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

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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

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[4] The abbreviations used are: FKBP, FK506-binding protein; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3′-kinase; PIKK, PI3K-related kinases; $p70^{S6K}$, ribosomal p70 S6 kinase; eIF, eukaryotic initiation factor; FBS, fetal bovine serum; IL, interleukin; HEK, human embryonic kidney; HA, hemagglutinin; mAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; PDK, phosphoinositide-dependent kinase; PKC, protein kinase C.

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 *Eco*RI and *Xba*I 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_2SO) 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 CDTNAKGNKRSRTR<u>T</u>D<u>S</u>YS. 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_3VO_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_3VO_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_3VO_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. 1*A*, *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. 1*B* 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

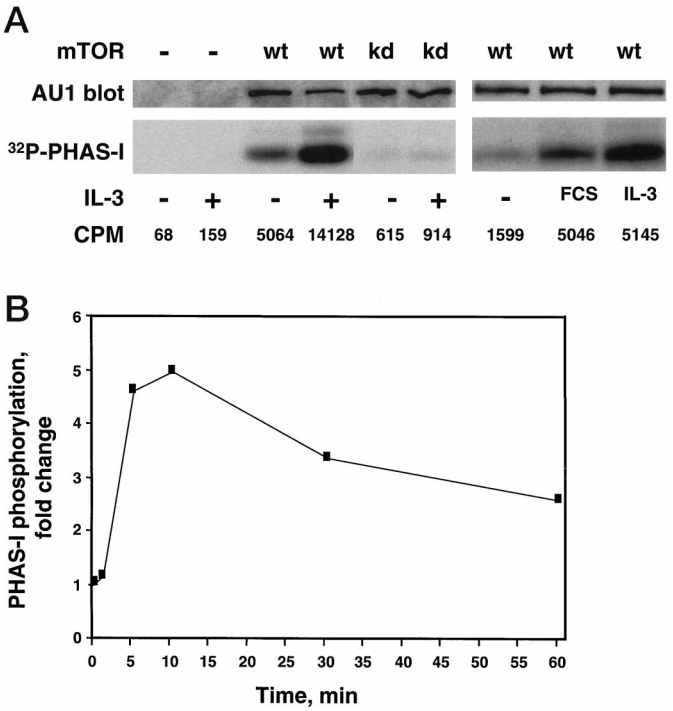

Fig. 1. Stimulation of mTOR kinase activity by IL-3 or serum. FDC-P1 cells were stably transfected with wild-type AU1-tagged mTOR or a catalytically inactive version of AmTOR. Transfected clones were cultured for 6 h in medium without serum and IL-3. A, *left panel*, factor-deprived FDC-P1 cells were 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. 1*A*, *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 $\leq$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 immunoreactivity 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. 1*B*). 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

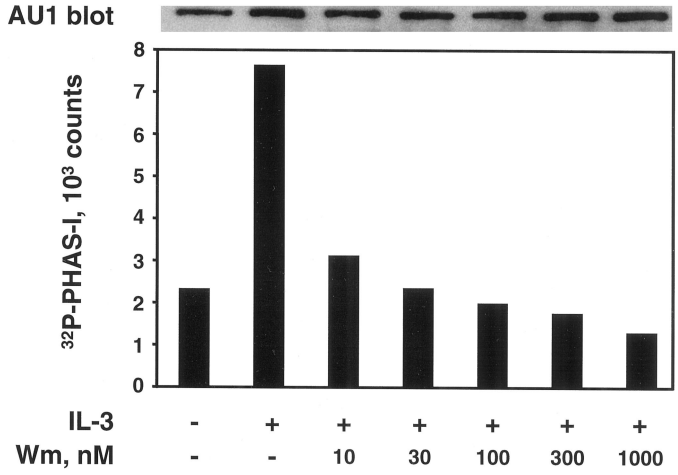

Fig. 2. Inhibition of IL-3 dependent mTOR activation by cellular treatment with wortmannin. AmTOR^{wt}-transfected FDC-P1 cells were deprived of growth factors as described in Fig. 1. AmTOR^{wt}-expressing cells were treated for 30 min with the indicated concentrations of wortmannin (*Wm*). After stimulation 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*).

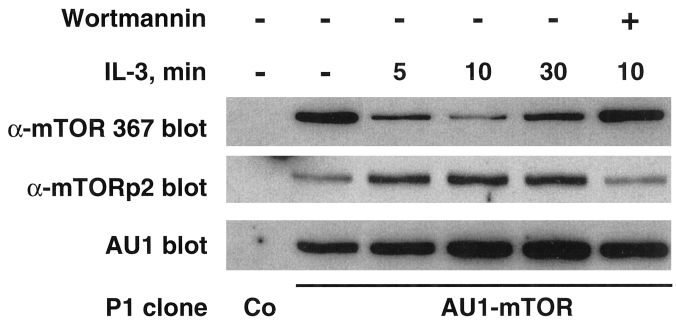

Fig. 3. IL-3-dependent alterations in the reactivity of AmTOR^{wt} with R domain-directed α-mTOR antibodies. AmTOR^{wt}-expressing FDC-P1 cells were deprived of growth factors and then stimulated for the indicated times with medium only (−) or with IL-3. Wortmannin (100 nM) was added to the indicated 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.

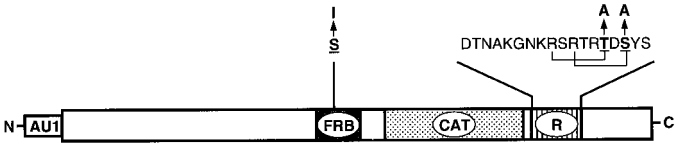

Fig. 4. Schematic diagram of mTOR functional domains and mutants used in this study. The NH_2-terminal AU1 epitope tag is followed by an extended NH_2-terminal region of unknown function. The FKBP12·rapamycin binding (*FRB*) domain is labeled, as is the Ser (2035)→Ile mutation used to generate the rapamycin-resistant 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. 5*A*, 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. 5*B* 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. 6*A*). 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

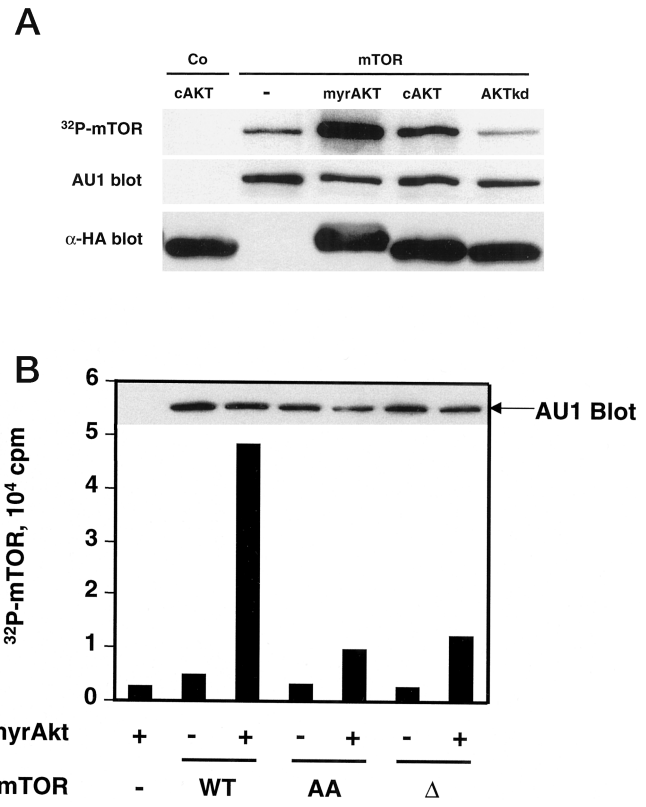

Fig. 5. Phosphorylation of mTOR by AKT *in vitro*. *A*, separate populations of HEK 293 cells were transiently transfected with expression plasmids encoding HA-tagged wild-type AKT (*cAKT*), myristylated AKT (*myrAKT*), catalytically inactive AKT (*AKtkd*), or $AmTOR^{wt}$ (*mTOR*). Extracts from the $AmTOR^{wt}$- 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.

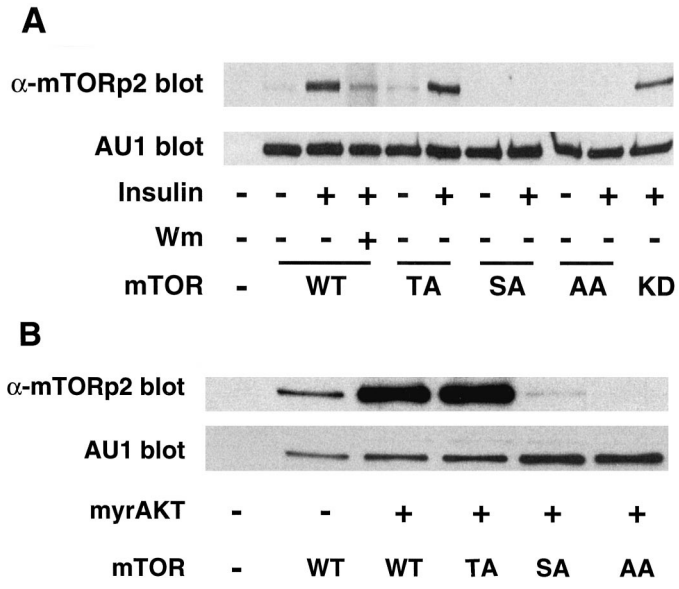

Fig. 6. Phosphorylation of mTOR at Ser^{2448} in HEK 293 cells. *A*, insulin-stimulated mTOR phosphorylation. HEK 293 cells were transfected with expression plasmids encoding AmTOR-SI (*WT*), catalytically inactive AmTOR-SI (*kd*), or with AmTOR-SI constructs containing single or double Ala substitutions at Thr^{2446} and Ser^{2448} (denoted as TA, SA, and AA, respectively). At 24 h after transfection, the cells were transferred into low serum (0.5% FBS) medium and were cultured for an additional 17 h, followed by a 1-h culture in serum-free DMEM. The indicated samples were treated for 10 min with 1 μM insulin prior to the preparation of cellular extracts. One sample was treated with 1 μM wortmannin (*Wm*) before the addition of insulin. AmTOR-SI polypeptides were immunoprecipitated with α-AU1 mAb, and proteins were resolved by SDS-PAGE. The samples were immunoblotted sequentially with phosphospecific α-mTORp2 antibodies (*upper panel*) and α-AU1 mAb (*lower panel*). *B*, phosphorylation of mTOR in myrAKT-expressing cells. HEK 293T cells were transfected with the indicated AmTOR-SI constructs, together with either empty plasmid (−) or a myrAKT expression plasmid (+). At 20 h after transfection, the cells were transferred into DMEM containing 2% serum and were cultured for 2 h prior to cell harvest. The AmTOR-SI proteins were immunoprecipitated and immunoblotted as described in *A*.

with AmTOR-SI expression constructs containing single or double mutations at Ser^{2448} and Thr^{2446} (designated SA, TA, and AA, respectively). Stimulation of serum-starved HEK 293 cells for 10 min with insulin caused a wortmannin-sensitive increase in the reactivity of AmTOR-SI (designated WT in the figure) with α-mTORp2 antibodies. Virtually identical results were obtained when the TA mutant was immunoblotted with α-mTORp2 antibodies, indicating that Thr^{2446} was not a major phosphorylation site in insulin-stimulated cells. In contrast, both the SA and AA mutants failed to react with α-mTORp2 antibodies, either before or after insulin stimulation. To determine whether the kinase activity of mTOR itself was required for the phosphorylation of Ser^{2448}, we transfected HEK 293 cells with the catalytically inactive AmTOR-SI/kd double mutant. As shown in the final lane of Fig. 6*A*, insulin treatment also triggers the phosphorylation of AmTOR-SI/kd, which indicates that this modification is not explained by the previously reported autokinase activity of mTOR (44).

In subsequent studies, AmTOR-SI (WT), and its mutated TA, SA, and AA derivatives were individually expressed in HEK-293T cells, together with myrAKT (Fig. 6*B*). The α-mTORp2 reactivities of both the WT and TA forms of AmTOR-SI were markedly increased in myrAKT-expressing cells. Once again, mutation of Ser^{2448} to Ala in the SA and AA mutants caused a near-total loss of the myrAKT-dependent increase in α-mTORp2 binding. Although we consistently observed a slight increase in α-mTORp2 reactivity after coexpression of the SA mutant with myrAKT, the physiological relevance of this residual Thr^{2446} phosphorylation remains unclear.

Impact of AKT-mediated Phosphorylation on mTOR Signaling Functions. To examine the role of Ser^{2448} phosphorylation in mTOR signaling functions, we focused on the two known downstream targets in this pathway, p70^{S6K} and the translational repressor, PHAS-I. Because qualitatively similar results were obtained with both of these downstream reporters for mTOR activity, only the data obtained with p70^{S6K} will be presented in this report. The studies were performed by transfection of HEK 293 cells with the various AmTOR-SI constructs, together with a FLAG-tagged p70^{S6K} expression plasmid. As explained above, the Ser (2035) to Ile mutation (SI) renders the recombinant AmTOR-SI resistant to inhibition by the FKBP12·rapamycin complex. Thus, when the transfected cells are treated with rapamycin, endogenous mTOR activity is strongly suppressed, and insulin-mediated p70^{S6K} activation is contingent on the function of the transiently expressed AmTOR-SI protein (32). In the control sample, transiently transfected wild-type (WT) AmTOR failed to support insulin-stimulated activation of p70^{S6K} in the presence of rapamycin (Fig. 7*A*). In contrast, cells transfected with the rapamycin-resistant mTOR-SI mutant displayed a robust increase in p70^{S6K} activity in response to insulin. Surprisingly, AmTOR-SI constructs containing mutations at

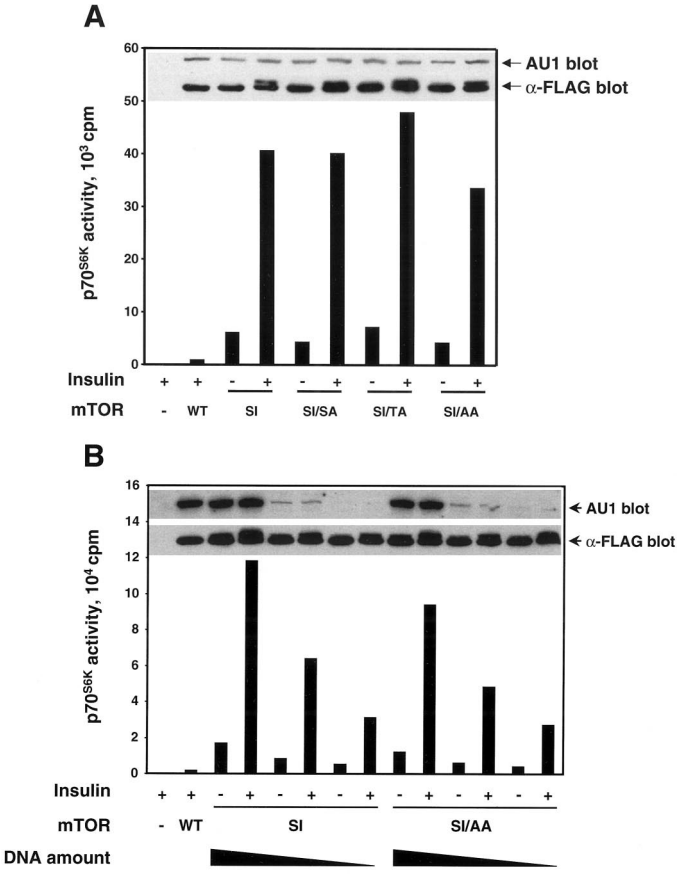

Fig. 7. Signaling functions of AmTOR phosphorylation site mutants in HEK 293 cells. HEK 293 cells were transfected with expression plasmids encoding wild-type AmTOR (*WT*), AmTOR-SI (*SI*), or the indicated AmTOR-SI constructs containing Ala (*A*) at Thr^{2446} and/or Ser^{2448}. In *B*, the amounts of AmTOR plasmid DNAs used were: WT, 1 μg; SI and the SI/AA double mutant, 1 μg, 0.3 μg, or 0.1 μg. The cells were cotransfected with a reporter plasmid encoding FLAG-p70^{S6K}. Controls (−) received empty expression vector (pcDNA3) only. After a 24-h transfection, the cells were deprived of serum for 18 h and then treated for 1 h with 10 nM rapamycin. The rapamycin-treated cells were stimulated for 15 min with 1 μM insulin prior to cell lysis. The cells were lysed in detergent-containing buffer, and FLAG-p70^{S6K} was immunoprecipitated with α-FLAGM2 affinity gel. Protein kinase activity was determined by measuring the incorporation of radiolabeled phosphate into the S6 peptide substrate. The amount of AmTOR expressed in each cell population was determined by immunoblotting with α-AU1 mAb (*inset, upper panel*), and the amount of FLAG-p70^{S6K} immunoprecipitated in each sample was determined by immunoblotting with α-FLAGM2 (*inset, lower panel*).

either Ser^{2448} (SI/SA) or Thr^{2446} (SI/TA) or both sites (SI/AA) were as effective as the intact mTOR-SI in supporting insulin-stimulated p70^{S6K} activation. Similar results were obtained when cotransfected myrAKT, rather than exogenous insulin, served as the stimulus for p70^{S6K} activation or PHAS-I phosphorylation (data not shown).

A trivial explanation for these results is that overexpression of the AmTOR-SI mutants in HEK 293 cells might simply override a normal requirement for phosphorylation at Ser^{2446} or Thr^{2448}. To address this possibility, HEK 293 cells were transfected with progressively decreasing amounts of mTOR-SI or mTOR-SI/AA plasmid DNA, and after serum deprivation, the cells were restimulated with insulin (Fig. 7*B*). The results clearly indicated that, even under conditions in which AmTOR-SI expression was limiting for p70^{S6K} activation, loss of the Thr^{2446} and/or Ser^{2448} phosphoacceptor sites had no effect on the coupling of insulin receptor occupancy to p70^{S6K} activation. Once again, similar results were obtained when the titration study was repeated with myrAKT as the stimulus for p70^{S6K} activation (results not shown).

Amino Acids 2430–2450 of mTOR Define a "Repressor" Domain. Although mutations of the AKT phosphorylation sites in mTOR failed to interfere with either p70^{S6K} activation or PHAS-I phosphorylation in HEK 293 cells, we remained intrigued by the observation that antibody binding to amino acids 2433–2450 of mTOR significantly increased the protein kinase activity of mTOR *in vitro* (38). To further investigate the biochemical basis of this phenomenon, we deleted amino acids 2430–2450 from both the AmTOR and AmTOR-SI constructs to create the corresponding "Δ" mutants. AmTOR-Δ was expressed in HEK 293 cells, and the PHAS-I kinase activity of this protein was determined under linear *in vitro* reaction conditions (Fig. 8*A*). The AmTOR-Δ mutant consistently displayed an elevated level of protein kinase activity relative to the full-length AmTOR^{wt} protein. When corrected for the background activity present in α-AU1 immunoprecipitates from mock-transfected cells (*first lane* in Fig. 8*A*), the protein kinase activity of AmTOR-Δ was 3.5–10-fold higher than that of wild-type AmTOR over five independent trials.

To determine whether the AmTOR-Δ mutant also displayed enhanced signaling function *in vivo*, we compared the abilities of AmTOR-SI and AmTOR-SI/Δ to support p70^{S6K} activation in rapamycin-treated HEK 293 cells. Over a broad range of expression levels, the AmTOR-SI/Δ deletion mutant consistently drove p70^{S6K} activity in serum-starved HEK 293 cells to at least a 3-fold higher level than was obtained in cells expressing full-length AmTOR-SI (Fig. 8*B*). The increase in FLAG-p70^{S6K} activity induced by AmTOR-SI/Δ was paralleled by the appearance of a more slowly migrating form of FLAG-p70^{S6K}, indicating that expression of this activated form of mTOR was sufficient to increase the phosphorylation of p70^{S6K} in serum-starved cells (Fig. 8*B*, *inset*). Despite the increase in basal p70^{S6K} activity provoked by AmTOR-SI/Δ, insulin stimulation was still required for maximal phosphorylation and activation of p70^{S6K}. Qualitatively similar results were obtained when PHAS-I was substituted for p70^{S6K} as the reporter for signaling through mTOR. The fact that expression of AmTOR-SI/Δ does not fully replace the requirement for insulin is consistent with models suggesting that both mTOR-dependent and -independent signaling pathways contribute to p70^{S6K} activation and PHAS-I phosphorylation in hormone-stimulated cells (18–21, 34).

Phosphorylation of mTOR in Prostate Cancer Cell Lines. The finding that AKT is an upstream protein kinase for mTOR prompted speculation that cancer cells bearing mutations leading to autonomous activation of the PI3K-AKT pathway might show constitutive phosphorylation of mTOR at Ser^{2448}. The human prostate cancer cell line, PC-3, exhibits constitutive activation of the PI3K-AKT pathway be-

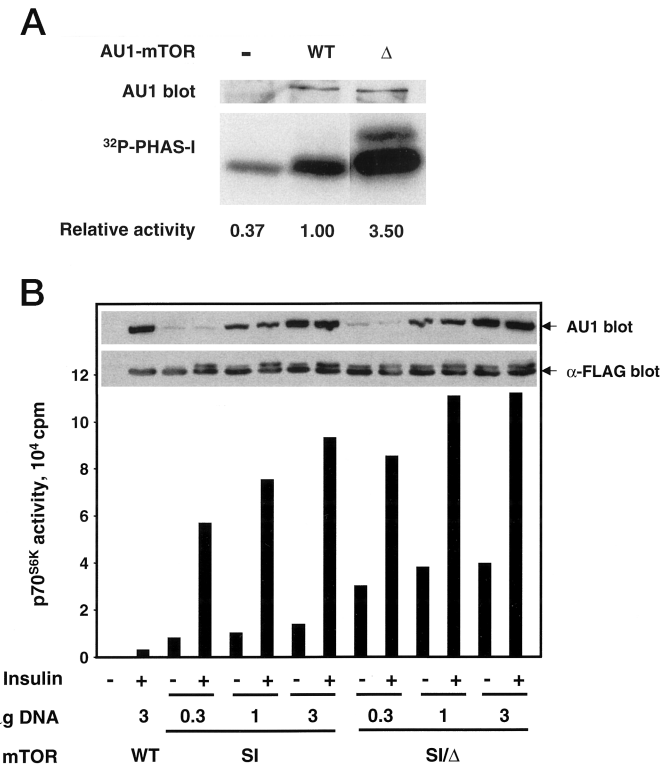

Fig. 8. Activation of mTOR by deletion of the α-mTOR 367-binding region. *A*, protein kinase activities of AmTOR proteins *in vitro*. HEK 293 cells were transfected with expression plasmids encoding AmTOR-WT (*WT*) or AmTOR-Δ (Δ). The transfected cells were incubated for 24 h in DMEM containing 10% serum, followed by serum deprivation for 18 h. The cells were lysed in Tween 20-containing buffer, and AmTOR proteins were immunoprecipitated with α-AU1 mAb. Protein kinase activities were determined with PHAS-I as the substrate, as described in the Fig. 1 legend. The quantities of immunoprecipitated mTOR were assessed by immunoblotting with AU1 mAb (*upper panel*). Incorporation of $^{32}P_i$ into PHAS-I was normalized to that obtained in the WT-containing immune complex kinase assay. *B*, activation of p70^{S6K} by AmTOR-SI/Δ. HEK 293 cells were cotransfected with wild-type AmTOR (*WT*), AmTOR-SI (*SI*), or AmTOR-SI/Δ (*SI*/Δ), together with a FLAG-p70^{S6K} expression plasmid. The activity of FLAG-p70^{S6K} was assayed as described in the Fig. 7 legend.

cause of loss of function mutations in the tumor suppressor gene, *PTEN*, together with overexpression of the AKT3 isoform (45). A second prostate cancer cell line, DU 145, retains functional PTEN but displays a 20–40-fold increase in AKT3 protein expression and enzyme activity (45). The growth of these cell types, as measured in clonogenic assays, is strongly inhibited by treatment with 20 nM rapamycin.[6] In the present studies, we examined the phosphorylation of mTOR at Ser^{2448} under serum starvation conditions and after restimulation with serum. In HEK 293 cells, phosphorylation of endogenous mTOR at Ser^{2448} was strongly dependent on cellular stimulation with insulin or serum (Fig. 9). In contrast, Ser^{2448} phosphorylation in DU 145 and PC-3 cells was substantially resistant to serum starvation. Thus, prostate cancer cell lines that contain overexpressed and/or persistently activated AKT also maintain the phosphorylation of Ser^{2448} in mTOR in the absence of serum-derived growth factors.

DISCUSSION

Accumulating evidence supports the idea that uncontrolled activation of the PI3K-dependent signaling cascade contributes to the development and progression of many human cancers. The lipid second messengers generated by PI3K activity stimulate a series of PDKs,

[6] C. C. Hudson, P. Yin, and R. T. Abraham, unpublished data.

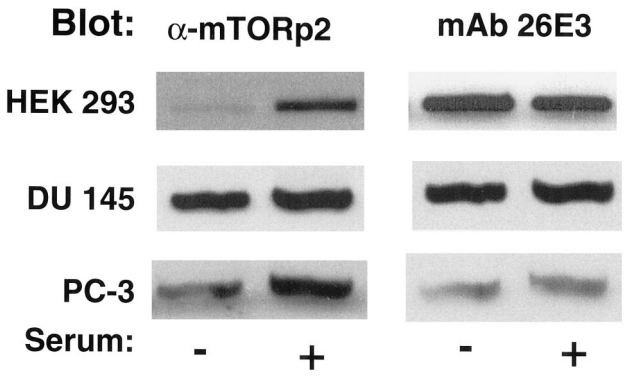

Fig. 9. Phosphorylation of endogenous mTOR in prostate cancer cell lines. HEK 293, DU 145, and PC-3 cells were deprived of serum for 20 h, and then the indicated samples were stimulated for 15 min with fresh 10% FBS. The cells were lysed, and 100 μg of soluble protein from each sample were separated by SDS-PAGE. The proteins were transferred onto an Immobilon P membrane, and the membrane was probed sequentially with α-mTORp2 antibodies and α-mTOR 26E3 mAb.

including PDK-1, PDK-2, and AKT, which in turn, transmit signals that stimulate a host of cellular responses, including survival, growth, migration, protein synthesis, and transport. Several studies have implicated mTOR as a downstream target of one or more of these PDKs in insulin- or growth factor-stimulated cells. The present report substantiates the linkage between AKT and mTOR by demonstrating that mTOR is phosphorylated by AKT at Thr^{2446} and Ser^{2448} *in vitro*, and that one of these sites, Ser^{2448}, is predominantly phosphorylated in response to stimuli that activate AKT in intact cells. Moreover, we found that Ser^{2448} is constitutively phosphorylated in prostate cancer cell lines that express deregulated AKT activity. Thus, the anticancer as well as immunosuppressive activity of rapamycin may be related to the disruption of the mTOR-dependent limb of the PI3K-AKT signaling pathway during G_1 phase of the cell cycle.

In the initial experiments, we examined the effect of IL-3 stimulation on mTOR kinase activity in FDC-P1 myeloid progenitor cells that stably expressed either $AmTOR^{wt}$ or $AmTOR^{kd}$. Like the parental cell line, the transfected cells require IL-3 and serum for survival and proliferation, and the cell growth rate is reduced by 50–60% in the presence of rapamycin.[7] Restimulation of $AmTOR^{wt}$-expressing cells with either IL-3 or serum triggered a rapid increase in the protein kinase activity of $AmTOR^{wt}$, as measured in immune complex kinase assays with PHAS-I as the substrate. The time course of IL-3-dependent mTOR activation was nearly identical to that reported with insulin-stimulated 3T3-L1 adipocytes (39) or serum-stimulated HEK 293 cells (33). An essential role for PI3K in this pathway was clearly suggested by the sensitivity of IL-3-dependent mTOR kinase activation to a low concentration (10 nM) of the PI3K inhibitor, wortmannin. The potency of wortmannin as an inhibitor of IL-3-dependent mTOR activation is considerably greater than that reported for the mTOR kinase itself (44), which supports the conclusion that a wortmannin-sensitive PI3K activity mediates the activation of mTOR in response to IL-3 receptor occupancy.

The first evidence supporting a direct role for a PI3K-dependent kinase in the regulation of mTOR function was reported by Scott *et al.* (39), who showed that AKT activation triggered a modification of mTOR that resulted in a decrease in the reactivity of mTAb1 antibodies with the full-length protein. We confirmed this result with our own α-mTOR 367 antibodies, which bind to the same region of mTOR (residues 2433–2450) as that recognized by mTAb1. The peptide sequence recognized by the mTAb1 and α-mTOR 367 antibodies is notable in several respects. Among the members of the TOR protein family, the mTAb1/α-mTOR 367 target sequence is unique to the mammalian TOR protein. The absence of this stretch of amino acids from the yeast orthologues, TOR1p and TOR2p, suggested that these residues may have been inserted during metazoan evolution to receive a regulatory, hormonal input not present in single yeast cells. Furthermore, we surmised that the α-mTOR 367 epitope, although located downstream of the catalytic domain, may allosterically regulate the protein kinase activity of mTOR, because the binding of mTAb1 or α-mTOR 367 antibodies stimulated mTOR kinase activity (38).[4] Finally, the presence of two consensus phosphorylation sites for AKT in this region suggested that amino acid residues 2433–2450 of mTOR conferred responsiveness to an afferent regulatory signal(s) triggered by the activation of PI3K.

Our studies with the phosphospecific α-mTORp2 antibodies demonstrate that Ser^{2448} is rapidly phosphorylated in both IL-3- and insulin-stimulated cells. In contrast to Ser^{2448}, the second candidate AKT phosphorylation site, Thr^{2446}, was detectably modified only in HEK 293T cells that overexpressed activated myrAKT. These results strongly suggest that cytokine-dependent phosphorylation of mTOR is carried out, at least in part, by AKT, and that Ser^{2448} is the major site of modification, whereas Thr^{2446} is at best a minor phosphorylation site in insulin-stimulated cells. It should be noted, however, that our conclusion concerning the phosphorylation site preference of AKT is based on studies with mTOR mutants containing Ala substitutions at either Thr^{2446} or Ser^{2448}. At this point, we cannot rule out the possibility that mTOR phosphorylation during hormone or growth factor stimulation is processive, with Ser^{2448} phosphorylation setting the stage for Thr^{2446} phosphorylation by AKT or another protein kinase. While this manuscript was in preparation, Nave *et al.* (46) also identified Ser^{2448} in mTOR as a phosphorylation site for AKT in insulin-stimulated HEK 293 cells; however, this study did not address the functional significance of Ser^{2448} phosphorylation in any biological assays. Nonetheless, two independent studies now suggest that hormonally activated AKT regulates mTOR function through phosphorylation of the COOH-terminal region at Ser^{2448}. The exact nature of this AKT-regulated function remains a centrally important question. Our results indicate that mutations of Ser^{2448} and/or Thr^{2446} to nonphosphorylatable alanine residues had no detectable effect on the abilities of the resulting AmTOR-SI mutants to support insulin- or myr-AKT-stimulated $p70^{S6K}$ activation and PHAS-I phosphorylation in rapamycin-treated HEK 293 cells. Although we cannot completely rule out artifacts attributable to overexpression of the recombinant AmTOR-SI proteins in HEK 293 cells, titration studies with plasmids encoding the AmTOR-SI phosphorylation site mutants failed to uncover any quantitative defect attributable to the lack of Ser^{2448} and/or Thr^{2446} phosphorylation.

A more provocative explanation for the present findings is that phosphorylation of mTOR by AKT is required for the transmission of signals other than those leading to modulation of $p70^{S6K}$ or PHAS-I activities. A relevant example stems from recent findings that mTOR kinase activity is involved in regulation of the activities of novel PKC isoforms (PKC-δ and PKC-ϵ; Ref. 47). These studies identified a hydrophobic sequence located in the PKC-δ and PKC-ϵ COOH-terminal regions, the phosphorylation of which is rapamycin sensitive and mTOR dependent in serum-stimulated cells. Interestingly, the activation of PKC-δ and PKC-ϵ requires at least one additional phosphorylation event, which appears to be executed by PDK1. An intriguing possibility is that PI3K also governs the mTOR-dependent limb of the PKC activation pathway by triggering the phosphorylation of mTOR at Ser^{2448}. Given the reported contributions of these PKC isotypes to mitogenic signaling (48, 49), it will be important to determine whether this AKT-mediated phosphorylation event contrib-

[7] A. Sekulic and R. T. Abraham, unpublished results.

utes to the regulation of PKC-δ and PKC-ϵ activities by polypeptide growth factors.

Indirect evidence supporting a regulatory function for Ser^{2448} phosphorylation stems from the discovery that deletion of the region (amino acids 2430–2450) surrounding these sites generates a hyperactivated version of mTOR. The AmTOR-Δ deletion mutant displayed a 3.5–10-fold elevation in protein kinase activity *in vitro* relative to the full-length $AmTOR^{wt}$ protein. In intact cells, expression of AmTOR-SI/Δ significantly increased the basal levels of $p70^{S6K}$ activity and PHAS-I phosphorylation (results not shown) in serum-starved and rapamycin-treated HEK 293 cells and enhanced both responses when the starved cells were stimulated with insulin. On the basis of the biochemical and phenotypic consequences of deleting amino acids 2430–2450 from mTOR, we propose that this region serves as a "repressor domain," the function of which is normally modulated by growth factors and other stimuli that affect mTOR-dependent signaling. The present findings suggest that Ser^{2448} phosphorylation is not sufficient to relieve the inhibitory activity of the repressor domain on mTOR kinase activity. However, the presence of six serine or threonine residues in the repressor domain hints that this region might channel inputs from multiple upstream kinases to the mTOR catalytic domain. The generation of mTOR constructs bearing additional point mutations and internal deletions within the repressor domain will help to unravel its roles in the regulation of mTOR kinase activity and function in intact cells.

Rapamycin displays potent cytostatic activities against certain tumors and may augment tumor cell killing by certain anticancer agents (50). Early observations demonstrated that different tumor cell lines displayed highly variable sensitivities to rapamycin. The phenotypic parameters that govern tumor cell sensitivity *versus* resistance to rapamycin are poorly understood; however, the ability to predict tumor responsiveness to rapamycin therapy becomes more critical as the rapamycin analogue, CCI-779, moves into Phase II clinical trials as an anticancer agent. The present report offers preliminary evidence to support the notion that the status of the PI3K-AKT signaling pathway in cancer cells is an important determinant of the cellular response to rapamycin. The prostate cancer cell lines, DU 145 and PC-3, exhibit hyperactivation of the PI3K-AKT pathway, either through loss of PTEN (PC-3) and/or increased protein expression and enzymatic activity of AKT (DU 145). In each case, we found that the phosphorylation of mTOR at the AKT phosphorylation site (Ser^{2448}) was abnormally persistent under serum starvation conditions. These findings raise the possibility that tumor cells bearing activating mutations in the PI3K pathway are "hard-wired" through mTOR (and hence sensitive to rapamycin) for the stimulation of G_1-phase progression. The finding that serum-independent S-phase entry in rodent fibroblasts transformed with activated AKT is exquisitely sensitive to rapamycin supports this model (51). Clearly, additional experiments are required to establish the relationship between deregulated PI3K-AKT activity and rapamycin sensitivity in human cancer cells. If this hypothesis proves correct, then screening of tumor biopsy specimens for PTEN loss, AKT activation, and mTOR phosphorylation may provide a rational basis for identification of those cancer patients most likely to benefit from therapy with mTOR inhibitors, such as rapamycin or CCI-779.

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