A Direct Linkage between the Phosphoinositide 3-Kinase-AKT Signaling Pathway and the Mammalian Target of Rapamycin in Mitogen-stimulated and Transformed Cells[1]

Aleksandar Sekulić,[2] Christine C. Hudson,[2] James L. Homme, Peng Yin, Diane M. Otterness, Larry M. Karnitz, and Robert T. Abraham[3]

Department of Immunology [A. S.], Mayo School of Medicine [J. L. H.], and Division of Oncology Research [L. M. K.], Mayo Clinic, Rochester, Minnesota 55905, and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710 [C. C. H., P. Y., D. M. O., R. T. A.]

ABSTRACT

The microbially derived antiproliferative agent rapamycin inhibits cell growth by interfering with the signaling functions of the mammalian target of rapamycin (mTOR). In this study, we demonstrate that interleukin-3 stimulation induces a wortmannin-sensitive increase in mTOR kinase activity in a myeloid progenitor cell line. The involvement of phosphoinositide 3'-kinase (PI3K) in the regulation of mTOR activity was further suggested by findings that mTOR was phosphorylated in vitro and in vivo by the PI3K-regulated protein kinase, AKT/PKB. Although AKT phosphorylated mTOR at two COOH-terminal sites (Thr^{2446} and Ser^{2448}) in vitro, Ser^{2448} was the major phosphorylation site in insulin-stimulated or -activated AKT-expressing human embryonic kidney cells. Transient transfection assays with mTOR mutants bearing Ala substitutions at Ser^{2448} and/or Thr^{2446} indicated that AKT-dependent mTOR phosphorylation was not essential for either PHAS-I phosphorylation or p70^{S6K} activation in HEK cells. However, a deletion of amino acids 2430–2450 in mTOR, which includes the potential AKT phosphorylation sites, significantly increased both the basal protein kinase activity and in vivo signaling functions of mTOR. These results demonstrate that mTOR is a direct target of the PI3K-AKT signaling pathway in mitogen-stimulated cells, and that the identified AKT phosphorylation sites are nested within a "repressor domain" that negatively regulates the catalytic activity of mTOR. Furthermore, the activation status of the PI3K-AKT pathway in cancer cells may be an important determinant of cellular sensitivity to the cytostatic effect of rapamycin.

INTRODUCTION

Rapamycin is a potent immunosuppressive drug and investigational anticancer agent, the major mechanism of action of which involves the inhibition of lymphoid or tumor cell proliferation, through interference with an event(s) required for G_1-to-S phase progression in cycling cells. The block to G_1 phase progression imposed by rapamycin occurs prior to the "restriction point," based on the observations that rapamycin inhibits the phosphorylation of the retinoblastoma protein and that rapamycin-treated cells are not fully committed to enter S-phase of the cell cycle after release from drug-induced G_1 arrest (1–3). The sensitivity of certain tumor cell lines to the cytostatic effects of rapamycin has prompted considerable interest in the possibility that this drug might be a useful cancer chemotherapeutic agent. Indeed, a rapamycin analogue (CCI-779; Wyeth-Ayerst) is now in Phase I clinical trials in cancer patients in the United States and Europe.

The molecular pharmacology underlying the cellular effects of rapamycin is now understood in considerable detail. The principal rapamycin "receptor" is a widely expressed intracellular protein termed FKBP[4]-12. In mammalian cells, the interaction of rapamycin with FKBP12 generates a pharmacologically active complex that binds with high affinity to the mTOR [Ref. 4; also named FRAP (5), RAFT1 (6), or RAPT1 (7) by others]. This rapamycin target protein is a member of a recently described family of protein kinases, termed PIKKs. The PIKK family members share a COOH-terminal catalytic domain that bears significant sequence homology to the lipid kinase domains of PI3Ks (8). Other members of the PIKK family include TOR1p and TOR2p, the budding yeast orthologues of mTOR. The finding that rapamycin interacts with FPR1p, the budding yeast orthologue of FKBP12, to arrest yeast cell growth in G_1 phase (9) suggests that the TOR signaling pathway has been at least partially conserved during eukaryotic evolution.

The specificity of rapamycin as an inhibitor of mTOR function facilitated the identification of the downstream signaling events governed by mTOR in mitogen-stimulated cells. To date, the rapamycin-sensitive signaling activities ascribed to mTOR impinge primarily on the translational machinery. Rapamycin treatment triggers the rapid dephosphorylation and inactivation of p70^{S6K} in mitogen-stimulated cells (10–14). The overall effect of p70^{S6K} activation is to stimulate ribosome biogenesis, and, in turn, to increase the capacity of the translational machinery, which allows cells to meet the increased demand for protein synthesis imposed by cell cycle progression (15–17). Although p70^{S6K} activation involves a complex series of phosphorylation events catalyzed by multiple protein kinases (18–21), the prompt reversal of p70^{S6K} activation by rapamycin (11, 14) suggests that this protein kinase requires continuous signaling through mTOR to both achieve and maintain the activated state. The exact nature of the input supplied by mTOR remains unclear; however, recent findings suggest that mTOR phosphorylates and suppresses the activity of a type 2A protein phosphatase bound directly to p70^{S6K} (22). Hence, rapamycin treatment may inactivate p70^{S6K} by removing a mTOR-dependent inhibitory constraint on the activity of a p70^{S6K}-targeted type 2A protein phosphatase PP2A.

A second downstream protein targeted by mTOR is the translational repressor, PHAS-I, also termed 4E-BP1. PHAS-I represses translation initiation through association with eIF-4E, the mRNA cap-binding subunit of the eIF-4F complex. The binding of PHAS-I to eIF-4E blocks assembly of the eIF-4F complex at the 5'-cap structure of the mRNA template, thereby decreasing the efficiency of translation initiation (23). Stimulation of cells with insulin or growth factors

causes the phosphorylation of PHAS-I at five sites, which leads to the release of eIF-4E, and, in turn, an increase in eIF-4F-dependent translation initiation (24–29). The phosphorylation of PHAS-I induced by hormonal stimuli is strongly inhibited by rapamycin (27, 30, 31). Earlier results suggested that mTOR is directly responsible for the phosphorylation of PHAS-I in intact cells (32, 33), although additional proline-directed kinases appear to be required for full phosphorylation of PHAS-I in insulin-stimulated cells (34).

The signaling pathway that couples growth factor receptor occupancy to mTOR activation is only partially understood. However, accumulating evidence places mTOR downstream of both PI3K and the PI3K-regulated protein kinase, AKT (also termed PKB), in growth factor-stimulated cells. This model is based in part on genetic and pharmacological evidence that links activation of PI3K and/or AKT to the two intracellular events known to be governed by mTOR, the activation of p70^{S6K} and the phosphorylation of PHAS-I (35). The notion that mTOR participates in signaling downstream from PI3K may be particularly relevant to the antitumor activity of rapamycin. Recent studies have identified a negative regulator of PI3K-mediated signaling, PTEN, as a tumor suppressor gene product (36). The tumor suppressor function of PTEN is attributed to its activity as a phosphoinositide 3-phosphatase, which effectively terminates PI3K-mediated signaling via dephosphorylation of the second messengers, phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate. Thus, loss of PTEN function leads to hyperactivation of the PI3K signaling cascade, which promotes abnormal cell growth, survival, and migration.

The importance of PI3K signaling during tumorigenesis is underscored by observations that mutations in the PTEN gene occurs frequently in a variety of human cancers, including prostate cancer and glioblastoma (37). If mTOR also resides downstream of PI3K and/or AKT, then mTOR activity should also be deregulated in PTEN-deficient tumor cells, and consequently, PTEN status might be an important predictor of cancer cell sensitivity to the mTOR inhibitor, rapamycin. Given these speculative arguments, it becomes increasingly important to define the interactions among PI3K, AKT, and mTOR as the rapamycin analogue, CCI-779, moves into clinical trials in patients with different types of cancer.

At the inception of this study, the most direct evidence for epistatic relationships among PI3K, AKT, and mTOR stems from results obtained with a polyclonal antibody, termed mTAb1, which recognizes a COOH-terminal peptide sequence in mTOR (residues 2433–2450; Ref. 38). The authors noted that cellular stimulation with insulin, or expression of mutationally activated AKT, caused a decrease in the immunoreactivity of mTOR in anti-mTAb1 immunoblot analyses (39). The loss of mTAb1 binding activity was reversed by treatment of the immunoprecipitated mTOR with a protein phosphatase prior to immunoblot analysis. Collectively, these results suggested that insulin or AKT stimulation caused the phosphorylation of mTOR at a site(s) that resulted in a decrease in the recognition of this protein by the mTAb1 antibody.

The goal of the present study was to further understand the role of the PI3K-AKT signaling pathway in the regulation of mTOR function by extracellular stimuli. We demonstrate that stimulation of myeloid progenitor cells with IL-3 triggers a rapid increase in the protein kinase activity of mTOR. The IL-3-dependent increase in mTOR activity is blocked by low concentrations of the PI3K inhibitor, wortmannin. Furthermore, we provide *in vitro* and *in vivo* evidence that AKT phosphorylates mTOR at a site(s) located in a region that represses the catalytic activity of the mTOR kinase domain. Deletion of this repressor domain generates a mTOR mutant bearing a constitutively elevated level of protein kinase activity. These findings outline a direct linkage between the PI3K-AKT pathway and mTOR and

suggest that deregulated signaling through mTOR may contribute to the transformed phenotype of PTEN-deficient cancer cells.

MATERIALS AND METHODS

Plasmids, Reagents, and Antibodies. The expression vectors encoding AU1-tagged wild-type mTOR (AmTOR^{wt}) and catalytically inactive ("kinase-dead") mTOR (AmTOR^{kd}) were described previously (32). The rapamycin-resistant versions of mTOR contain an additional Ser (2035)→Ile mutation and are usually designated with the suffix "SI" (e.g., AmTOR-SI). PCR-based mutagenesis was used to construct single and double mTOR point mutants in which Thr^{2446} and Ser^{2448} were changed to alanine (A) residues. The internal deletion mutant, AmTORΔ, which lacks amino acids 2430–2450, was prepared by the PCR-based SOEing technique (40). The expression vectors for HA-tagged wild-type AKT (cAKT), catalytically inactive AKT (AKT-kd) and the constitutively active myristylated form of AKT (myrAKT), were kind gifts from P. N. Tsichlis (Fox-Chase Cancer Center, Philadelphia, PA). The cDNA encoding p70^{S6K} (kindly provided by Dr. Naohiro Terada, National Jewish Medical and Research Center, Denver, CO) was appended with nucleotides encoding an NH$_2$-terminal FLAG epitope tag and was cloned into pcDNA3 using EcoRI and XbaI restriction sites. All PCR products were subcloned and then sequenced to ensure the fidelity of the amplification step.

Recombinant murine IL-3 was purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant human insulin (Recombulin) and G418 (Geneticin) were obtained from Life Technologies, Inc. (Gaithersburg, MD), and FuGene transfection reagent was purchased from Boehringer Mannheim (Indianapolis, IN). Wortmannin (Sigma) was dissolved in DMSO (Me$_2$SO) to yield a 1.2 mM stock solution. The wortmannin stock solution was aliquoted and stored at −70°C. Rapamycin (Sigma) was prepared as a 10 μM stock solution in ethanol and aliquoted and stored as described above.

The α-AU1 and 12CA5 (α-HA) mAbs were purchased from Babco (Richmond, CA), and the α-mTOR monoclonal antibody, 26E3, was a generous gift from Dr. Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN). Peptides corresponding to amino acid residues 2433–2450 in mTOR were synthesized (Research Genetics, Huntsville, AL) with or without phosphate at either or both of the underlined residues in the sequence CDTNAKGNKRSR-TRTDSYS. Polyclonal antibodies directed against the nonphosphorylated peptide were raised by immunizing rabbits with the peptide coupled to keyhole limpet hemocyanin. The antiserum (designated α-mTOR 367) was affinity-purified over a peptide-coupled Sulfolink bead column according to the manufacturer's procedure (Pierce, Rockford, IL). Phosphospecific antibodies were prepared in a similar fashion, except that a keyhole limpet hemocyanin-coupled, dually phosphorylated peptide (containing phosphate at both Thr^{2446} and Ser^{2448} served as the immunogen. The resulting antiserum was first passed over a column consisting of nonphosphorylated peptide immobilized on Sulfolink beads, and the flow-through fraction was then passed through a second column containing the immobilized, dually phosphorylated peptide. The bound antibodies (designated α-mTORp2) were eluted at low pH and were stored in PBS containing 0.05% azide.

Cell Culture and Transfections. The murine IL-3-dependent myelomonocytic progenitor cell line, FDC-P1, was cultured in standard growth medium [RPMI 1640 supplemented with 10% (v/v) FBS (Hyclone, Logan, UT), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES (pH 7.2), and 10% (v/v) WEHI-3 cell-conditioned medium as a source of IL-3]. Stably transfected FDC-P1 cells expressing AmTOR^{wt} were prepared by suspending 1×10^7 exponentially growing cells in 350 μl of standard growth medium at 4°C. The cells were mixed with a total of 45 μg of plasmid DNA suspended in the same medium. Mock transfectants received 45 μg of pcDNA3 only, whereas mTOR transfectants were electroporated with 25 μg of mTOR-encoding plasmid plus 20 μg of pcDNA3 as filler. Prior to electroporation, the cell-DNA mixtures were incubated for 10 min at room temperature. The cells were electroporated with a BTX model T820 square-wave electroporator (San Diego, CA) at a setting of 350 V (10-ms pulse duration). The electroporated cells were mixed gently and then allowed to stand at room temperature for an additional 10 min. The cells were then diluted into 20 ml of standard growth medium and cultured for 24 h, at which time the cells were transferred into fresh growth medium containing 800 μg of G418/ml. Stable clones that expressed AmTOR^{wt} and AmTOR^{kd} were isolated by limiting dilution, and expression levels of the transfected proteins were assessed by immunoblotting with the AU1 mAb.

For experiments, exponentially growing FDC-P1 cells (2×10^7 cells/sample were washed twice in PBS. The cells were resuspended in 20 ml of starvation medium [RPMI 1640 containing 100 μg/ml BSA, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, buffered to pH 7.2 with 10 mM HEPES]. After 4–6 h in culture, the factor-deprived cells were treated for 30 min with the indicated pharmacological inhibitors and then were restimulated with either 30 ng/ml IL-3 or 20% FBS.

HEK 293 and 293T cells were maintained in monolayer cultures in DMEM (Life Technologies, Inc.) supplemented with 10% FBS or 5% FBS, respectively. Prior to transfection, 6×10^5 cells were seeded into a 60-mm tissue culture dish. The cells were cultured for 24 h under standard conditions and then were transfected with a total of 5 μg of plasmid DNA mixed with 8 μl of FuGene transfection reagent/dish. The standard amounts of plasmid DNAs used for each transfection were: AKT, 0.25 μg; mTOR, 3 μg; and p70^{S6K}, 2 μg. When necessary, the total amount of plasmid DNA was brought to 5 μg by addition of the empty pcDNA3 expression vector. The transfected cells were cultured for 16 h, washed one time in PBS, and arrested for 24 h in serum-free DMEM. The serum-deprived cells were pretreated for 30 min with wortmannin or rapamycin and then stimulated with insulin for the indicated times. The procedures for transfection of HEK 293T cells were similar, except that the cells were deprived of serum for 2 h prior to drug treatment.

DU 145 and PC-3 prostate cancer cells were maintained in monolayer cultures in RPMI 1640 supplemented with 10% FBS. Prior to assay, 2×10^5 cells were seeded in 60-mm tissue culture dishes. After 24 h, the cells were transferred into serum-free RPMI 1640 and were cultured for an additional 20 h. The cells were washed in PBS and lysed in LB buffer [25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% (w/v) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 20 nM microcystin- LR, 100 μg/ml PMSF, and protease inhibitor cocktail (5 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin)]. The lysates were cleared of insoluble material, and the cleared extracts were assayed for total protein to equalize sample loading prior to SDS-PAGE.

Immunoprecipitations. Mock-transfected or AmTOR-transfected FDC-P1 cells (2×10^7 cells/sample) were growth factor deprived and restimulated as described above. The cells were washed in PBS and lysed by sonication in 1 ml of buffer L [50 mM Tris-HCl, 50 mM β-glycerophosphate, 100 mM NaCl (pH 7.4), containing 10% glycerol, 0.2% Tween 20, 1 mM DTT, 1 mM Na$_3$VO$_4$, 1 mM MgCl$_2$, 50 nM microcystin-LR, 1 mM PMSF, and protease inhibitor cocktail]. The lysates were cleared of insoluble material by centrifugation, and the extracts were immunoprecipitated with 1 μl of α-AU1 mAb for 2 h at 4°C. The immunoprecipitates were washed three times in buffer W [50 mM Tris-HCl, 50 mM β-glycerophosphate, 100 mM NaCl (pH 7.4), containing 10% glycerol, 0.2% Tween 20, and 1 mM DTT] and twice in buffer K [10 mM HEPES, 50 mM NaCl, 50 mM β-glycerophosphate (pH 7.4), 50 nM microcystin-LR, and the protease inhibitor cocktail].

Immunoblot Analyses. For immunoblot analyses with α-mTOR 367 or α-mTORp2 antibodies, recombinant AmTOR was immunoprecipitated with the tag-specific α-AU1 mAb from transfected FDC-P1, HEK 293, or HEK 293T cells. The immunoprecipitated proteins were separated by SDS-PAGE through 6% polyacrylamide gels. After transfer to Immobilon-P, the membranes were blocked and probed with 5 μg per ml affinity-purified antibodies in Tris-buffered saline-0.2% Tween 20 (TBST) containing 2% (w/v) BSA (for α-mTORp2 antibodies) or 5% milk (for α-mTOR 367 antibodies). Immunoreactive proteins were detected with horseradish peroxidase-conjugated to protein A and the Renaissance reagent (New England Nuclear, Boston, MA). The blots were then stripped and reprobed with the α-AU1 mAb in TBST-milk solution. The phosphorylation state of endogenous mTOR in DU 145 or PC-3 prostate cancer cells was analyzed by immunoblotting with α-mTORp2 as described above, followed by reblotting of the same membrane with the α-mTOR mAb, 26E3, in TBST-milk solution.

Kinase Assays. The protein kinase activity of immunoprecipitated mTOR was assayed with recombinant PHAS-I as the substrate (Stratagene, La Jolla, CA; Ref. 32). The samples were separated by SDS-PAGE, and radiolabeled PHAS-I was detected by autoradiography. Incorporation of ^{32}P into PHAS-I was quantitated with a Molecular Dynamics Storm 840 Phosphorimager (Sunnyvale, CA) and ImageQuant software.

Phosphorylation of mTOR by AKT in vitro was performed by transfection of AmTOR^{wt} and HA-tagged myrAKT, c-AKT, or catalytically inactive AKT into separate populations of HEK 293 cells. The cells were plated in 60-mm culture dishes and were transfected as described above. After 16 h, the transfected cells were transferred into serum-free DMEM and cultured for 24 h. Cellular extracts were prepared by removal of the culture medium, followed by addition of 400 μl of buffer P per dish [50 mM Tris-HCl, 100 mM NaCl, 50 mM β-glycerophosphate (pH 7.4), containing 10% (w/v) glycerol, 1% Triton X-100, 1 mM DTT, 50 nM microcystin, 1 mM PMSF, and protease inhibitor cocktail]. The detached cells were disrupted by sonication, and cleared extracts from the HA-tagged AKT-expressing cells and the AmTOR^{wt}-expressing cells were mixed at a total protein ratio of 1:9. The epitope-tagged mTOR and AKT proteins were coimmunoprecipitated with 1 μl of AU1 mAb and 5 μg of 12CA5 mAb bound to protein A-Sepharose beads that had been precoupled to rabbit antimouse immunoglobulin antibodies. The immunoprecipitates were washed three times in buffer N [25 mM HEPES (pH 7.6), 0.5 M NaCl, 10% glycerol, 1 mM Na$_3$VO$_4$, and 0.2% Tween 20] and two times in kinase buffer F [50 mM Tris (pH 7.5), 10 mM MgCl$_2$, 50 mM Na$_3$VO$_4$, and 1 mM DTT]. The coimmunoprecipitated proteins were incubated for 50 min at 30°C in 20 μl of kinase buffer F supplemented with 10 μM ATP and 20 μCi of [γ-^{32}P]ATP (specific activity, 4500 Ci/mmol). The reaction products were separated by SDS-PAGE and transferred to an Immobilon-P membrane. The incorporation of ^{32}P into wild-type or mutated forms of AmTOR was detected by autoradiography and quantitated by phosphorimager analysis as described above.

To measure the activity of transiently transfected FLAG-p70^{S6K}, serum-deprived HEK 293 cells were prepared as described above. The cells were stimulated with 1 μM insulin and then lysed in TNEE buffer [50 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA, 2 mM EGTA, 25 mM β-glycerophosphate, 25 mM NaF (pH 7.5), containing, 0.5% Triton X-100, 100 μM sodium orthovanadate, 2 mM DTT, and protease inhibitor cocktail]. The epitope-tagged p70^{S6K} was immunoprecipitated from cellular extracts with anti-FLAG M2 affinity resin (Sigma Chemical Co., St. Louis, MO), and protein kinase activity was determined with a p70^{S6K} assay kit (Upstate Biotechnology, Inc., Lake Placid, NY).

RESULTS

Stimulation of mTOR Catalytic Activity by Serum or IL-3. Our initial objective was to determine whether mTOR activity was regulated in a PI3K-dependent fashion by IL-3, a cytokine that promotes the proliferation and survival of myeloid lineage progenitor cells. To facilitate the analyses of mTOR kinase activity, we stably expressed AU1-tagged wild-type or catalytically inactive ("kinase-dead") versions of mTOR (AmTOR^{wt} and AmTOR^{kd}, respectively) in IL-3-dependent FDC-P1 cells. Importantly, the stable cell lines selected for these studies were not overexpressing the recombinant protein, as indicated by immunoblot analyses of the transfected clones for total mTOR protein levels with antibodies that recognize both the endogenous and transfected proteins (results not shown). The tagged AmTOR proteins therefore serve as a "tracer" subpopulation, the behavior of which in response to physiological stimuli should reflect that of the endogenous mTOR.

In the initial studies, AmTOR^{wt}-expressing cells were deprived of serum and IL-3 for 6 h and then were restimulated for 10 min with IL-3 prior to the preparation of cellular extracts. The extracts were immunoprecipitated with α-AU1 mAb, and mTOR kinase activities were determined with PHAS-I as the substrate. Parallel samples were prepared from identically treated cells that expressed the AmTOR^{kd} mutant. Stimulation of AmTOR^{wt}-expressing FDC-P1 cells with IL-3 significantly increased the in vitro kinase activity of the immunoprecipitated AmTOR^{wt} but did not change the amount of AmTOR^{wt} in these immunoprecipitates (Fig. 1A, left panel). The activation of mTOR by IL-3 was maximal at 5–10 min after cytokine stimulation and then dropped to a lower, but still elevated, plateau level of activity that was sustained for at least 4 h after cytokine addition (Fig. 1B and data not shown). In contrast, AU1 immunoprecipitates from either mock-transfected or AmTOR^{kd}-expressing cells contained low levels of background protein kinase activity that was not substantially increased by cellular stimulation with IL-3. Interestingly, serum-starved
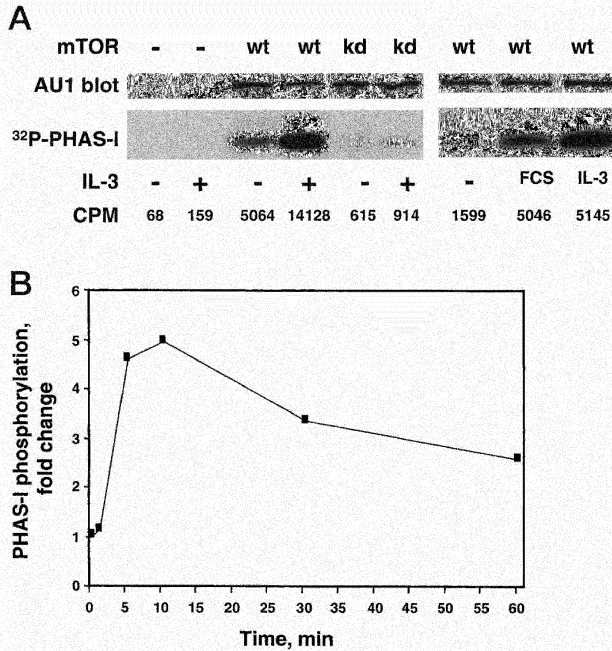
Fig. 1. Stimulation of mTOR kinase activity by IL-3 or serum. FDC-P1 cells were stably transfected with wild-type AU1-tagged mTOR or a catalytically inactive version of AmTOR. Transfected clones were cultured for 6 h in medium without serum and IL-3. A, left panel, factor-deprived FDC-P1 cells were stimulated for 10 min with IL-3 or medium only (−). The protein kinase activities of wild-type (wt) AmTOR or the "kinase-dead" (kd) AmTOR were determined in immune complex kinase assays with [γ-^{32}P]ATP and recombinant PHAS-I as the substrate (lower panels). Incorporation of ^{32}P$_i$ into PHAS-I was quantitated with a Molecular Dynamics Storm 840 phosphorimaging system and reported as cpm. The amounts of immunoprecipitated mTOR were assessed by immunoblotting with AU1 mAb (upper panels). Right panel, AmTOR^{wt}-expressing FDC-P1 cells were restimulated for 10 min with IL-3 or serum, and AmTOR^{wt} kinase activities were measured as above. B, mTOR^{wt}-expressing FDC-P1 cells were restimulated for the indicated times with IL-3. The protein kinase activities in AmTOR^{wt} immunoprecipitates were determined as described in A. Incorporation of ^{32}P$_i$ into PHAS-I was normalized to the basal level of phosphorylation in the immunoprecipitate prepared from unstimulated cells.

FDC-P1 cells also displayed a clear increase in AmTOR^{wt} activity after a 10-min exposure to fresh serum (Fig. 1A, right panel). Thus, ligation of receptors for IL-3, as well as undefined serum components (possibly insulin-like growth factors), initiates a signaling pathway leading to mTOR activation in FDC-P1 cells.

Role of PI3K in IL-3-dependent mTOR Activation. Earlier studies implicated the PI3K pathway in the activation of mTOR-dependent signaling events in HEK 293 cells and 3T3-L1 preadipocytes (35, 39). Stimulation of the IL-3 receptor also triggers a rapid increase in PI3K activity (41), which suggested that PI3K might be responsible for the activation of mTOR in IL-3-stimulated FDC-P1 cells. If the activation of mTOR by IL-3 is dependent on PI3K, then this response should be inhibited by pretreatment of the cells with wortmannin at drug concentrations ≤100 nM (42). As shown in Fig. 2, the activation of AmTOR^{wt} by IL-3 was virtually abrogated by pretreatment of the FDC-P1 cells with 10 nM wortmannin. Thus, the sensitivity of IL-3-dependent mTOR activation to wortmannin strongly suggests that this response is dependent on the activation of PI3K.

Direct Phosphorylation of mTOR by the PI3K-regulated Kinase, AKT. An earlier report provided evidence that activation of the PI3K-AKT pathway led to the phosphorylation of the mTAb1 antibody epitope located in the COOH-terminal region of mTOR (39). Using independently derived polyclonal antibodies (α-mTOR 367) specific for the same region of mTOR (amino acid residues 2433–2450), we observed a similar time-dependent decrease in the immu-

noreactivity of AU1-tagged mTOR isolated from IL-3-stimulated FDC-P1 cells (Fig. 3, upper panel). Interestingly, the time course of the alteration in anti-mTOR antibody reactivity corresponded closely to the changes in mTOR kinase activity induced by IL-3 (Fig. 1B). The IL-3-dependent decrease in mTOR immunoreactivity was abrogated by pretreatment of the cells with 100 nM wortmannin, suggesting that this alteration was mediated through the activation of PI3K. As will be described below, parallel immunoblot analyses with a phospho-mTOR-specific antibody (α-mTORp2) indicated that the decrease in α-mTOR 367 reactivity induced by IL-3 stimulation is attributable to the phosphorylation of at least one amino acid (Ser^{2448}) located within the α-mTOR 367 epitope (Fig. 3, middle panel; see below for description).

Examination of the peptide sequence recognized by α-mTOR 367 antibodies revealed that this region contained two consensus phosphorylation sites (Thr^{2446} and Ser^{2448}) for AKT (Fig. 4). To determine whether mTOR was an in vitro substrate for AKT, we expressed AmTOR^{wt}, HA-tagged wild-type AKT (cAKT), activated AKT (myrAKT), or a catalytically inactive AKT (AKTkd) in different populations of HEK 293 cells. Cellular extracts were then mixed, and
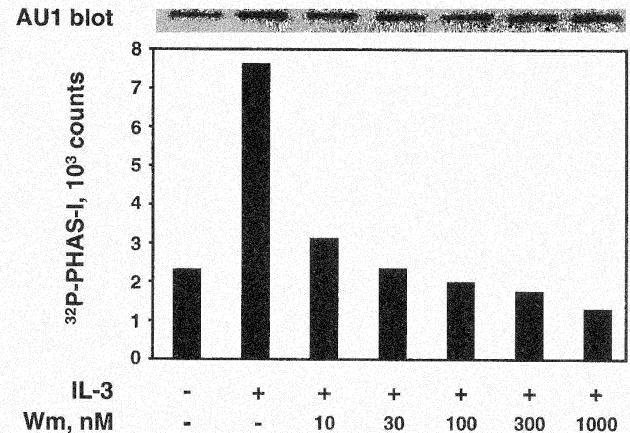

Fig. 2. Inhibition of IL-3 dependent mTOR activation by cellular treatment with wortmannin. AmTOR^{wt}-transfected FDC-P1 cells were deprived of growth factors as described in Fig. 1. AmTOR^{wt}-expressing cells were treated for 30 min with the indicated concentrations of wortmannin (Wm). After stimulation of the cells for 10 min with IL-3, AmTOR^{wt} was immunoprecipitated, and immune complex kinase assays were performed as described in the Fig. 1 legend. The amount of AmTOR^{wt} in each immunoprecipitate was determined by α-AU1 immunoblotting (upper panel).
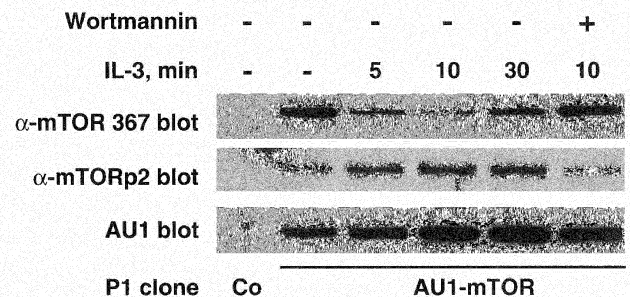

Fig. 3. IL-3-dependent alterations in the reactivity of AmTOR^{wt} with R domain-directed α-mTOR antibodies. AmTOR^{wt}-expressing FDC-P1 cells were deprived of growth factors and then stimulated for the indicated times with medium only (−) or with IL-3. Wortmannin (100 nM) was added to the indicated sample 30 min prior to IL-3 stimulation. The cells were lysed, and AmTOR^{wt} was immunoprecipitated from cleared extracts with α-AU1 mAb. The control lane (Co) represents an α-AU1 immunoprecipitate from mock-transfected (empty plasmid only) FDC-P1 cells. The immunoprecipitates were resolved by SDS-PAGE and sequentially immunoblotted with α-mTOR 367 antibodies, phosphospecific α-mTORp2 antibodies (see Figs. 4 and 5 for details), and α-AU1 mAb.
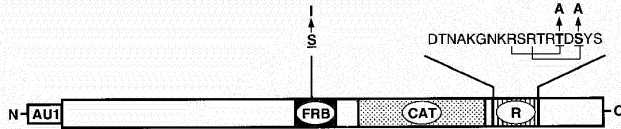
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S

A A
DTNAKGNKRSRTRTDSYS

N—AU1 FRB CAT R —C

Fig. 4. Schematic diagram of mTOR functional domains and mutants used in this study. The NH$_2$-terminal AU1 epitope tag is followed by an extended NH$_2$-terminal region of unknown function. The FKBP12·rapamycin binding (FRB) domain is labeled, as is the Ser (2035)→Ile mutation used to generate the rapamycin-resistant AmTOR-SI mutants. The FRB domain is followed by the catalytic region (CAT). Finally, the putative "repressor" (R) domain (residues 2430–2450) is shown together with the newly identified AKT phosphorylation sites at Thr^{2446} and Ser^{2448}.

AmTOR^{wt} was coimmunoprecipitated with each form of AKT. The mixed immune complexes were then subjected to in vitro kinase assays. As shown in Fig. 5A, a relatively low level of phosphorylation was observed in immunoprecipitates containing AmTOR^{wt} only. This background phosphorylation reflects the in vitro autokinase activity of mTOR (43, 44). The incorporation of radiolabeled phosphate into AmTOR^{wt} was strongly increased by coimmunoprecipitation with the activated form of AKT (myrAKT) and, to a lesser extent, with wild-type AKT. The increase in mTOR phosphorylation was dependent on the protein kinase activity of AKT, because coimmunoprecipitation of mTOR with a catalytically inactive form of AKT failed to enhance the phosphorylation of mTOR in the immune complex kinase assay. Identical results were obtained when the different AKT proteins were coimmunoprecipitated with a catalytically inactive version of AmTOR, indicating that mTOR kinase activity was not required for the in vitro phosphorylation of the mTOR polypeptide by AKT (results not shown).

To determine which, if any, of the putative AKT phosphorylation sites within the α-mTOR 367 epitope were modified by AKT in vitro, we coimmunoprecipitated myrAKT with mutated mTOR polypeptides containing single or double Ala substitutions at Thr^{2446} and Ser^{2448}. The results presented in Fig. 5B demonstrate that Ala substitutions at both Thr^{2446} and Ser^{2448} (the "AA" mutant) almost completely eliminated the in vitro phosphorylation of AmTOR by myrAKT. Single alanine substitutions at Thr^{2446} ("TA" mutant) or Ser^{2448} ("SA" mutant) each showed an approximate 50% reduction in phosphorylation by myrAKT (data not shown). Hence, both of the identified sites within the α-mTOR 367 binding site were targeted for modification by myrAKT in vitro. Deletion of the entire α-mTOR 367 target sequence (residues 2430–2450) decreased the phosphorylation of the resulting "mTOR-Δ" mutant to a level similar to that obtained with the AA double mutant. Thus, the in vitro phosphorylation of mTOR by myrAKT occurs largely, if not entirely, at two closely spaced residues (Thr^{2446} and Ser^{2448}) located within the peptide sequence recognized by α-mTOR 367 and mTAb1 (39) antibodies.

Phosphorylation of mTOR at Ser^{2448} in Intact Cells. The observation that AKT phosphorylates the COOH terminal region of mTOR prompted efforts to determine whether these residues underwent reversible modification in growth factor- or hormone-stimulated cells. To examine the phosphorylation states of Thr^{2446} and Ser^{2448} in intact cells, we generated phospho-mTOR-specific (α-mTORp2) antibodies (see "Materials and Methods" for details). These antibodies specifically recognized phosphopeptides corresponding to amino acids 2433–2450 of mTOR and containing phosphate at either Thr^{2446} or Ser^{2448}, as well as the doubly phosphorylated peptide.[5] To determine whether physiological stimuli trigger the phosphorylation of these sites, we first returned to the protein blot shown in Fig. 3. When this blot was stripped and reprobed with α-mTORp2 antibodies, we

observed that IL-3 stimulation caused a prompt increase in the reactivity of mTOR with these phosphospecific antibodies (Fig. 3, middle panel). Notably, the time-dependent increase in α-mTORp2 immunoreactivity mirrored precisely the decrease in α-mTOR 367 binding provoked by IL-3 stimulation (Fig. 3, upper panel). Pretreatment of these cells with 100 nM wortmannin blocked the increase in α-mTORp2 binding stimulated by IL-3, indicating that this alteration was dependent on the activation of PI3K.

Transient transfection studies in HEK 293 cells revealed that insulin stimulation also provoked a rapid increase in the α-mTORp2 reactivity of the rapamycin-resistant AmTOR-SI mutant (Fig. 6A). AmTOR-SI contains a single amino acid substitution [Ser (2035)→Ile] that renders the hormone-dependent signaling functions of this protein kinase resistant to rapamycin in intact cells (see below for additional explanation). It should be noted that this upstream "SI" mutation did not influence the binding of α-mTORp2 to the ectopically expressed mTOR, because identical results were obtained in HEK 293 cells transfected with the AmTOR-WT-encoding plasmid (results not shown). To determine whether Thr^{2446} or Ser^{2448}, or both sites, were targeted for phosphorylation in vivo, cells were transfected
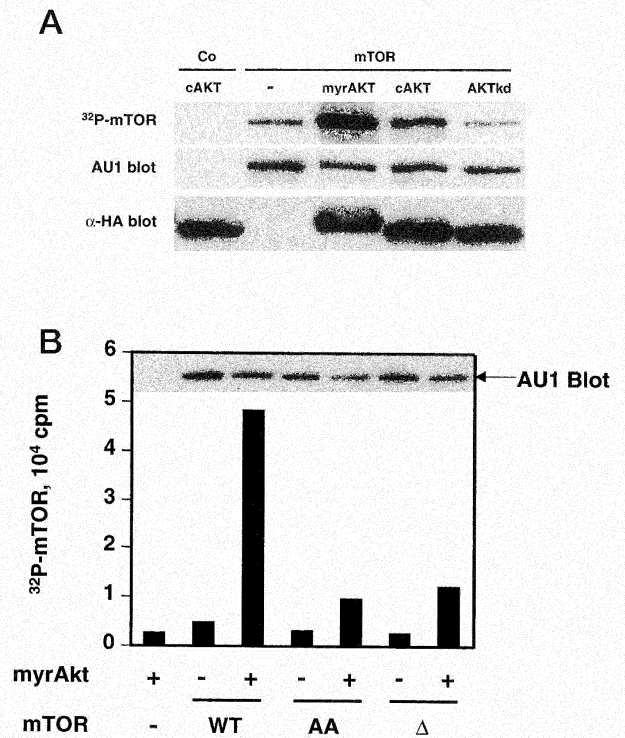
A

B

Fig. 5. Phosphorylation of mTOR by AKT in vitro. A, separate populations of HEK 293 cells were transiently transfected with expression plasmids encoding HA-tagged wild-type AKT (cAKT), myristylated AKT (myrAKT), catalytically inactive AKT (AKt kd), or AmTOR^{wt} (mTOR). Extracts from the AmTOR^{wt}- and HA-AKT-expressing cells were mixed, and the tagged proteins were captured on protein A-Sepharose beads armed with both α-AU1 and α-HA antibodies. The immunoprecipitates were washed and incubated in phosphorylation buffer containing [γ-^{32}P]ATP, and the reaction products were separated by SDS-PAGE. Incorporation of radiolabeled phosphate into AmTOR^{wt} was detected by autoradiography (upper panel). The amounts of immunoprecipitated AmTOR^{wt} and HA-AKT polypeptides in each sample were determined by immunoblotting with α-AU1 or α-HA mAb, respectively (lower panels). B, separate populations of HEK 293 cells were transfected with the AmTOR^{wt} (WT), AmTOR containing Ala substitutions at Thr^{2446} and Ser^{2448} (AA), or AmTOR with a deletion of residues 2430–2450 (Δ). Cellular extracts containing the indicated AmTOR proteins were mixed with extracts from myrAKT expressing (+) or mock-transfected cells (−). The myrAKT and AmTOR polypeptides were coimmunoprecipitated and subjected to immune complex kinase assays as described in A. In the inset, the amount of AmTOR in each sample was determined by immunoblotting.

[5] C. C. Hudson and R. T. Abraham, unpublished results.

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