptor- and GβL binding sites.

taining an active mTOR pathway in vivo. Furthermore, GβL is necessary for the formation of a nutrient- and rapamycin-sensitive interaction between raptor and mTOR. We propose a regulatory mechanism in which GβL and raptor function together to modulate the mTOR kinase activity.

Results

Identification of GβL as a Subunit of the mTOR-Signaling Complex

Using cell lysis conditions that preserve the raptor-mTOR complex, we isolated from HEK-293T cells growing in nutrient-rich media a 36 kDa protein that specifically interacts with mTOR (Figure 1A). As expected for an mTOR-containing complex purified from cells in nutrient-rich conditions (Kim et al., 2002), raptor was found in substoichiometric amounts with mTOR (Figure 1A). Mass spectrometric analysis identified the 36 kDa protein as GβL (*G* protein β-subunit-*l*ike protein, pronounced "Gable"), a widely expressed protein of unknown function (Rodgers et al., 2001). The structure of GβL consists almost entirely of seven WD40 repeats with high sequence similarity to those in the β subunits of heterotrimeric G proteins (Rodgers et al., 2001) (see Supplemental Figures S1 and S2 at http://www.molecule.org/cgi/content/full/11/4/895/DC1). Like mTOR and raptor, GβL is conserved among all eukaryotes, including *D. melanogaster*, *S. pombe*, *S. cerevisiae*, *C. elegans*, and *A. thaliana* (Ochotorena et al., 2001; Roberg et al., 1997). In complementary work, Loewith et al. (2002) reported that Lst8p, the budding yeast homolog of GβL, interacts with Tor1p and Tor2p and showed that transiently expressed recombinant versions of mTOR and GβL can interact with each other. Genetic analyses show that Lst8p regulates cell growth, the localization of amino acid transporters, and the expression of RTG genes, processes in which the TOR pathway plays a role (Roberg et al., 1997; Liu et al., 2001; Loewith et al., 2002). The fission yeast homolog of GβL, Wat1p, has functions in maintaining the stability of the genome and the integrity of microtubules (Ochotorena et al., 2001).

GβL Binds to the mTOR Kinase Domain Specifically and Independently of Raptor

An anti-GβL antibody generated against residues 298–312 detects GβL in immunoprecipitates prepared from HEK-293T cells using an mTOR but not control antibod-

ies (Figure 1B). Unlike raptor, GβL remains bound to mTOR in buffers containing the detergent Triton X-100, suggesting that the GβL-mTOR interaction does not require raptor. In addition to HEK-293T cells, we also detected the endogenous mTOR-GβL complex in mouse NIH-3T3 and C2C12 cell lines (data not shown). Furthermore, when overexpressed in HEK-293T cells, recombinant myc-GβL interacts with endogenous mTOR (Figure 1C) and coexpressed HA-mTOR (Figure 1D). Myc-tagged raptor overexpressed in HEK-293T cells also interacts with endogenous GβL in buffers containing the detergent CHAPS (Figure 1E). However, we can barely detect the interaction between raptor and GβL when analyzed in buffers containing Triton X-100 or NP-40 (data not shown), conditions previously shown to disrupt the raptor-mTOR interaction (Kim et al., 2002). Thus, the interaction between raptor and GβL might not be strong enough to survive cell lysis conditions containing such detergents, or the proteins may not interact directly and only bind through mTOR.

To identify the GβL binding site(s) on mTOR, we tested the capacity of full-length HA-GβL to interact with myc-tagged fragments of mTOR coexpressed in HEK-293T cells (Figure 1F). GβL interacts strongly with the C-terminal half of mTOR (amino acids 1348–2549), a region that binds weakly but specifically to raptor (Kim et al., 2002). On the other hand, GβL did not interact at all with the N-terminal half of mTOR (amino acids 1–1480), the region containing the principal binding site for raptor, the mTOR HEAT repeats. Further delineation of the GβL binding site reveals that GβL interacts with the mTOR kinase domain (amino acids 2115–2431) but not the adjacent FRB domain (amino acids 2000–2115), the known binding site for FKBP12-rapamycin (Chen et al., 1995). Truncation of the mTOR kinase domain destroyed its strong interaction with GβL but revealed that two separate regions of mTOR, amino acids 2185–2254 and 2255–2431, exhibit weak but specific binding to GβL (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/11/4/895/DC1). This suggests that the mTOR kinase domain might make multiple contacts with GβL to form a stable interaction or that GβL binding requires the intact kinase domain.

The kinase domain of mTOR shows high sequence similarity to those of other PIK family members, such as ATM, ATR, and DNA-PKcs (Keith and Schreiber, 1995). However, we could not detect an interaction between GβL and any of these proteins (data not shown), suggesting that GβL binding requires a structural motif present only in the mTOR kinase domain.

GβL Positively Regulates the mTOR Pathway

To determine the role of GβL in the mTOR pathway, we investigated the effects on mTOR signaling of decreasing the expression level of GβL using small interfering RNAs (siRNAs). Consistent with a critical role for GβL in mTOR function, a decrease in the expression of GβL reduced the in vivo phosphorylation state of endogenous S6K1 to a similar extent as a decrease in the expression of mTOR (Figure 2A). The siRNA targeting GβL or mTOR specifically decreased the phosphorylation levels of Thr389 and Thr421/Ser424 of S6K1, sites known to be phosphorylated by mTOR in vitro (Burnett et al., 1998; Isotani et al., 1999). The GβL siRNA did not significantly affect the expression of S6K1 or ATM, or the phosphorylation state or amount of PKB1/Akt1, a downstream effector of PI 3-Kinase. Unlike a decrease in raptor expression (Kim et al., 2002), a reduction in GβL expression did not also reduce mTOR levels. We confirmed the positive role of GβL in maintaining an active mTOR pathway in individual cells using an immunofluorescence assay that detects phosphorylated S6, the downstream target of S6K1. In HEK-293T cell monolayers transfected with siRNAs targeting mTOR, raptor, or GβL but not lamin, many fewer cells had phospho-S6 staining (Figure 2B), suggesting that these pathway components have a necessary role in mediating S6 phosphorylation. Similar results were also obtained in HeLa cells (data not shown).

Downstream effectors of mTOR, such as S6K1 and 4E-BP1, respond to upstream signals derived from both nutrients and growth factors (reviewed in Gingras et al., 2001; Kozma and Thomas, 2002; Schmelzle and Hall, 2000). To test whether GβL plays a role in transmitting growth factor signals downstream of mTOR, we cotransfected HEK-293T cells with an expression plasmid encoding myc-S6K1 and siRNAs targeting either lamin, GβL, mTOR, or raptor. After serum starvation, we stimulated the cells for 1 hr with dialyzed serum and measured the phosphorylation state of the myc-S6K1 reporter. Serum increased the phosphorylation of S6K1 in the cells cotransfected with the control siRNA but had only small effects in the cells cotransfected with the siRNAs targeting GβL, mTOR, and raptor (Figure 2C). In our previous study (Kim et al., 2002), we showed that raptor plays an important role in nutrient-sensitive signaling to S6K1 and cell growth. To investigate whether GβL also plays a role in nutrient-sensitive signaling to S6K1, we transfected HEK-293T cells with the siRNAs and the myc-S6K1 reporter as in the above serum stimulation experiment. Seventy-two hours after transfection, we deprived the cells of leucine for 40 min. When the leucine-deprived cells were stimulated with leucine for 10 min, the increase in the phosphorylation state of the myc-S6K1 reporter was significantly less in the cells transfected with siRNAs for GβL, mTOR, and raptor than in those transfected with the control lamin siRNA (Figure 2D). Each of the siRNAs targeting the mTOR pathway components had slightly different effects on the phosphorylation state of the myc-S6K1 reporter, perhaps reflecting their different efficacies in knocking down their respective targets. These results indicate that GβL, as well as mTOR and raptor, are important for signaling both growth factor and nutrient conditions to the S6K1 growth regulator.

The mTOR pathway, and S6K1 in particular, plays a critical role in regulating cell growth and determining mammalian cell size (Kim et al., 2002; Fingar et al., 2002). Consistent with a positive role for GβL in cell size control, actively growing HEK-293T cells transfected with siRNAs targeting GβL or mTOR underwent comparable reductions in size compared to cells transfected with a control siRNA (Figure 2E). Taken together, our loss-of-function studies indicate that GβL has a positive, likely essential, role in the mTOR pathway and participates in transmitting both growth factor and nutrient signals to downstream effectors of mTOR. Consistent with this

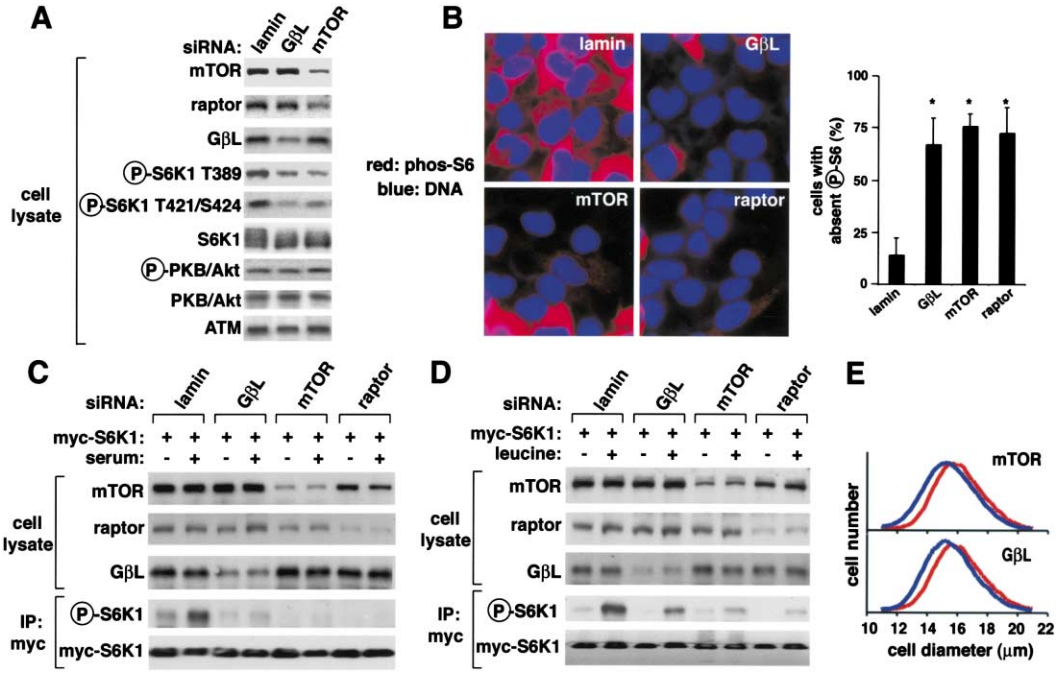

Figure 2. GβL has a Positive Function in the mTOR Pathway

(A) Inhibition of endogenous S6K1 phosphorylation in cells transfected with siRNAs targeting GβL. Cell lysates prepared from HEK-293T cells transfected with the lamin, mTOR, or GβL siRNAs were analyzed with immunoblotting for the indicated proteins or phosphorylated residues of S6K1.

(B) Transfection of the siRNA targeting GβL eliminates S6 phosphorylation. HEK-293T cells transfected with the indicated siRNAs were immunostained with an antibody recognizing S6 phosphorylated at residues 235 and 236 (red channel) and stained with Hoechst to detect cell nuclei (blue channel). Bar graph shows mean ± SD number of cells without phospho-S6 staining in wells transfected with the indicated siRNAs (n = 10 experiments). For each experiment, 100 cells were counted in a randomly selected area of the slide. *$p < 0.001$ when compared to lamin control.

(C) Inhibition of serum-mediated stimulation of S6K1 phosphorylation in cells transfected with an siRNA targeting GβL. HEK-293T cells transfected with myc-S6K1 and the indicated siRNAs were starved for serum for 24 hr two days after transfection. Dialyzed fetal bovine serum was added as indicated for 1 hr before harvesting the cells. The phosphorylation level of myc-S6K1 in myc-immunoprecipitates was analyzed by immunoblotting using the phospho-Thr389 antibody. Cell lysates prepared from these cells were also analyzed with immunoblotting for the indicated proteins.

(D) Inhibition of leucine-stimulated S6K1 phosphorylation in cells transfected with the GβL siRNA. HEK-293T cells transfected as in (C) were placed in leucine-free RPMI for 40 min three days after transfection and then stimulated as indicated with leucine for 10 min (52 μg/ml). Immunoprecipitation and immunoblotting were performed as in (C).

(E) GβL plays a positive role in cell size control. Shown are distributions of cell diameters of actively growing HEK-293T cells 3 days after transfection with siRNAs targeting lamin (red line), mTOR, or GβL (blue line). The mean ± SD (μm) of the cell diameters are: lamin siRNA 16.02 ± 0.05 (n = 4); mTOR siRNA 15.47 ± 0.05 (*) (n = 4); GβL siRNA 15.45 ± 0.06 (*) (n = 4). *$p < 0.05$ when compared to lamin control.

finding, a genome-wide RNAi screen in *C. elegans* (Fraser et al., 2000) shows that a knock-down in expression of ceGβL, the worm homolog of GβL, causes a growth defect (www.wormbase.org/db/seq/rnai?name=JA%3AC10H11.8;class=RNAi).

GβL Stimulates the mTOR Kinase Activity

In testing potential mechanisms by which GβL might exert its positive function, we found that coexpressing HA-GβL with myc-mTOR strongly increased the kinase activity of mTOR toward S6K1 and 4E-BP1 and its capacity for autophosphorylation (Figures 3A and 3B). Using phospho-specific antibodies against S6K1, we found the GβL increased the phosphorylation of both the Thr389 and Ser421/Thr424 sites of S6K1 (Figure 2A), the same sites reduced in cells with decreased GβL and mTOR expression (Figure 3A). We observed a dose-dependent relationship between the amount of coexpressed GβL and the level of mTOR activation (Figure 3C). GβL-mediated stimulation of the mTOR kinase does not require raptor, as it occurs even when myc-mTOR is washed with buffers containing Triton X-100, conditions that remove endogenous raptor (Kim et al., 2002). Control kinase assays using a kinase-dead version of mTOR confirm that the observed activity is that of mTOR and not of a contaminating kinase associated with GβL or of GβL-stimulated autophosphorylation of S6K1 (Figure 3D). These results suggest that stimulation of the mTOR kinase activity is likely an important mechanism by which GβL positively regulates the mTOR pathway.

By changing conserved residues in the WD-40 repeats of GβL, we generated two GβL mutants (F320S and S72D) that interact only weakly with mTOR and another (G192D) that does not bind mTOR at all (Figure 3E). GβL mutants F320S and S72D, but not G192D, partially stimulate the kinase activity of coexpressed myc-mTOR,

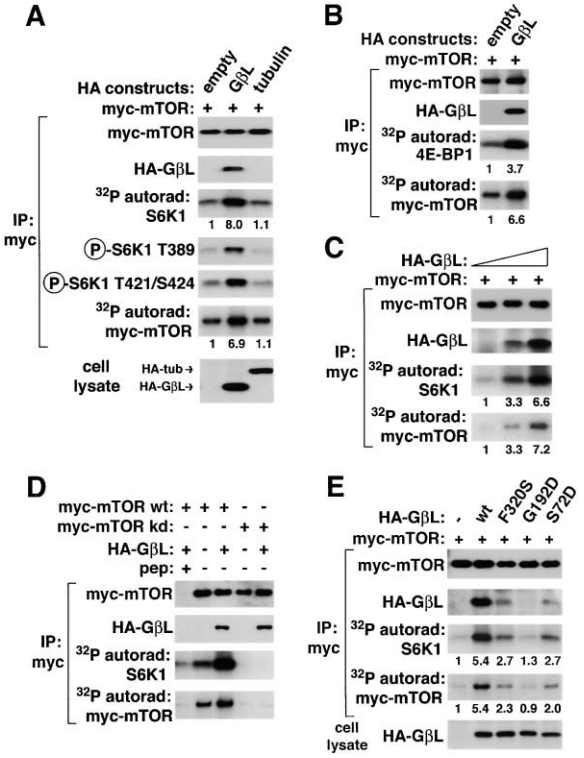

Figure 3. GβL Stimulates the In Vitro Kinase Activity of mTOR

(A) The binding of GβL to mTOR increases the kinase activity of mTOR toward itself and S6K1. mTOR kinase assays were performed as described (Kim et al., 2002) using anti-myc immunoprecipitates prepared from HEK-293T cells transfected with the indicated plasmids. Phosphorylation of S6K1 was also detected by immunoblotting using the indicated phospho-specific antibodies.

(B) GβL increases mTOR kinase activity toward 4E-BP1. mTOR activity was determined as in (A).

(C) GβL-mediated stimulation of the mTOR kinase activity correlates with the amount of GβL bound to mTOR and is independent of raptor. Myc-immunoprecipitates were prepared from HEK-293T cells expressing myc-mTOR and increasing amounts of HA-GβL and lysed in the Triton X-100-containing buffer A (Kim et al., 2002). mTOR autophosphorylation and activity toward GST-S6K1 were determined as in (A).

(D) GβL stimulation of the kinase activity in myc-mTOR immunoprecipitates depends on the presence of mTOR and its wild-type (wt) kinase domain. The activity is absent in the D2357E mutant (kd) or when immunoprecipitates are prepared in the presence of the peptide recognized by the anti-myc monoclonal antibody (9E10).

(E) GβL stimulation of the mTOR kinase activity depends on its capacity to bind mTOR. The kinase activity was determined (Kim et al., 2002) in myc immunoprecipitates prepared from cells transfected with the indicated plasmids and lysed in the Triton X-100-containing buffer A. In parts (A), (B), (C), and (E), the numbers below the autoradiographs indicate the fold changes in ^{32}P incorporation as determined with a phospho-imager.

indicating a correlation between the capacity of a GβL mutant to bind mTOR and to activate the mTOR kinase (Figure 3E).

The Binding of GβL to mTOR Is Necessary for Nutrients to Regulate the Raptor-mTOR Association

The stability of the GβL-mTOR interaction, unlike that of raptor-mTOR, is unaffected by nutrient conditions,

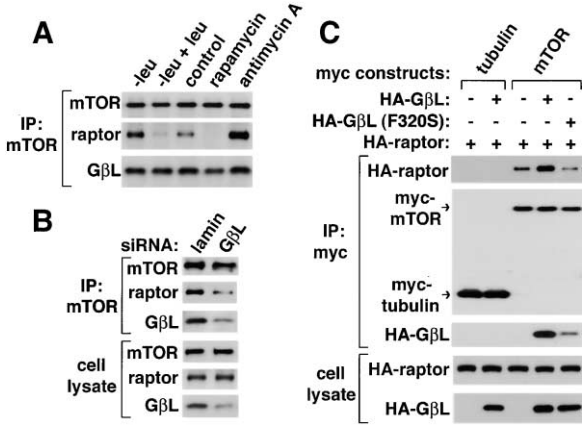

Figure 4. GβL Stabilizes the Raptor-mTOR Interaction

(A) The GβL-mTOR interaction is unaffected by nutrient conditions, rapamycin, and mitochondrial function. HEK-293T cells were not treated (control), deprived of leucine for 50 min (−leu), or deprived of leucine for 50 min and restimulated for 10 min with 52 μg/ml leucine (−leu +leu), or treated for 10 min with 20 nM rapamycin or 5 μM antimycin A. mTOR immunoprecipitates were prepared and analyzed by immunoblotting for mTOR, raptor, and GβL.

(B) Destabilization of the raptor-mTOR interaction by a reduction in GβL expression. mTOR immunoprecipitates and cell lysates prepared from cells transfected with siRNAs targeting lamin or GβL were analyzed by immunoblotting for mTOR, raptor, and GβL.

(C) GβL stabilizes the mTOR-raptor association. Immunoblotting was used to analyze the amounts of HA-raptor and HA-GβL recovered in myc immunoprecipitates prepared from HEK-293T cells expressing myc-mTOR or myc-γ-tubulin and HA-raptor with wild-type or F320S HA-GβL.

such as leucine stimulation or deprivation, or by treatment with rapamycin or the mitochondrial inhibitor, antimycin A (Figure 4A). In addition, the GβL-mTOR association is resistant to detergents like Triton X-100 (Figure 1B) and Nonidet P-40 and high salt concentrations (data not shown), suggesting that the interaction is not only constitutive but also more stable than that of mTOR with raptor. We noted that an siRNA-mediated decrease in the expression level of GβL reduced the amount of *both* GβL and raptor bound to mTOR, implying that GβL has a role in stabilizing the raptor-mTOR interaction (Figure 4B). Consistent with this idea, coexpression of HA-GβL with myc-mTOR increased the amount of coexpressed raptor that coimmunoprecipitates with myc-mTOR compared to when myc-mTOR was expressed alone or with a GβL mutant (F320S) that weakly binds mTOR (Figure 4C). The GβL mutant may compete with endogenous wild-type GβL for binding to mTOR, as its overexpression slightly decreases the amount of raptor recovered with myc-mTOR. Thus, GβL, through its constitutive interaction with mTOR, seems to play an important role in stabilizing the raptor-mTOR association.

Before the discovery of GβL, we hypothesized that both a constitutive interaction and a nutrient-sensitive interaction mediate the association between raptor and mTOR (Kim et al., 2002). We proposed that the nutrient-sensitive interaction strengthens under nutrient-poor conditions and stabilizes the mTOR-raptor complex so that it survives in vitro isolation. Because GβL stabilizes

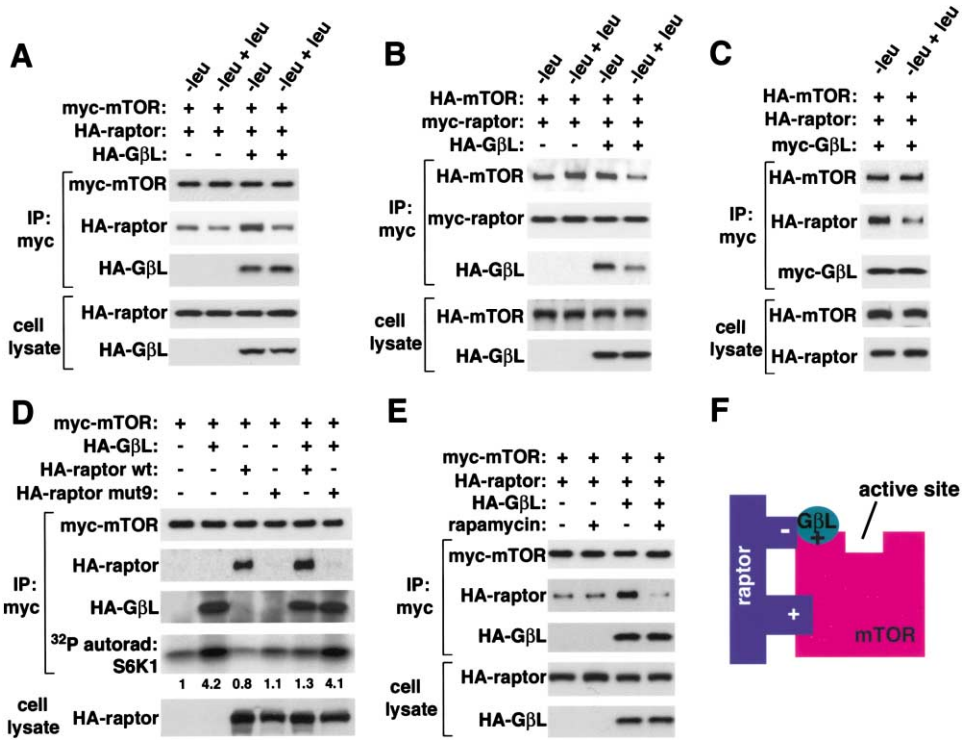

Figure 5. GβL Is Required for the Formation of a Nutrient-Sensitive Complex between Raptor and mTOR

(A) GβL is needed for the nutrient-mediated regulation of the raptor-mTOR interaction. HEK-293T cells transfected with the indicated plasmids were treated with different leucine conditions as in Figure 4A. Anti-myc-mTOR immunoprecipitates were prepared and analyzed by immunoblotting for the indicated proteins.

(B) GβL mediates the nutrient-sensitive interaction between raptor and mTOR. HEK-293T cells were transfected with the indicated plasmids, and anti-myc-raptor immunoprecipitates were prepared and analyzed as in (A).

(C) Complexes isolated through a myc-tag on GβL also show a leucine-sensitive interaction between mTOR and raptor.

(D) GβL and raptor have opposite effects on the mTOR kinase activity. Myc-mTOR kinase activity toward GST-S6K1 was determined as in Figure 3A from HEK-293T cells growing in nutrient-rich conditions and coexpressing myc-mTOR with the indicated HA-tagged GβL and/or raptor variants.

(E) Rapamycin disrupts the GβL-mediated interaction between raptor and mTOR. HEK-293T cells were transfected with myc-mTOR and HA-raptor or together with HA-GβL and treated with rapamycin (100 nM) for 20 min. The amounts of myc-mTOR, HA-raptor, and HA-GβL in anti-myc-mTOR immunoprecipitates were analyzed by immunoblotting.

(F) Model for regulation of mTOR function by GβL and raptor.

the raptor-mTOR association, we considered the possibility that GβL contributes to the nutrient-sensitive mechanism that mediates the interaction between raptor and mTOR. To test this idea, we investigated whether GβL is necessary for raptor and mTOR to form a nutrient-sensitive interaction. In HEK-293T cells, we coexpressed recombinant raptor and mTOR with or without GβL and analyzed the nutrient sensitivity of the raptor-mTOR association (Figure 5A). In the absence of GβL, the leucine concentration of the cell medium did not affect the amount of HA-raptor bound to myc-mTOR. However, when we coexpressed GβL with raptor and mTOR, the presence of leucine in the medium lowered the amount of HA-raptor bound to myc-mTOR, just as we see for endogenous raptor-mTOR pair (Figure 4A). As is the case with endogenous GβL and mTOR (Figure 4A), the interaction between the recombinant GβL and mTOR was not sensitive to leucine. Thus, these results suggest that GβL is necessary for the formation of a nutrient-sensitive association between mTOR and raptor.

To confirm such a role for GβL, we expressed recombinant, tagged versions of mTOR, raptor, and GβL and isolated the resulting complexes using epitope tag antibodies specific to raptor or GβL instead of mTOR. Consistent with the results in Figure 5A, the leucine concentration in the cell medium affected the amount of HA-mTOR bound to myc-raptor only when HA-GβL was also present (Figure 5B). In addition, the amount of HA-GβL recovered in the myc-raptor immunoprecipitates correlated with the amount of HA-mTOR. Lastly, when we isolated the complexes through myc-GβL, the amount of coimmunoprecipitating HA-raptor was also sensitive to the presence of leucine in the cell medium (Figure 5C). Thus, independent of the component used to isolate the mTOR signaling complex, the mTOR-raptor association is nutrient sensitive only if GβL is present. These findings suggest that GβL might be part of a nutrient-sensitive interaction between raptor and the mTOR-GβL complex or that its binding to mTOR induces a conformational change that makes the raptor-mTOR interaction nutrient sensitive.

We previously reported that the binding of raptor to endogenous mTOR induced by nutrient-poor conditions

decreases the mTOR kinase activity (Kim et al., 2002). Because GβL stabilizes the mTOR-raptor association and is necessary for its nutrient sensitivity (Figures 5A–5C), we predicted that GβL might be necessary for raptor to inhibit the mTOR kinase activity. As expected, in the absence of overexpressed GβL, HA-raptor had only a small inhibitory effect on the kinase activity of coexpressed myc-mTOR (Figure 5D). On the other hand, when the three proteins were expressed together to form a heterotrimeric complex, HA-raptor almost completely inhibited the HA-GβL-stimulated increase in myc-mTOR kinase activity without changing the amount of HA-GβL bound to myc-mTOR. Raptor mutant 9, which is incapable of interacting with mTOR (Kim et al., 2002), did not affect the basal or the GβL-stimulated mTOR kinase activity. Thus, the GβL plays a critical role in both the nutrient-sensitive interaction of raptor with mTOR and in raptor-mediated inhibition of mTOR kinase activity.

The mTOR Signaling Complex Must Contain GβL for Rapamycin to Destabilize the Raptor-mTOR Association

Rapamycin destabilizes the interaction between raptor and mTOR regardless of nutrient availability (Kim et al., 2002). Because FKBP12-rapamycin binds directly N-terminal to the mTOR kinase domain, the docking site for GβL, we thought that FKBP12-rapamycin binding might perturb the capacity of GβL to stabilize the mTOR-raptor interaction. To test this possibility, we investigated whether the rapamycin sensitivity of the raptor-mTOR interaction is dependent upon GβL. Interestingly, unlike the endogenous proteins (Kim et al., 2002), rapamycin did not destabilize the interaction between recombinant mTOR and raptor coexpressed in HEK-293T cells (Figure 5E). However, when we coexpressed GβL with them, the interaction between the recombinant proteins was destabilized by rapamycin treatment. This result suggests that FKBP12-rapamycin perturbs the raptor-mTOR interaction mediated by GβL.

Discussion

GβL: A Regulatory Subunit of the mTOR Signaling Complex

Our findings indicate that GβL is a subunit of the mTOR signaling complex that positively regulates the mTOR pathway. GβL interacts constitutively with the mTOR kinase domain, activates the mTOR kinase, and is necessary for mTOR to form a nutrient-sensitive interaction with raptor. Between GβL and raptor, the mTOR signaling complex contains 14 WD-40 repeats, which are found in numerous proteins involved in diverse cellular processes including signal transduction, cell cycle progression, vesicular trafficking, and RNA processing (Neer et al., 1994; Smith et al., 1999). In many cases, proteins containing WD-40 repeats form multimeric complexes with other proteins, in which the repeats serve as scaffolds for building the complexes (Smith et al., 1999). In a few cases, domains containing WD-40 repeats play a role in recruiting phosphorylated proteins to the catalytic sites of enzymes (Nash et al., 2001; Yaffe and Elia, 2001). Interestingly, WD-40 repeat domains are also found in proteins that interact with PP2A, a phosphatase for which substantial evidence suggests that it has a major role in regulating downstream signaling by the mTOR pathway (Moreno et al., 2000; Peterson et al., 1999). Either as scaffolds or adaptors for recruiting substrates, the WD-40 repeat domains of GβL and raptor are likely to play important roles in regulating the mTOR pathway.

Studies in fission yeast hint that GβL is likely to participate in other signaling systems besides the TOR pathway. Mutations in Wat1p, the fission yeast homolog of GβL, lead to genomic instability and cell morphological changes, phenotypes not necessarily associated with TOR pathway dysfunction (Kemp et al., 1997; Ochotorena et al., 2001). Furthermore, Wat1p interacts with Prp2p, the large subunit of the essential splicing factor U2AF (Ochotorena et al., 2001). Nevertheless, our loss-of-function experiments strongly indicate that GβL plays an essential, positive role in controlling cell growth by activating the mTOR kinase.

mTOR, Raptor and GβL Are the Minimal Components of the Nutrient-Sensitive Complex (NSC)

We readily observe a nutrient-sensitive interaction between endogenous mTOR and raptor or between endogenous mTOR and recombinant raptor expressed at low levels (Kim et al., 2002). However, until our current report, we had not been able to detect regulation by nutrients of the association between recombinant, overexpressed versions of mTOR and raptor, consistent with findings from other groups (Hara et al., 2002; Loewith et al., 2002). Thus, we hypothesized that the discrepant results between the endogenous and recombinant proteins might be due to the absence of a limiting cellular factor(s) that is required for the formation of a nutrient-sensitive complex. We report here that this appears to be the case. Coexpression of GβL with raptor and mTOR is necessary and sufficient to obtain a nutrient-sensitive interaction between recombinant versions of raptor and mTOR. Thus, mTOR, raptor, and GβL are likely to be the minimal elements needed for the formation of the nutrient-sensitive complex (NSC). In budding yeast the TOR proteins interact with other proteins (AVO1p, AVO2p, and AVO3p) besides KOG1p and Lst8p, the yeast homologs of raptor and GβL, respectively (Loewith et al., 2002). How these proteins function in the yeast TOR pathway is unknown, and mammalian homologs of AVO1, AVO2, and AVO3 have not been identified. Because mTOR forms a large protein complex (1.5–2.0 mDa) (our unpublished data), it seems probable that mTOR has partner proteins in addition to raptor and GβL, although our current work suggests that these hypothetical proteins are not required for the formation of a nutrient-sensitive complex.

GβL and Raptor Have Opposing Effects on the mTOR Kinase Activity

How GβL stimulates the mTOR kinase activity is currently unknown, but we have explored several options. One possibility is that GβL contributes to the stability or folding of the mTOR kinase domain. This notion is supported by the finding that several heat shock proteins bind the GβL-docking domain on mTOR when it is expressed in the absence of GβL (data not shown).

Furthermore, as GβL increases the capacity of mTOR to phosphorylate itself as well as S6K1 and 4E-BP1, it seems likely that GβL has a function independent of mTOR substrates, such as maintaining the structure of the mTOR active site. However, the complex of GβL and an mTOR fragment (amino acids 2000–2549) containing the kinase domain does not have activity (data not shown). This finding indicates that GβL is not sufficient to activate the isolated mTOR kinase domain and that other mTOR regions besides the kinase domain are important for mTOR activity. Other mTOR regions are likely not required for maintaining the structural integrity of the mTOR active site, because the isolated mTOR kinase domain, with or without GβL, binds ATP-agarose as well as the full-length protein (our unpublished data).

It is also possible that GβL, like raptor (Hara et al., 2002), plays a role in the recognition and recruitment of mTOR substrates. Consistent with this idea, we find that recombinant HA-GβL interacts with endogenous S6K1 independently of mTOR and raptor, but we have failed to detect a specific interaction between GβL and a recombinant epitope-tagged S6K1 (data not shown). Further work will be required to determine the physiological role of GβL and raptor in recruiting mTOR substrates, particularly as extremely low percentages of the total cellular substrates are bound to raptor or GβL (our unpublished data).

Irrespective of the mechanism by which GβL activates mTOR, we favor a model in which the binding of raptor to the complex of GβL and the mTOR kinase domain inhibits GβL-mediated activation of mTOR (Figure 5F). Although it has been reported that raptor stimulates the mTOR kinase activity (Hara et al., 2002), under our experimental conditions we do not see this effect of raptor when using S6K1 or 4E-BP1 as kinase substrates (data not shown). A possible explanation for this discrepancy is that co-overexpression of recombinant mTOR and raptor but not GβL might not generate a physiologically functional complex. Alternatively, different experimental or cellular conditions, such as the expression level of endogenous GβL and/or raptor, might account for the observed differences. Nevertheless, we find that, independently of raptor, GβL stimulates the mTOR kinase activity and that inhibition of the mTOR activity by raptor requires GβL. These findings suggest to us a model in which the opposing actions of GβL and raptor regulate the mTOR activity (Figure 5F). In this model, raptor forms a nutrient-sensitive interaction with GβL docked to the mTOR kinase domain. The binding of raptor to this site, which is induced by nutrient-poor conditions, might inhibit the positive function of GβL. Nutrient-rich conditions would weaken the raptor interaction, releasing GβL from the inhibitory effects of raptor.

Although the docking site for FKBP12-rapamycin, the FRB domain (Chen et al., 1995), is directly N-terminal to the GβL binding site on mTOR, we find that rapamycin does not significantly affect the amount of GβL bound to mTOR (Figure 4A). Instead, rapamycin destabilizes the interaction between mTOR and raptor (Kim et al., 2002), and this effect requires GβL (Figure 5E). Thus, the binding of FKBP12-rapamycin to mTOR, like nutrient-rich conditions, might weaken the interaction of raptor with the mTOR-GβL complex. However, unlike nutrient-rich conditions, the presence of FKBP12-rapamycin next to GβL might perturb the positive function of GβL, mimicking the inhibitory effects of raptor seen under nutrient-poor conditions. During the identification of mTOR (which we called RAFT1 at the time), we showed that when FKBP12-rapamycin is bound to mTOR, it is close enough to an unidentified 35 kDa protein that FKBP12 can be chemically cross-linked to it (Sabatini et al., 1994). This mysterious protein, which we named RAFT2, may be the 36 kDa GβL, a possibility consistent with the closely juxtaposed binding sites on mTOR for FKBP12-rapamycin and GβL.

Conclusions

Our current findings provide strong evidence that the interaction of GβL with mTOR is important for the nutrient-mediated regulation of the mTOR signaling complex. The molecular signals derived from nutrients that modulate the interactions remain to be explored in future work. It is possible that GβL or raptor might be modified by upstream nutrient-regulated mechanisms, which may change the affinity of the interactions between the proteins in the complex. In addition, we cannot exclude the possibility that GβL might recruit an unidentified cellular component to the mTOR kinase domain, which conveys the upstream nutrient-derived signals to the mTOR complex. Because growth factors do not change the stability of the raptor-mTOR interaction (Kim et al., 2002), the PI3K/PKB pathway is likely to affect mTOR function in a different way than nutrients, perhaps, as has been suggested, by affecting the phosphorylation state of mTOR (Scott et al., 1998; Sekulic et al., 2000). Our finding that by coexpressing three recombinant proteins (mTOR, raptor, and GβL) we can reconstitute the nutrient-sensitive complex opens the door to the molecular dissection of the signals involved in its regulation. We propose that the opposing effects on mTOR activity of the interactions mediated by GβL and raptor provide a mechanism by which cellular conditions, such as nutrient levels, can positively and negatively regulate mTOR signaling to the cell growth machinery. The balance between the actions of the two regulators may be perturbed in human diseases, such as cancer and diabetes, and could be artificially manipulated for potentially therapeutic benefits.

Experimental Procedures

Materials

Reagents were obtained from the following sources: DSP and protein G-Sepharose from Pierce; [γ-^{32}P]ATP from NEN; mTOR, S6K1, lamin, and Rho antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; phospho-S6, phospho-T389 S6K1, phospho-T421/S424 S6K1, and phospho-S473 PKB/Akt antibodies from Cell Signaling; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; myc rabbit polyclonal antibody from Upstate Biotechnology; DMEM, leucine, glucose, RPMI, and RPMI without leucine from Life Technologies; rapamycin and antimycin A from Calbiochem. The rabbit polyclonal anti-GβL antibody, recognizing residues 298–312 of human GβL, was produced and purified using the custom antibody service from Covance.

Purification and Identification of GβL

mTOR immunoprecipitates prepared from 200 million HEK293T cells were prepared as described (Kim et al., 2002) and resolved by SDS-

PAGE, and proteins were visualized by Coomassie blue staining. The band corresponding to GβL was excised and trypsinized (Erdjument-Bromage et al., 1994). A hundred percent of the generated peptides were subjected to a micro-clean-up procedure using 2 μl bed-volume of Poros 50 R2 (PerSeptive) reversed-phase beads packed in an Eppendorf gel-loading tip. Mass spectrometry (MALDI-ReTOF) was then carried out on two peptide pools (16% and 30% MeCN) recovered from the RP-microtip column using a Bruker REFLEX III instrument with delayed extraction. For mass fingerprinting, top major experimental masses (*m/z*) combined from both MALDI-ReTOF experiments were used to search a nonredundant human protein database (NR; ~66,605 entries; NCBI; Bethesda, MD), using the PeptideSearch (M. Mann, University of Southern Denmark) algorithm. A molecular weight range twice the predicted weight was covered with a mass accuracy restriction better than 40 ppm, and a maximum of one missed cleavage site was allowed per peptide. Alternatively, mass spectrometric-based sequencing (ESI-MS/MS) of selected peptides from partially fractionated pools was carried out using a PE-SCIEX API300 triple quadrupole instrument, fitted with a continuous flow nano-electrospray source (JaFIS). All peptide masses in pools were obtained by DE-MALDI-reTOF MS (BRUKER Reflex III). Peptide sequences were obtained by nano-electrospray tandem MS (JaFIS source with SCIEX API300 triple quadrupole).

Immunoprecipitations

10×10^6 HEK-293T cells growing in 10 cm dishes were rinsed once with PBS and lysed in 1 ml of ice-cold buffer B (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, 0.3% CHAPS, and one tablet EDTA-free protease inhibitors [Roche] per 10 ml). After clearing the lysates by centrifugation at 10,000 × g for 10 min, 30 μl of a 50% slurry of protein G-Sepharose and 4 μg of the immunoprecipitating antibody was added to the supernatant. After a 3 hr incubation at 4°C, immunoprecipitates were washed four times with buffer B and once with wash buffer 1 (50 mM HEPES [pH 7.5], 40 mM NaCl, and 2 mM EDTA). Samples were resolved by SDS-PAGE, proteins transferred to PVDF and used for immunoblotting as described (Burnett et al., 1998). When Triton X-100 was used to eliminate raptor binding to mTOR, immunoprecipitates were prepared as above except that buffer A (buffer B with 1% Triton X-100 instead of CHAPS) was used to lyse the cells.

Cloning of the GβL cDNA, DNA Manipulations, and Mutagenesis

The human GβL clone from the mammalian gene collection (MGC5518) was obtained from Incyte, subcloned into myc- and HA-prk5 vectors by PCR, and transfected into HEK-293T cells using lipofectamine 2000 as described by the manufacturer (Invitrogen). The mTOR fragments indicated in Figure 1 and Supplemental Figure S3 (at http://www.molecule.org/cgi/content/full/11/4/895/DC1) were expressed from cDNAs subcloned into the myc-prk5 vectors. The GβL open reading frame in pBluescript II SK(+) was mutagenized using the QuickChange mutagenesis kit (Stratagene) as described by the manufacturer, subcloned into the SalI and NotI sites of myc- and HA-prk5, and confirmed by DNA sequencing. The GβL mutants used in this study are S72D, G192D, and F320S. The mutated sites are indicated in the alignment of the WD40 repeats (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/11/4/895/DC1) and the structural model of GβL (see Supplemental Figure S2 at http://www.molecule.org/cgi/content/full/11/4/895/DC1). All other epitope tagged constructs have been described (Burnett et al., 1998; Kim et al., 2002).

Plasmid and siRNA Transfections

3 million HEK293T cells in 6 cm dishes were transfected with plasmid constructs indicated in the figure legends using the Lipofectamine 2000 transfection reagent (Life Technology). 24 hr after DNA addition, cells were rinsed once with PBS and lysed in 300 μl of ice-cold buffer B. Immune complexes were prepared from cleared supernatants using 2 μg monoclonal anti-myc or anti-HA antibodies and 20 μl of a 50% slurry protein G-Sepharose. After a 3 hr incubation, immuneprecipitates were washed five times with buffer B. Bound proteins were eluted in 1× sample buffer, and mTOR or HA- or myc-tagged proteins were detected by immunoblotting as described (Burnett et al., 1998). Twenty-one-nucleotide complementary RNAs with two-nucleotide overhangs (Elbashir et al., 2001) were designed to target bases 188–210 of the human GβL open reading frame. The sequences for the siRNAs for lamin, mTOR, and raptor, the transfection conditions, and the procedures for determining cell size have been described (Kim et al., 2002).

Immunofluorescence

HEK-293T or HeLa cells transfected with siRNA targeting lamin, GβL, mTOR, or raptor were harvested one day after transfection and seeded onto 1.5 cm diameter gelatin-coated glass coverslips. 48 hr after seeding, the cells were fixed in 3.7% paraformaldehyde for 20 min at room temperature, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing twice with PBS and blocking with 1% BSA for 1 hr, the cells were incubated overnight with the anti-phospho S6 antibody. The cells were then washed twice with PBS, incubated with an anti-rabbit cy3-labeled secondary antibody (Jackson Immunolabs) and Hoechst for 30 min, washed with PBS, mounted in glycerol containing 0.1% p-phenylenediamine, and visualized with fluorescence microscopy.

Sequence Alignments and Model Building

The WD40 repeat sequences of GβL were aligned with ClustalX v1.81 (Thompson et al., 1997) using the Gonnet series weight matrix. Pairwise gap opening and gap extension penalties were set at 10.00 and 0.10, respectively. Multiple alignment gap opening and gap extension penalties were set at 10.00 and 0.20. The GβL model was built and optimized with Modeler (Sali and Blundell, 1993) using the coordinates of the TUP1 β chain (pdb: 1ERJ accession: 1ERJ_B) as the template.

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