Phosphorylation of the Translational Repressor PHAS-I by the Mammalian Target of Rapamycin

Gregory J. Brunn, Christine C. Hudson, Aleksandar Sekulić, Josie M. Williams, Hajime Hosoi, Peter J. Houghton, John C. Lawrence Jr., Robert T. Abraham*

The immunosuppressant rapamycin interferes with G_1-phase progression in lymphoid and other cell types by inhibiting the function of the mammalian target of rapamycin (mTOR). mTOR was determined to be a terminal kinase in a signaling pathway that couples mitogenic stimulation to the phosphorylation of the eukaryotic initiation factor (eIF)-4E–binding protein, PHAS-I. The rapamycin-sensitive protein kinase activity of mTOR was required for phosphorylation of PHAS-I in insulin-stimulated human embryonic kidney cells. mTOR phosphorylated PHAS-I on serine and threonine residues in vitro, and these modifications inhibited the binding of PHAS-I to eIF-4E. These studies define a role for mTOR in translational control and offer further insights into the mechanism whereby rapamycin inhibits G_1-phase progression in mammalian cells.

Stimulation of quiescent cells with growth factors leads to a dramatic increase in the translation of a subset of mRNAs whose protein products are required for progression through the G_1 phase of the cell cycle (*1*, *2*). Translational control usually occurs at the level of initiation, an event influenced by regulatory elements located in the 5′-untranslated regions (UTRs) of many eukaryotic mRNAs. Efficient initiation of translation on mRNAs bearing a long, highly structured 5′-UTR is dependent on eIF-4F, a multisubunit complex containing a N^7-methylguanosine cap–binding subunit, eIF-4E; an RNA helicase, eIF-4A; and a multifunctional scaffolding protein, eIF-4G (*3*). The RNA helicase activity of the eIF-4F complex is believed to melt secondary structure in the 5′-UTR of capped mRNAs, thereby facilitating ribosome binding to the AUG initiation codon (*4*).

The association of eIF-4E with eIF-4G is inhibited by the eIF-4E–binding proteins PHAS-I and PHAS-II (*5*, *6*). In quiescent cells, PHAS-I is relatively underphosphorylated and binds tightly to eIF-4E. Stimulation of cells with growth factors markedly increases the phosphorylation of PHAS-I, which promotes the dissociation of the PHAS-I·eIF-4E complex. Hence, the pathway leading to PHAS-I phosphorylation couples growth factor receptor occupancy to the stimulation of eIF-4E–dependent protein synthesis. A link between eIF-4E function and cell-cycle progression is strongly suggested by the mitogenic and transforming effects of eIF-4E overexpression in fibroblasts (*7*).

PHAS-I is phosphorylated in vitro by the mitogen-activated protein (MAP) kinase (*6*, *8*). However, pharmacologic data indicate that MAP kinase is not responsible for growth factor–induced phosphorylation of PHAS-I in intact cells (*9*, *10*). The sensitivity of this event to rapamycin implicates a MAP kinase–independent pathway involving the rapamycin target protein mTOR (*11*) [also called FRAP or RAFT1 (*12*)]. mTOR and its budding yeast homologs Tor1p and Tor2p are members of the family of phosphoinositide 3-kinase–related kinases (*13*). Whether the TOR proteins function as lipid kinases or protein kinases remains uncertain. Here we show that mTOR functions as a protein kinase in the pathway leading to PHAS-I phosphorylation.

Human embryonic kidney (HEK) 293 cells were transfected with expression vectors encoding rat PHAS-I and either wild-type mTOR (mTOR-wt) or a rapamycin-resistant mTOR (mTOR-rr) mutant. This mutant contains a single amino acid substitution (Ser^{2035}→Ile) that generates a catalytically active kinase that has a lower binding affinity for the inhibitory FK506-binding protein-12 (FKBP12)·rapamycin complex (*14*). Both mTOR-wt and mTOR-rr contained an NH_2-terminal epitope tag recognized by the monoclonal antibody (mAb) AU1. Alterations in the phosphorylation state of PHAS-I were detected by immunoblot analysis with antibody to PHAS-I (anti–PHAS-I). Phosphorylation of PHAS-I decreases its electrophoretic mobility during SDS–polyacrylamide gel electrophoresis (PAGE) (*6*). Rapamycin inhibited the insulin-stimulated phosphorylation of PHAS-I in mock-transfected or mTOR-wt–transfected 293 cells, as indicated by the decrease in the intensity of the uppermost band (γ) and by the increase in the higher mobility band (α) that corresponds to a less phosphorylated form of PHAS-I (Fig. 1A). In contrast, PHAS-I phosphorylation in mTOR-rr–ex-

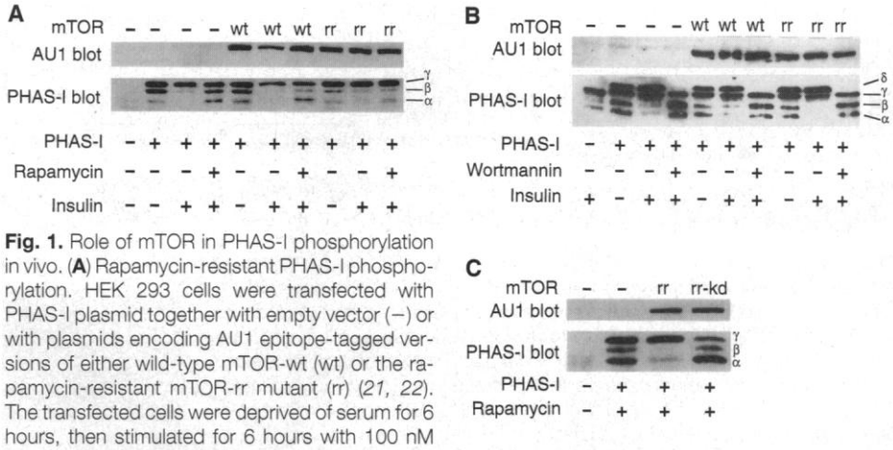

Fig. 1. Role of mTOR in PHAS-I phosphorylation in vivo. (**A**) Rapamycin-resistant PHAS-I phosphorylation. HEK 293 cells were transfected with PHAS-I plasmid together with empty vector (−) or with plasmids encoding AU1 epitope-tagged versions of either wild-type mTOR-wt (wt) or the rapamycin-resistant mTOR-rr mutant (rr) (*21*, *22*). The transfected cells were deprived of serum for 6 hours, then stimulated for 6 hours with 100 nM insulin. Rapamycin (5 nM) was added 1 hour before cell harvest. Detergent-soluble proteins were resolved by SDS-PAGE and were immunoblotted with mAb AU1 or anti–PHAS-I. The labels α, β, γ, and δ are arbitrary designations for immunoreactive bands that represent different phosphorylated forms of PHAS-I. (**B**) Effect of wortmannin on PHAS-I phosphorylation. HEK 293 cells were transfected with the PHAS-I plasmid together with either mTOR-wt or mTOR-rr. The cells were stimulated with insulin as described in (A), and were treated for 30 min with 1 μM wortmannin before preparation of cellular extracts. (**C**) Role of mTOR protein kinase activity in PHAS-I phosphorylation in vivo. HEK 293 cells were transfected with expression vectors encoding mTOR-rr or a catalytically inactive mTOR-rr-kd mutant (*24*). The transfected cells were stimulated with insulin, and 1 nM rapamycin was added 1 hour before the preparation of cellular extracts.

G. J. Brunn and J. C. Lawrence Jr., Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Charlottesville, VA 22908, USA.
C. C. Hudson, A. Sekulić, J. M. Williams, R. T. Abraham, Division of Oncology Research, Mayo Clinic, Rochester, MN 55905, USA.
H. Hosoi and P. J. Houghton, Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA.
*To whom correspondence should be addressed.

pressing 293 cells was only slightly attenuated by rapamycin. These results suggest that mTOR is the rapamycin-sensitive component of the signaling pathway leading to PHAS-I phosphorylation.

Wortmannin irreversibly inhibits the autophosphorylation of mTOR in vitro, with half-maximal and maximal inhibitions of this activity observed at drug concentrations of 0.2 and 1 μM, respectively (*10*). Because wortmannin targets the adenosine triphosphate (ATP)–binding site of mTOR, this drug should inactivate the kinase domains of both mTOR-wt and mTOR-rr. Treatment of mTOR-wt– or mTOR-rr–expressing 293 cells with 1 μM wortmannin resulted in a decrease in PHAS-I phosphorylation (Fig. 1B). Although mTOR may not be the only wortmannin-sensitive target in the PHAS-I phosphorylation pathway (*15*), these results suggested that the phosphotransferase activity of mTOR was important for signaling through this pathway.

The role of the kinase activity of mTOR in insulin-dependent PHAS-I phosphorylation was examined by expressing a catalytically inactive version of mTOR-rr (mTOR-rr-kd) in 293 cells (Fig. 1C). The mTOR-rr-kd double mutant contains an additional Asp^{2338}→Ala substitution that abrogates phosphotransferase activity (*10*) (Fig. 2B). The transfected cells were treated with rapamycin to inhibit endogenous mTOR, thereby allowing a direct comparison of the abilities of drug-resistant mTOR-rr and mTOR-rr-kd mutants to support this response in vivo. Whereas the highly phosphorylated γ form of PHAS-I predominated in mTOR-rr–expressing cells, accumulation of the underphosphorylated α and β forms of PHAS-I was clearly evident in the mTOR-rr-kd–expressing cells. The inability of the catalytically inactive mTOR-rr-kd mutant to drive the phosphorylation of PHAS-I in rapamycin-treated cells indicates that this response is dependent on the kinase activity of mTOR.

To determine whether mTOR itself functions as a PHAS-I kinase, we immunoprecipitated mTOR from rat brain extracts and incubated the immunoprecipitates in kinase buffer containing recombinant PHAS-I as the substrate. The anti-mTOR immunoprecipitates catalyzed the formation of at least three phosphorylated forms of PHAS-I that were distinguished on the basis of their electrophoretic mobilities (Fig. 2A). This PHAS-I kinase activity was inhibited by FKBP12·rapamycin but not by FKBP12·FK506. The PHAS-I kinase activity found in mTOR immunoprecipitates was inhibited by FKBP12·rapamycin, but not by FKBP12·FK506, which does not target mTOR in vivo (*16*). Furthermore, wortmannin inhibited the in vitro phosphorylation of PHAS-I at drug concentrations (0.1 to 1 μM) identical to those required for inhibition of mTOR autophosphorylation (*10*). These pharmacologic characteristics suggested that the PHAS-I kinase activity present in mTOR immunoprecipitates is due to mTOR itself.

We investigated this possibility further by testing the ability of recombinant mTOR to phosphorylate PHAS-I in immune complex kinase assays. AU1-tagged mTOR-wt and a catalytically inactive mTOR-kd mutant (Asp^{2338}→Ala) were expressed in K562 erythroleukemia cells. The mAb AU1 immunoprecipitates from mTOR-wt–expressing cells phosphorylated PHAS-I on both serine and threonine residues (Fig. 2, B and C). The PHAS-I kinase activity of recombinant mTOR-wt was sensitive to FKBP12·rapamycin or wortmannin, but not to FKBP12·FK506. In contrast, only background levels of PHAS-I kinase activity were present in mAb AU1 immunoprecipitates from mTOR-kd–expressing cells.

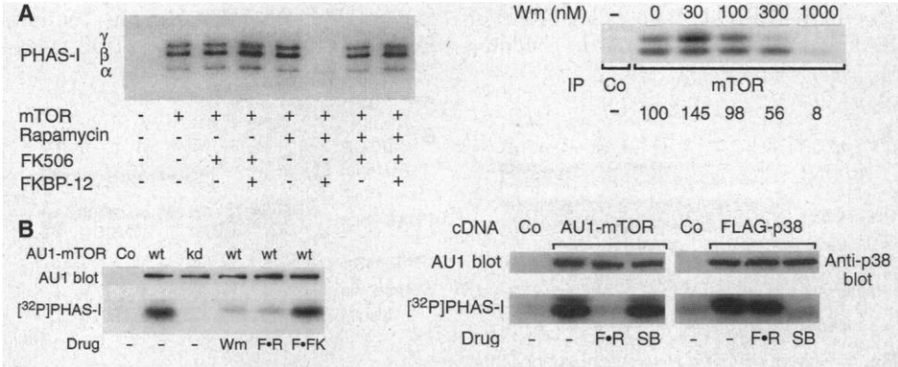

Fig. 2. Phosphorylation of PHAS-I by immunopurified mTOR. (**A**) Rat brain extracts were immunoprecipitated with anti-mTOR (+) or with nonimmune rabbit immunoglobulin G (−). Immune complex kinase reactions were done with recombinant PHAS-I as the substrate (*23*). The kinase reaction products were separated by SDS-PAGE, and ^{32}P-labeled PHAS-I was detected by autoradiography. In the right panel, rat brain extracts were immunoprecipitated (IP) with rabbit immunoglobulin G (Co) or with anti-mTOR, and immune complex kinase reactions were done as described above. The numbers below the sample lanes represent [^{32}P]phosphate incorporated into PHAS-I, as a percentage of that in the non-wortmannin (Wm)-treated control. (**B**) AU1-tagged mTOR-wt and mTOR-kd (left panel) or FLAG-tagged p38 (right panel) were immunoprecipitated from transfected K562 cells (*25*). Control transfections (Co) were performed with pcDNA3 only. The immunoprecipitates were treated with GST-FKBP12 plus rapamycin (F·R) or FK506 (F·FK), or 5 μM SB203508 (SB), and immune complex kinase assays were performed with PHAS-I as the substrate. ^{32}P-labeled PHAS-I (bottom panels) was detected by autoradiography. The immunoprecipitated mTOR and p38 were visualized by immunoblotting with mAb AU1 and anti-p38, respectively (top panels). (**C**) Phosphoamino acid analysis of PHAS-I phosphorylated in vitro by recombinant mTOR. Two-dimensional separation of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) was done as described (*26*).

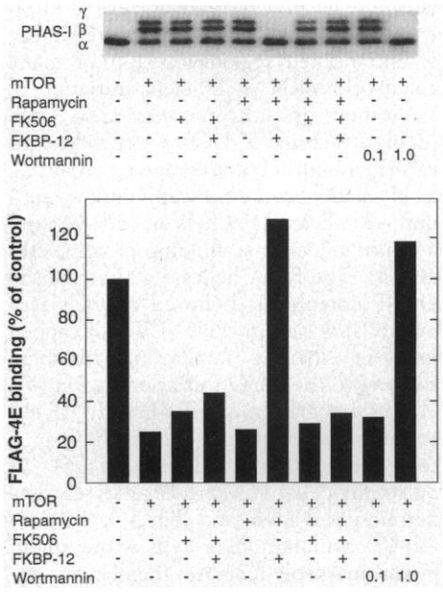

Fig. 3. The eIF-4E–binding activity of phosphorylated PHAS-I. Nonimmune rabbit immunoglobulin G (−) or anti-mTOR (+) immunoprecipitates from rat brain were incubated with the indicated agents, and immune complex kinase assays were done with recombinant PHAS-I and nonradioactive ATP (*23*). The concentrations of wortmannin are given in micromolar. (**Top**) Reaction products were separated by SDS-PAGE, and phosphorylated forms of PHAS-I were detected by immunoblotting. (**Bottom**) The eIF-4E–binding activity of PHAS-I was determined by Far-Western analysis with ^{32}P-labeled FLAG–eIF-4E as a probe (*6*). Radioactivity bound to PHAS-I was quantitated and normalized to the mock-phosphorylated control.

As a control for drug specificity, we examined the effect of rapamycin on the protein kinase activity of p38, which, like the related MAP kinases ERK1 and ERK2 (6, 8), phosphorylates PHAS-I in vitro. The phosphorylation of PHAS-I by recombinant p38 was not inhibited by FKBP12·rapamycin at concentrations that blocked the kinase activity of mTOR. Conversely, the p38 inhibitor SB203508 (17) blocked the phosphorylation of PHAS-I by p38 but not by mTOR-wt. The inhibitory effect of FKBP12·rapamycin on PHAS-I phosphorylation in vitro therefore appears specific for the kinase activity found in anti-mTOR immunoprecipitates.

The effect of phosphorylation by mTOR on the eIF-4E–binding activity of PHAS-I was assessed by Far-Western analysis. Recombinant PHAS-I was phosphorylated in vitro by rat brain–derived mTOR and was then subjected to SDS-PAGE and protein blotting. The binding activity of PHAS-I was determined by probing the membrane with ^{32}P-labeled eIF-4E (Fig. 3). Phosphorylation of PHAS-I by mTOR markedly reduced the ability of PHAS-I to interact with eIF-4E. Both the phosphorylation of PHAS-I and the loss of eIF-4E binding were blocked by FKBP12·rapamycin or wortmannin, but not by FKBP12·FK506. Indeed, a molar excess of FK506 antagonized the inhibitory effects of FKBP12·rapamycin by competing with rapamycin for the available FKBP12 (16).

The results of this study support the conclusion that mTOR functions as a PHAS-I kinase both in vitro and in vivo. Furthermore, mTOR phosphorylated PHAS-I in vitro at serine and threonine residues identical to those phosphorylated in insulin-stimulated adipocytes (18). These insulin-stimulated phosphorylation events are inhibited by rapamycin (18), which presumably explains the suppressive effect of this drug on eIF-4E–dependent translation. A homologous situation may exist in budding yeast, in which the rapamycin-sensitive functions of the TOR proteins that promote progression through G_1 have been linked to the stimulation of cap-dependent protein synthesis (19).

Accumulating evidence suggests that the rate of progression of mammalian cells through the G_1 phase is governed in part by the ratio of the translational stimulator eIF-4E to the repressor protein PHAS-I (7, 20). Thus, the phosphorylation of PHAS-I by mTOR may represent a critical step in the pathway that couples growth factor receptor occupancy to an increase in eIF-4E–dependent translation initiation. Inhibition of the PHAS-I kinase activity of mTOR may be the mechanism whereby rapamycin interferes with G_1 transit and S-phase commitment in both antigen-activated lymphocytes and transformed cells.

REFERENCES AND NOTES

1. C. Norbury and P. Nurse, *Annu. Rev. Biochem.* **61**, 441 (1992).
2. E. J. Brown and S. L. Schreiber, *Cell* **86**, 517 (1996).
3. N. Sonenberg, *Gene Expr.* **3**, 317 (1993).
4. V. Pain, *Eur. J. Biochem.* **236**, 747 (1996).
5. A. Pause *et al.*, *Nature* **371**, 762 (1994); C. Hu, S. Pang, X. Kong, M. Velleca, J. C. Lawrence Jr., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3730 (1994).
6. T.-A. Lin *et al.*, *Science* **266**, 653 (1994).
7. A. Lazaris-Karatzas, K. S. Montine, N. Sonenberg, *Nature* **345**, 544 (1990); M. R. Smith *et al.*, *New Biol.* **2**, 648 (1990); A. Lazaris-Karatzas *et al.*, *Genes Dev.* **6**, 1631 (1992).
8. T. A. J. Haystead, C. M. M. Haystead, C. Hu, T.-A. Lin, J. C. Lawrence Jr., *J. Biol. Chem.* **269**, 23185 (1994).
9. T.-A. Lin, X. Kong, A. R. Saltiel, P. J. Blackshear, J. C. Lawrence Jr., *ibid.* **270**, 18531 (1995); L. M. Graves *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7222 (1995); S. R. von Manteuffel, A.-C. Gingras, X.-F. Ming, N. Sonenberg, G. Thomas, *ibid.* **93**, 4076 (1996).
10. G. J. Brunn *et al.*, *EMBO J.* **15**, 5256 (1996).
11. C. J. Sabers *et al.*, *J. Biol. Chem.* **270**, 815 (1995).
12. E. J. Brown *et al.*, *Nature* **369**, 756 (1994); D. M. Sabatini, H. Erdjument-Bromage, M. Lui, P. Tempst, S. H. Snyder, *Cell* **78**, 35 (1994).
13. C. T. Keith and S. L. Schreiber, *Science* **270**, 50 (1995); T. Hunter, *Cell* **83**, 1 (1995); R. T. Abraham, *Curr. Opin. Immunol.* **8**, 412 (1996).
14. J. Chen, X.-F. Zheng, E. J. Brown, S. L. Schreiber, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4947 (1995).
15. J. Downward, *Nature* **371**, 378 (1994).
16. R. T. Abraham and G. J. Wiederrecht, *Annu. Rev. Immunol.* **14**, 483 (1996).
17. A. Cuenda *et al.*, *FEBS Lett.* **364**, 229 (1995).
18. P. Fadden, T. A. J. Haystead, J. C. Lawrence Jr., *J. Biol. Chem.* **272**, 10240 (1997); G. J. Brunn, P. Fadden, T. A. J. Haystead, J. C. Lawrence Jr., unpublished results.
19. M. N. Hall, *Biochem. Soc. Trans.* **24**, 234 (1996); N. C. Barbet *et al.*, *Mol. Biol. Cell* **7**, 25 (1996); C. J. Di Como and K. T. Arndt, *Genes Dev.* **10**, 1904 (1996).
20. D. Rousseau, A. C. Gingras, A. Pause, N. Sonenberg, *Oncogene* **13**, 2415 (1996).
21. The cDNAs encoding wild-type mTOR (mTOR-wt) and a rapamycin-resistant mTOR mutant (mTOR-rr) were cloned into pcDNA3 (Invitrogen). The mTOR-rr mutant contains a Ser^{2035}→Ile substitution that was created with the Transformer kit (Clontech). Both cDNAs were tagged at their 5′-termini with nucleotide sequences encoding the Asp-Thr-Tyr-Arg-Tyr-Ile sequence recognized by mAb AU1 (Babco). The PHAS-I expression vector, pCMV4-PHAS-I, is described by J. Lawrence (*Advances in Enzyme Regulation*, in press).
22. HEK 293 cells were seeded into 60-mm dishes at 6×10^5 cells per dish and were cultured for 24 hours in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were transfected with 2 μg of pCMV4-PHAS-I and 4 μg of pcDNA3 only or the mTOR-wt or mTOR-rr expression plasmids. Transfections were done with the TransIT reagent (Pan Vera, Madison, WI). After 12 hours, the cells were rested in DMEM containing 0.1% FBS and were stimulated with insulin and treated with drugs as indicated in the figure legends. Cells were washed with phosphate-buffered saline (PBS), then scraped into lysis buffer [50 mM β-glycerophosphate (pH 7.4), 1.5 mM EGTA, supplemented with 1% NP-40, 1 mM dithiothreitol, 20 nM microcystin-LR, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 μg/ml), aprotinin (5 μg/ml), and pepstatin (5 μg/ml)]. After centrifugation at 10,000*g*, extracts were equalized for protein content and subjected to SDS-PAGE through 12.5 or 7.5% gels for PHAS-I or mTOR immunoblots, respectively. PHAS-I immunoblots were performed as described [T. A. Lin and J. C. Lawrence, *J. Biol. Chem.* **271**, 30199 (1996)]. AU1-tagged mTOR proteins were blotted with mAb AU1 followed by rabbit antibodies to mouse immunoglobulin G (Pierce). The blots were developed with horseradish peroxidase coupled to protein A and the Enhanced Chemiluminescence reagent (Amersham).
23. Rat brain–derived mTOR was purified by immunoprecipitation. Samples (1 mg protein) of rat brain extract (*11*) were mixed with affinity-purified rabbit polyclonal antibodies to a peptide sequence corresponding to residues 2433 to 2450 of mTOR. Immune complexes were precipitated with protein A–Sepharose beads, and immunoprecipitates were washed twice in TGN buffer [50 mM tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 100 mM NaCl, containing 10% glycerol, 20 nM microcystin-LR, leupeptin (10 μg/ml), aprotinin (5 μg/ml), pepstatin A (5 μg/ml), and 600 μM PMSF], once in high-salt buffer [100 mM tris-HCl (pH 7.4), 500 mM LiCl], and then twice in kinase buffer [10 mM Hepes (pH 7.4), 50 mM β-glycerophosphate, 50 mM NaCl containing the phosphatase and protease inhibitors described above]. Immunoprecipitates were treated with wortmannin or with 10 μg of glutathione-S-transferase (GST)–FKBP12 fusion protein and, where indicated, 10 μM rapamycin or 100 μM FK506. After 40 min at 23°C, the beads were washed twice in kinase buffer and then mixed with 50 μl of kinase buffer containing 10 mM $MnCl_2$, 200 μM ATP, 1 μg of purified recombinant PHAS-I, and, when indicated, 10 μCi of [γ-^{32}P]ATP. Kinase reactions were incubated for 40 min at 30°C. After separation by SDS-PAGE, proteins were transferred to Immobilon-P (Millipore), and radiolabeled PHAS-I was detected by autoradiography. Incorporation of ^{32}P into PHAS-I was quantitated with an Ambis Imaging system.
24. The rapamycin-resistant, catalytically inactive mTOR double mutant (Ser^{2035}→Ile, Asp^{2338}→Ala) was prepared in pcDNA3 as described in (*21*). The protocols for 293 cell transfections with mTOR-rr– and mTOR-rr-kd–encoding plasmids and sample preparation are given in (*22*).
25. K562 cells (10^7 cells per sample) were transfected with 25 μg of the indicated plasmid DNAs plus 20 μg of pcDNA3 as filler. The DNA was introduced with a BTX model T820 square-wave electroporator at settings of 350 V and 10-ms pulse duration. After 16 hours, the cells were washed with PBS and sonicated in lysis buffer [50 mM tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 100 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 1 mM dithiothreitol, 0.2% Tween-20, with phosphatase and protease inhibitors]. Immunoprecipitations were done with mAb AU1, and kinase assays were done as described (*22, 23*). For p38 MAP kinase assays, K562 cells were electroporated with a FLAG-p38–encoding plasmid. The cells were osmotically shocked for 10 min with 0.4 M sorbitol before the preparation of cell extracts. Recombinant p38 was immunoprecipitated with anti–FLAG M1 affinity gel (Eastman Kodak). p38 MAP kinase activity was assayed as described [L. M. Karnitz, L. A. Burns, S. L. Sutor, J. Blenis, R. T. Abraham, *Mol. Cell. Biol.* **15**, 3049 (1995)], with PHAS-I as the substrate. Immunoprecipitated p38 was visualized by immunoblotting with a p38-specific antibody (New England Biolabs).
26. W. J. Boyle, P. van der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991).

27. We thank E. O'Neill for providing the FLAG-p38 expression plasmid, S. Sutor for technical assistance, and K. Jensen for assistance with the preparation of this manuscript. Supported by NIH grants DK28312, AR41189, and DK50628 (to J.C.L.), CA23099 (to P.J.H.), and CA52995 (to R.T.A.) and by American Cancer Society grants RPG-95-031-03-DHP (to P.J.H.) and RPG-95-040-03 (to R.T.A.). R.T.A. is a Leukemia Society of America Scholar.

17 March 1997; accepted 2 June 1997