Estrogen-Induced Activation of Mammalian Target of Rapamycin Is Mediated via Tuberin and the Small GTPase Ras Homologue Enriched in Brain

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Abstract

Inhibitors of the mammalian target of rapamycin (mTOR) are currently in clinical trials for the treatment of breast cancer. The mechanisms through which mTOR are activated in breast cancer and the relationship of mTOR activation to steroid hormones, such as estrogen, that are known to influence breast cancer pathogenesis, are not yet understood. Using MCF-7 cells as a model, we found that 17-β estradiol (E_2) rapidly increased the phosphorylation of downstream targets of mTOR: p70 ribosomal protein S6 kinase, ribosomal protein S6, and eukaryotic initiation factor 4E-binding protein 1. The phosphoinositide-3-kinase inhibitor, wortmannin, and the mTOR inhibitor, rapamycin, blocked E_2-induced activation of p70 ribosomal protein S6 kinase. We hypothesized that tuberin and the small GTPase Ras homologue enriched in brain (Rheb), regulators of the mTOR pathway, mediate E_2-induced activation of mTOR. Consistent with this hypothesis, E_2 rapidly (within 5 minutes) stimulated tuberin phosphorylation at T1462, a site at which Akt phosphorylates and inactivates tuberin. E_2 also rapidly decreased the inactive, GDP-bound form of Rheb. Finally, we found that small interfering RNA down-regulation of endogenous Rheb blocked the E_2-stimulated proliferation of MCF-7 cells, demonstrating that Rheb is a key determinant of E_2-dependent cell growth. Taken together, these data reveal that the TSC/Rheb/mTOR pathway plays a critical role in the regulation of E_2-induced proliferation, and highlight Rheb as a novel molecular target for breast cancer therapy. (Cancer Res 2006; 66(19): 9461-6)

Introduction

Mammalian target of rapamycin (mTOR) is a kinase that integrates signals from nutrients and growth factors to regulate many processes, including autophagy, ribosome biogenesis, and metabolism (reviewed in refs. 1–4). Recently, mTOR has become increasingly recognized as a potential target for cancer therapy. Activation of mTOR targets has been observed in breast cancer tissue specimens (5), and clinical trials of mTOR inhibitors in breast cancer patients are currently ongoing (6–8).

The small GTPase Ras homologue enriched in brain (Rheb) directly activates mTOR (9–11). Rheb is inhibited by the GTPase-activating protein (GAP) domain of the tuberous sclerosis complex 2 (*TSC2*) gene product, tuberin (10, 12–15). Activation of mTOR as a result of loss or inactivation of tuberin leads to the phosphorylation of downstream targets including p70 ribosomal protein S6 kinase (S6K), ribosomal protein S6 (S6), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), promoting increased protein translation and cell growth. At least three kinases are known to directly phosphorylate and inactivate tuberin: Akt (10, 16–19), p90 ribosomal S6 kinase (20), and Erk2 (21).

Recently, the activation of mTOR targets was observed in MCF-7 cells overexpressing aromatase (an enzyme catalyzing the conversion of testosterone and androgen to estradiol) in estrogen-producing cells of the adrenal glands, ovaries, placenta, testicles, adipose (fat) tissue, and brain, 4 hours after exposure to 17-β estradiol (E_2; ref. 22). However, the pathways that mediate this activation are not known. E_2 triggers nuclear transcriptional (genomic) events as well as rapid, "nongenomic" signaling cascades, both of which contribute to growth, survival, and migration. These rapid events occur in seconds to minutes, and can be activated by estrogen receptors (ER) that lack a nuclear localization signal or are targeted to the plasma membrane, thereby dissociating them from nuclear transcriptional activity (reviewed in refs. 23–25). The nongenomic effects of E_2 include rapid activation of phosphatidylinositol-3-kinase leading to the activation of Akt (26–28) and p42/44 mitogen-activated protein kinases (MAPK; refs. 29, 30).

We report here that within minutes of E_2 treatment of MCF-7 cells, tuberin was phosphorylated at a site that is associated with loss of GAP activity toward Rheb, resulting in an increase in Rheb activity and activation of downstream targets of mTOR. The rapid nature of these events is consistent with nongenomic E_2–induced signaling. In addition, down-regulation of endogenous Rheb using small interfering RNA (siRNA) completely blocked E_2-induced proliferation of MCF-7 cells. These data indicate that the TSC/Rheb/mTOR pathway regulates E_2-induced signaling and cell proliferation.

Materials and Methods

Cell culture and reagents. MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Prior to experiments, cells were serum-starved for 24 hours in serum-free and phenol red–free medium. E_2 (1 or 10 nmol/L; Sigma, St. Louis, MO), insulin-like growth factor I (IGF-I; 20 ng/mL; CalBiochem, La Jolla, CA), wortmannin (50 nmol/L; CalBiochem), rapamycin (20 nmol/L; BioMol, Plymouth Meeting, PA), and FTI 277 (10 μmol/L; CalBiochem), were added to the cells as indicated. MCF-7 cells were transfected using FuGene 6 transfection reagent (Roche Allied Science, Indianapolis, IN).

Immunoblotting and antibodies. Cells were rinsed once in ice-cold 1× PBS, then lysed in PTY buffer [50 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, and 1% Triton X-100] supplemented with phosphatase inhibitor cocktail I and II (Sigma). Cell lysates were resolved by SDS-PAGE and transferred onto Immobilon

P membranes (Millipore, Bedford, MA). The following antibodies were used for Western blot analysis: anti-tuberin C-20 and anti-Rheb C-19 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-hamartin (Zymed Laboratories, Inc., San Francisco, CA); anti-phospho-T1462-tuberin, anti-S6K, anti-phospho-T389-S6K, anti-S6, anti-phospho-S235/236-S6, anti-phospho-S473-Akt, anti-Akt, anti-phospho-T70-4E-BP1, anti-4E-BP1, anti-phospho-T202/Y204-p42/44-MAPK, anti-p42/44-MAPK (Cell Signaling Technology, Danvers, MA), and anti-β-actin (Sigma).

siRNA treatment. Cells were transfected with 50 nmol/L of Rheb siRNA, or 20 nmol/L of control siRNA (Dharmacon, Lafayette, CO) using TKO transfection reagent (Mirus, Madison, WI) for 24 hours, followed by serum starvation for 24 hours for mTOR activation assay, or by steroid deprivation for 24 hours for cell proliferation and cell cycle analyses.

Immunoprecipitation and phosphatase treatment. Cells were lysed on ice in PTY buffer. One milligram of total cell lysates were incubated with tuberin antibody (C-20) at 4°C overnight with rotation. Fifty microliters of Protein A agarose bead slurry (Invitrogen, Carlsbad, CA) was added to the immunoprecipitation complexes and incubated at 4°C for 2 hours. The beads were washed twice in lysis buffer and boiled in 2× SDS Laemmli buffer (Bio-Rad, Hercules, CA). For phosphatase treatment, the beads of tuberin immunoprecipitates were washed with phosphatase buffer [50 mmol/L of Tris-HCl (pH 7.5), 1 mmol/L of MnCl$_2$, and 1 mmol/L of DTT], resuspended in 100 μL of phosphatase buffer, and incubated at 37°C for 10 minutes. Serine/threonine protein phosphatase 1 (0.5 units; Sigma) was added and the samples were incubated at 37°C for 20 minutes. The beads were pelleted, eluted in 2× SDS Laemmli buffer, and separated by electrophoresis for Western blot analysis.

Measurement of intracellular Rheb activation state. To measure the activation of endogenous Rheb, we used an assay originally developed for Ras (31) that we previously adapted to measure Rheb activation (32). Endogenous Rheb was immunoprecipitated with the C-19 anti-Rheb antibody (Santa Cruz Biotechnology) or goat IgG as a control, and incubated at 100°C to release bound nucleotides. Released GDP was converted to ^{32}P-GTP using nucleoside 5′-diphosphate kinase and ^{32}P-ATP. GTP was separated from ATP by thin-layer chromatography and radioactivity was quantitated by liquid scintillation counting (31).

Cell proliferation assay. MCF-7 cells were transfected with control or Rheb siRNA for 24 hours, seeded into 24-well plates in phenol red–free medium supplemented with 10% charcoal-stripped FBS for 24 hours, then stimulated with 10 nmol/L of E$_2$ for 24 hours. ^{3}H-Thymidine (1 μCi) was added to each well for 6 hours, cells were washed twice with PBS, fixed with 5% TCA, and lysed with 0.5 N NaOH. ^{3}H-Thymidine incorporation was determined using scintillation counting.

Cell cycle analysis. Cells were washed with PBS, collected by trypsinization, fixed in cold 70% ethanol, and stained with 20 μg/mL of propidium iodide (Sigma) containing 9.5 mg/mL of RNase A. Cell cycle distribution was determined using fluorescence-assisted cell sorting (FACS) on a Becton Dickinson flow cytometer. The percentages of cells in G$_1$, S, and G$_2$-M phases of the cell cycle were determined using CellQUEST DNA Acquisition software (Becton Dickinson, Franklin Lakes, NJ).

Statistics. Results are presented as mean ± SD of experiments done in triplicate. Statistical analysis was done using a two-tailed paired Student's t test. Significance was achieved at $P < 0.05$.

Results

E$_2$ rapidly activates mTOR targets. We first established the kinetics of E$_2$-induced mTOR activation in MCF-7 cells by examining the phosphorylation of two key downstream targets of mTOR, S6 (S235/236) and 4E-BP1 (T70). E$_2$ rapidly increased the phosphorylation of both mTOR targets with maximum levels detected at 15 minutes (Fig. 1A). Preincubation with 20 nmol/L of rapamycin or 50 nmol/L of wortmannin for 30 minutes blocked E$_2$-induced phosphorylation of S6K (4-fold) and phosphorylation of S6 (5-fold; Fig. 1B). In addition to E$_2$'s rapid activation of mTOR in MCF-7 cells, which to our knowledge, has not been previously

reported, we also observed the phosphorylation of Akt within 5 minutes and p42/44-MAPK phosphorylation within 2 minutes, reflecting nongenomic pathways activated by E$_2$ that have been established by other groups.

Tuberin mediates E$_2$ activation of mTOR. Because E$_2$ activates Akt, and tuberin is phosphorylated and inactivated by Akt (10, 17–19), we hypothesized that signals from E$_2$ to mTOR are transduced by Akt and tuberin. To test this hypothesis, we first used a phosphospecific tuberin antibody to one of the primary Akt sites, T1462. A 4-fold increase in tuberin phosphorylation at T1462 was clearly evident 5 minutes after E$_2$ treatment (Fig. 2A). A 10-fold increase in T1462 phosphorylation was also present after IGF-I stimulation, as expected based on work by other groups (14, 16, 17). Immunoreactivity with the phosphospecific tuberin antibody was reduced by treatment of the immunoprecipitate with serine/threonine phosphatase 1. These results show that E$_2$ induces the rapid phosphorylation of tuberin at T1462, a site which is associated with loss of tuberin's GAP activity (16–18). Total tuberin levels did not change after E$_2$ treatment, in contrast with a previous report (30).

Expression of wild-type tuberin decreased the E$_2$-induced phosphorylation of S6 at both 15 and 30 minutes (Fig. 2B). Consistent with the hypothesis that tuberin mediates E$_2$-induced mTOR activation, expression of TSC2-containing alanine mutations at the two primary Akt sites (S939A/T1462A) also inhibited
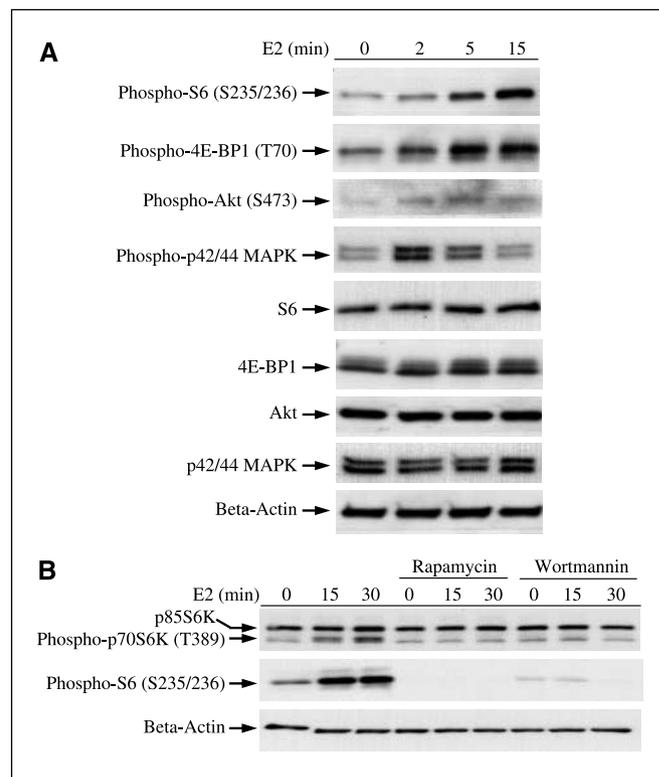

Figure 1. E$_2$ rapidly activates downstream targets of mTOR. *A,* MCF-7 cells were grown in phenol red–free and serum-free media for 24 hours, and then stimulated with 1 nmol/L of E$_2$ for 0 to 15 minutes. S6, 4E-BP1, Akt, and MAPK levels and phosphorylation status in response to E$_2$ were determined by Western blot. β-Actin immunoblot is provided as an additional loading control. *B,* MCF-7 cells were preincubated for 30 minutes with wortmannin or rapamycin followed by E$_2$ treatment for 0 to 30 minutes. S6K and S6 phosphorylation was determined by Western blot using phosphospecific antibodies. E$_2$ induced the phosphorylation of S6K and S6 by 4-fold and 5-fold, respectively, at 30 minutes.
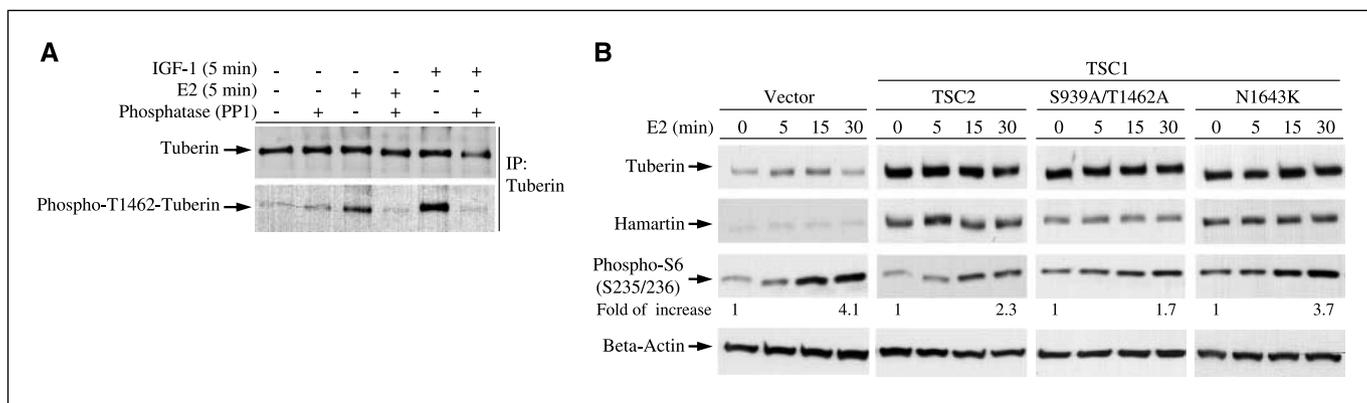
Figure 2. Tuberin mediates E_2 activation of mTOR. *A*, MCF-7 cells were grown in phenol red–free and serum-free media for 24 hours, and then stimulated with 10 nmol/L of E_2 or 20 ng/mL of IGF-I for 5 minutes. Endogenous tuberin was immunoprecipitated, and tuberin phosphorylation was detected using anti-phospho-T1462-tuberin. E_2 and IGF-I induced the phosphorylation of tuberin to a maximum of 4-fold and 10-fold, respectively. *B*, MCF-7 cells were transfected with TSC1 and either wild-type TSC2, TSC2AA (S939A/T1462A), or the patient-derived GAP mutant of TSC2 (N1643K) for 24 hours, starved in phenol red–free and serum-free media for 24 hours, and stimulated with 1 nmol/L of E_2 for the times indicated. S6 phosphorylation was determined by Western blot using phosphospecific antibodies. Numbers below phospho-S6 immunoblot indicate fold of increase in phosphorylation of S6 compared with $t = 0$.

E_2-induced activation of S6 to a similar extent. In contrast, expression of TSC2 N1643K, which carries a patient-derived TSC2 mutation and lacks GAP activity toward Rheb (32, 33), did not inhibit E_2-activated S6 phosphorylation (Fig. 2*B*). The fact that the TSC2 S939A/T1462A mutant did not completely prevent E_2-induced S6 phosphorylation may be due to the relatively low transfection efficiency (30%) in MCF-7 cells, although we cannot exclude additional phosphorylation sites on tuberin, and/or other pathways mediating E_2 signals to S6.

E_2 activation of mTOR is mediated via Rheb. To determine whether E_2 activates Rheb, the target of tuberin's GAP domain, we measured the inactive fraction of Rheb (Rheb-GDP) in E_2-treated MCF-7 cells. IGF-I stimulation was used as a positive control because IGF-I is known to activate Rheb (10). A 66% decrease in GDP-Rheb was present within 5 minutes after E_2 stimulation (Fig. 3*A*), reflecting the predicted increase in the active GTP-bound Rheb expected after tuberin inactivation. The magnitude of the changes in Rheb-GDP at 15 minutes was similar between IGF-I and E_2-stimulated cells.

Rheb is a farnesylated GTPase, and inhibition of farnesylation blocks Rheb-induced mTOR activation (9, 34). To determine whether E_2-mediated mTOR activation is farnesylation-dependent, MCF-7 cells were preincubated for 24 hours with 10 μmol/L of FTI 277, a farnesyl transferase inhibitor. FTI 277 blocked the E_2-stimulated phosphorylation of S6 (Fig. 3*B*). To further confirm the role of Rheb in mediating the E_2 activation of mTOR, we down-regulated endogenous TSC2 and Rheb using siRNA. Down-regulation of tuberin resulted in a constitutive phosphorylation of S6K, as expected (Fig. 3*C*). Stimulation with E_2 did not result in a further increase in S6K phosphorylation. This suggests that E_2 signals via the TSC2 pathway, which is maximally stimulated by the loss of tuberin. Consistent with this model, down-regulation of Rheb using Rheb siRNA reduced the phosphorylation of S6K after E_2 stimulation. Collectively, these results are consistent with a model in which E_2-induced activation of mTOR is mediated by Rheb.

Down-regulation of endogenous Rheb blocks E_2-induced cell proliferation and inhibits G_1 to S cell cycle progression in MCF-7 cells. We tested the effect of down-regulation of endogenous Rheb on E_2-induced cell proliferation and G_1 to S

cell cycle progression in MCF-7 cells. Rheb siRNA treatment completely abrogated the E_2-induced MCF-7 cell proliferation as measured by ^{3}H-thymidine incorporation (Fig. 4*A*). This result was confirmed by FACS. In control siRNA-treated cells, 24 hours of E_2 stimulation resulted in a redistribution of cells in G_1 phase, from 72% to 46%. In the absence of Rheb, 24 hours of E_2 had no effect on the cell cycle profile (Fig. 4*B* and *C*). Collectively, these results are consistent with a model in which E_2-induced activation of mTOR is mediated by Rheb.

Discussion

We show here that tuberin and Rheb, the target of tuberin's GAP domain, are key regulators of E_2-induced mTOR activation. To our knowledge, this is the first report to document E_2-induced activation of mTOR in breast cancer–derived cells with endogenous levels of aromatase. We observed a rapid effect of E_2 on both the phosphorylation of tuberin and the activation status of Rheb. In addition, down-regulation of Rheb completely blocked E_2-induced MCF-7 cell proliferation. These findings complement recent work from two groups examining the response of breast cancer cells to the mTOR inhibitor RAD001. In the first study, Treek et al. found that inhibition of mTOR with RAD001 and ER signaling with tamoxifen blocked mTOR activation and induced apoptosis in MCF-7 cells (35). However, the effect of the treatments on E_2-induced signaling, growth, or survival was not tested. In the second study, Boulay et al. found that the mTOR inhibitor, RAD001, in combination with the aromatase inhibitor, letrozole, induced apoptosis in MCF-7 cells overexpressing aromatase (22). The authors also showed the activation of mTOR targets after E_2 stimulation in MCF-7 cells overexpressing aromatase, but at later time points (4 hours) than the present study (22). Rodrik et al. found that rapamycin reversed the E_2-induced increases in Myc expression and cell survival in MCF-7 cells. Interestingly, however, they did not observe E_2-induced phosphorylation of S6K at later time points. It will certainly be important to determine whether the rapamycin-sensitive phenotypes observed by Rodrik et al. are Rheb-dependent (36).

We hypothesized that signals from E_2 to mTOR are transduced by Akt and tuberin, and used a phosphospecific tuberin antibody
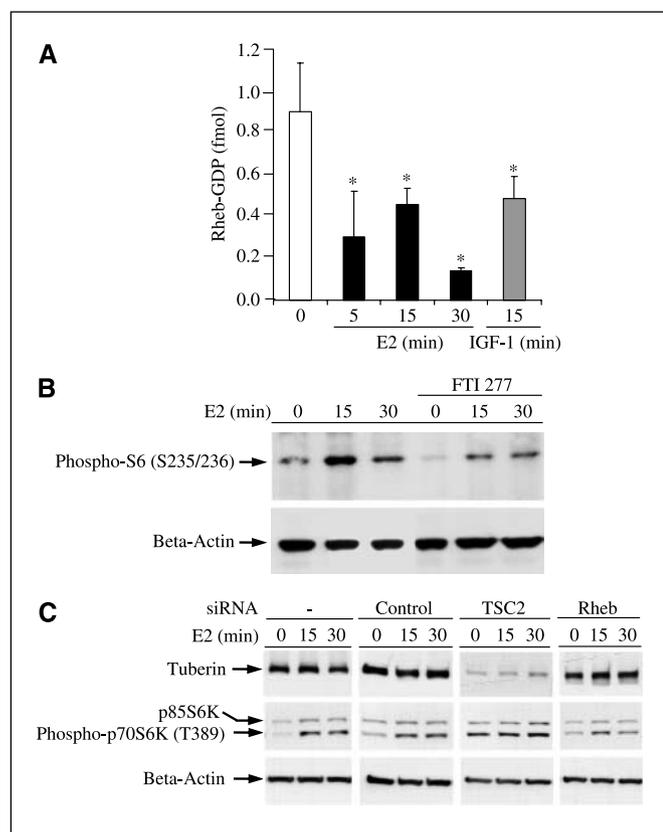
Figure 3. E_2 rapidly affects the activation status of endogenous Rheb. *A,* MCF-7 cells were grown in phenol red–free and serum-free media for 24 hours, and then stimulated with 1 nmol/L of E_2 (*black columns*) or 20 ng/mL of IGF-I (*gray column*) for the times indicated. Endogenous Rheb was immunoprecipitated, and inactive GDP-bound Rheb was measured (*, $P < 0.05$ relative to $t = 0$). *B,* MCF-7 cells were grown in phenol red–free and serum-free media for 24 hours, preincubated with or without FTI 277 for 24 hours, and stimulated with 10 nmol/L of E_2 for 0 to 30 minutes. S6 phosphorylation was determined by Western blot. β-Actin immunoblot is provided as a loading control. *C,* MCF-7 cells were transfected with tuberin siRNA or Rheb siRNA for 24 hours, serum was withdrawn for 24 hours, and then stimulated with 10 nmol/L of E_2 for 0 to 30 minutes. S6K phosphorylation was determined by Western blot.

to one of the primary Akt sites (T1462) to test this hypothesis. We observed rapid tuberin phosphorylation stimulated by E_2 at 5 minutes, with no effect on total tuberin levels. This is in contrast with a previous report that E_2 treatment increases tuberin degradation within 30 minutes in human lung fibroblasts and myofibroblasts (30). This disparity may reflect inherent cellular differences between MCF-7 cells and fibroblasts. Alternatively, it is possible that the phosphorylated forms of tuberin that we observed after E_2 or IGF-I treatment reduced the intensity of the 200 kDa tuberin band in the cell types studied by Flores-Delgado et al.

The proportion of breast cancers with activation of mTOR, and the relationship of mTOR activation to ER expression, are not yet fully understood. Bose et al. recently found that 72% of invasive breast cancers had hyperphosphorylation of S6, but surprisingly, only 24% had hyperphosphorylation of mTOR (5). Whether this difference reflects alternate mTOR-independent pathways through which S6 can be activated, or a difference in the sensitivities of the phospho-S6 and phospho-mTOR antibodies in archival formalin-fixed tissue specimens, is not known. Several studies have linked

hyperphosphorylation of Akt with breast cancer, including Kirkegaard et al. who found that a high level of phosphorylated Akt was associated with a decrease in overall survival in a study of 402 patients with ER-positive tumors (37). This study did not examine markers of mTOR activity in the tumors, which is critical because the relationship between Akt and mTOR is complex. The activation of mTOR activates a feedback mechanism through which Akt is inhibited (38–40). Therefore, it is difficult to predict the activation status of mTOR in a tumor in which Akt activation is detected.
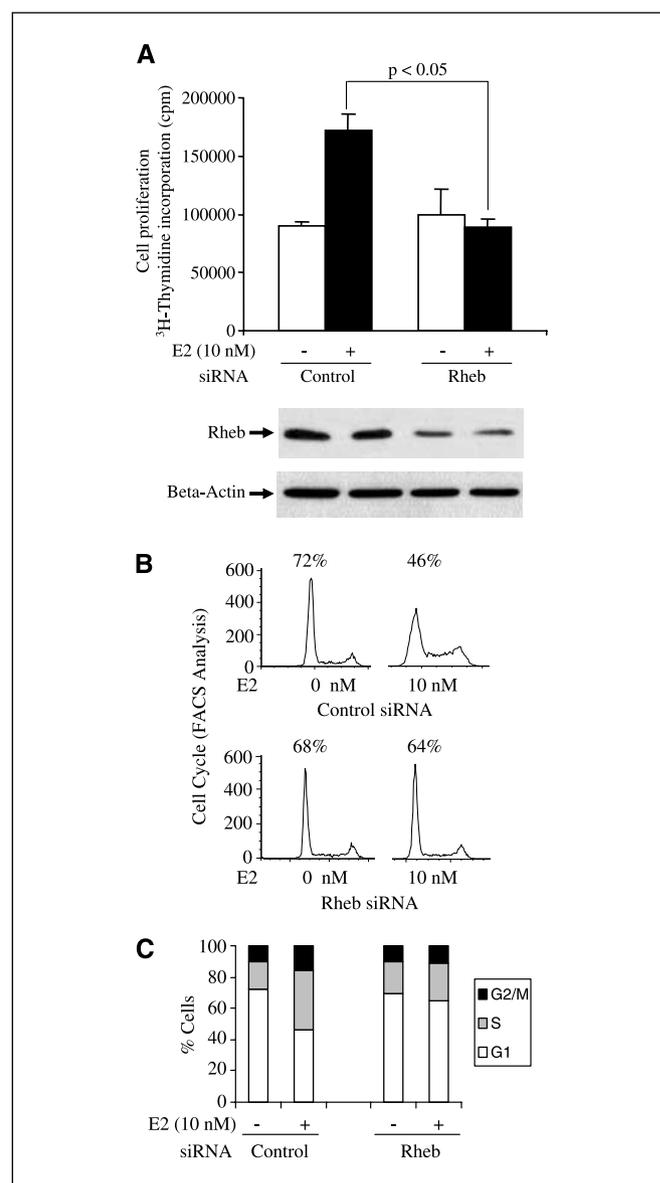

Figure 4. Down-regulation of endogenous Rheb inhibits E_2-induced cell proliferation and G_1 to S cell cycle progression in MCF-7 cells. *A,* MCF-7 cells were transfected with control or Rheb siRNA for 24 hours, seeded into 24-well plates in phenol red–free medium supplemented with 10% charcoal-stripped FBS for 24 hours, then stimulated with 10 nmol/L of E_2 for 24 hours. Cell growth was determined by ^{3}H-thymidine incorporation. Rheb expression was determined by Western blot. *B,* MCF-7 cells were transfected with control or Rheb siRNA for 24 hours, seeded into 10 cm plates, and stimulated with 0 or 10 nmol/L of E_2 for 24 hours. Cells were harvested and subjected to FACS analysis to determine cell cycle distribution. The percentage of cells in G_1 phase is indicated. *C,* summary of the FACS analyses in (*B*).

The association of tuberin and Rheb with E_2-induced signaling suggests that the TSC proteins may influence breast cancer pathogenesis. Two recent studies suggest that tuberin levels may predict the metastatic potential of breast cancer, although the results are contradictory. Jiang et al. found significantly lower levels of tuberin and hamartin in tumors from patients who developed recurrence and died from breast cancer compared with those who remained disease-free (41). In contrast, Liu et al. found a trend toward increased expression of tuberin transcript with decreased time to metastasis using a RNA expression microarray data set containing 295 breast carcinomas, although the results did not reach statistical significance, and metastasis as a specific end point was not included (42). At this point, it is difficult to resolve the results of these two studies. One possibility, given our results, is that the primary effect of tuberin in breast cancer metastasis will be observed in ER-positive tumors, and that reanalysis of these studies stratifying for ER expression and mTOR activation will be instructive.

Our data highlight Rheb as a novel target for breast cancer therapy. Rheb is a farnesylated protein, and farnesyl-deficient forms of Rheb are unable to activate mTOR (9). Interestingly, Yue et al. found that the farnesylation inhibitor, farnesylthisalicylic acid, inhibits the growth of MCF-7 cells (43). This is consistent with the data presented here, which would predict an inhibition of E_2-induced growth of MCF-7 cells by farnesylthisalicylic acid via loss of the farnesylated form of Rheb. Inhibition of mTOR has been shown to restore tamoxifen responsiveness in MCF-7 cells expressing constitutively active Akt (44). Similarly, therapies directed at Rheb could benefit patients in whom hormone resistance has developed. Further studies are needed to address the potential role and timing of inhibitors of Rheb and mTOR in breast cancer, either singly or in combination with inhibitors of intersecting pathway targets.

Whether the E_2/TSC2/Rheb/mTOR pathway studied here is related to the pathogenesis of the pulmonary manifestation of TSC, lymphangioleiomyomatosis, is not clear. Lymphangioleiomyomatosis occurs almost exclusively in women, leading to the hypothesis that dysregulated E_2 signaling plays a key role in the pathogenesis of lymphangioleiomyomatosis. We and others have shown that mutational inactivation of both copies of the *TSC1* or *TSC2* genes (consistent with the "two-hit" tumor suppressor gene model) could cause lymphangioleiomyomatosis (45, 46), but the proportion of lymphangioleiomyomatomas in which this occurs is unknown. It was recently proposed that in cells with mutational inactivation of one copy of TSC2, inactivation of the remaining wild-type tuberin could be mediated by tuberin phosphorylation rather than TSC2 mutation, leading to tumorigenesis (21, 47, 48). Given our results, phosphorylation and inactivation of tuberin as a result of E_2-stimulation could act as the "second hit," and account in part for the strong predisposition towards lymphangioleiomyomatosis in females. Further studies are needed to test this hypothesis in lymphangioleiomyomatosis-derived cells. Additional estrogen-linked mechanisms may also be involved in the pathogenesis of lymphangioleiomyomatosis because tuberin has been found to interact with ER-α and to function *in vitro* as a transcriptional corepressor of the ER (49–51).

In summary, using MCF-7 breast cancer cells as a model, we found that E_2 signals via tuberin and Rheb to activate mTOR and induce E_2-driven cell proliferation. The clinical significance of these data in breast cancer and lymphangioleiomyomatosis needs to be evaluated in further studies. The apparent role of Rheb in E_2-induced growth signaling positions Rheb as a potential novel molecular target for the treatment of hormone-dependent breast cancer.

Acknowledgments

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