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mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling[▽]

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The mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase that forms two functionally distinct complexes important for nutrient and growth factor signaling. While mTOR complex 1 (mTORC1) regulates mRNA translation and ribosome biogenesis, mTORC2 plays an important role in the phosphorylation and subsequent activation of Akt. Interestingly, mTORC1 negatively regulates Akt activation, but whether mTORC1 signaling directly targets mTORC2 remains unknown. Here we show that growth factors promote the phosphorylation of Rictor (rapamycin-insensitive companion of mTOR), an essential subunit of mTORC2. We found that Rictor phosphorylation requires mTORC1 activity and, more specifically, the p70 ribosomal S6 kinase 1 (S6K1). We identified several phosphorylation sites in Rictor and found that Thr1135 is directly phosphorylated by S6K1 *in vitro* and *in vivo*, in a rapamycin-sensitive manner. Phosphorylation of Rictor on Thr1135 did not affect mTORC2 assembly, kinase activity, or cellular localization. However, cells expressing a Rictor T1135A mutant were found to have increased mTORC2-dependent phosphorylation of Akt. In addition, phosphorylation of the Akt substrates FoxO1/3a and glycogen synthase kinase 3α/β (GSK3α/β) was found to be increased in these cells, indicating that S6K1-mediated phosphorylation of Rictor inhibits mTORC2 and Akt signaling. Together, our results uncover a new regulatory link between the two mTOR complexes, whereby Rictor integrates mTORC1-dependent signaling.

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved phosphatidylinositol 3-kinase (PI3K)-related Ser/Thr kinase that integrates signals from nutrients, energy sufficiency, and growth factors to regulate cell growth as well as organ and body size in a variety of organisms (reviewed in references 4, 38, 49, and 77). mTOR was discovered as the molecular target of rapamycin, an antifungal agent used clinically as an immunosuppressant and more recently as an anticancer drug (5, 20). Recent evidence indicates that deregulation of the mTOR pathway occurs in a majority of human cancers (12, 18, 25, 46), suggesting that rapamycin analogs may be potent antineoplastic therapeutic agents.

mTOR forms two distinct multiprotein complexes, the rapamycin-sensitive and -insensitive mTOR complexes 1 and 2 (mTORC1 and mTORC2), respectively (6, 47). In cells, rapamycin interacts with FKBP12 and targets the FKBP12-rapamycin binding (FRB) domain of mTORC1, thereby inhibiting some of its function (13, 40, 66). mTORC1 is comprised of the mTOR catalytic subunit and four associated proteins, Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with sec13 protein 8), PRAS40 (proline-rich Akt substrate of 40 kDa), and Deptor (28, 43, 44, 47, 59, 73, 74). The small GTPase Rheb (Ras homolog enriched in brain) is a key upstream activator of mTORC1 that is negatively regulated by the tuberous sclerosis complex 1 (TSC1)/TSC2 GTPase-activating protein complex (reviewed in reference 35). mTORC1 is activated by PI3K and Ras signaling through direct phosphorylation and inactivation of TSC2 by Akt, extracellular signal-regulated kinase (ERK), and p90 ribosomal protein S6 kinase (RSK) (11, 37, 48, 53, 63). mTORC1 activity is also regulated at the level of Raptor. Whereas low cellular energy levels negatively regulate mTORC1 activity through phosphorylation of Raptor by AMP-activated protein kinase (AMPK) (27), growth signaling pathways activating the Ras/ERK pathway positively regulate mTORC1 activity through direct phosphorylation of Raptor by RSK (10). More recent evidence has also shown that mTOR itself positively regulates mTORC1 activity by directly phosphorylating Raptor at proline-directed sites (20a, 75). Countertransport of amino acids (55) and amino acid signaling through the Rag GTPases were also shown to regulate mTORC1 activity (45, 65). When activated, mTORC1 phosphorylates two main regulators of mRNA translation and ribosome biogenesis, the AGC (protein kinase A, G, and C) family kinase p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and thus stimulates protein synthesis and cellular growth (50, 60).

The second mTOR complex, mTORC2, is comprised of mTOR, Rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated mitogen-activated protein kinase-interacting protein 1), mLST8, PRR5 (proline-rich region 5), and Deptor (21, 39, 58, 59, 66, 76, 79). Rapamycin does not directly target and inhibit mTORC2, but long-term treatment with this drug was shown to correlate with mTORC2 disassembly and cytoplasmic accumulation of Rictor (21, 39, 62, 79). Whereas mTORC1 regulates hydrophobic motif phosphorylation of S6K1, mTORC2 has been shown to phosphorylate other members of the AGC family of kinases. Biochemical and genetic evidence has demonstrated that mTORC2 phosphorylates Akt at Ser473 (26, 39, 68, 70), thereby contrib-

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uting to growth factor-mediated Akt activation (6, 7, 52). Deletion or knockdown of the mTORC2 components mTOR, Rictor, mSin1, and mLST8 has a dramatic effect on mTORC2 assembly and Akt phosphorylation at Ser473 (26, 39, 79). mTORC2 was also shown to regulate protein kinase Cα (PKCα) (26, 66) and, more recently, serum- and glucocorticoid-induced protein kinase 1 (SGK1) (4, 22). Recent evidence implicates mTORC2 in the regulation of Akt and PKCα phosphorylation at their turn motifs (19, 36), but whether mTOR directly phosphorylates these sites remains a subject of debate (4).

Activation of mTORC1 has been shown to negatively regulate Akt phosphorylation in response to insulin or insulin-like growth factor 1 (IGF1) (reviewed in references 30 and 51). This negative regulation is particularly evident in cell culture models with defects in the TSC1/TSC2 complex, where mTORC1 and S6K1 are constitutively activated. Phosphorylation of insulin receptor substrate-1 (IRS-1) by mTORC1 (72) and its downstream target S6K1 has been shown to decrease its stability and lead to an inability of insulin or IGF1 to activate PI3K and Akt (29, 69). Although the mechanism is unknown, platelet-derived growth factor receptor β (PDGF-Rβ) has been found to be downregulated in TSC1- and TSC2-deficient murine embryonic fibroblasts (MEFs), contributing to a reduction of PI3K signaling (80). Interestingly, inhibition of Akt phosphorylation by mTORC1 has also been observed in the presence of growth factors other than IGF-1, insulin, or PDGF, suggesting that there are other mechanisms by which mTORC1 activation restricts Akt activity in cells (reviewed in references 6 and 31). Recent evidence demonstrates that rapamycin treatment causes a significant increase in Rictor electrophoretic mobility (2, 62), suggesting that phosphorylation of the mTORC2 subunit Rictor may be regulated by mTORC1 or downstream protein kinases.

Herein, we demonstrate that Rictor is phosphorylated by S6K1 in response to mTORC1 activation. We demonstrate that Thr1135 is directly phosphorylated by S6K1 and found that a Rictor mutant lacking this phosphorylation site increases Akt phosphorylation induced by growth factor stimulation. Cells expressing the Rictor T1135A mutant were found to have increased Akt signaling to its substrates compared to Rictor wild-type- and T1135D mutant-expressing cells. Together, our results suggest that Rictor integrates mTORC1 signaling via its phosphorylation by S6K1, resulting in the inhibition of mTORC2 and Akt signaling.

MATERIALS AND METHODS

DNA constructs and recombinant proteins. The plasmids encoding hemagglutinin (HA)-tagged murine Rictor and myc-tagged human Rictor were provided by Estela Jacinto (UMDNJ, NJ) and David Sabatini (MIT, MA), respectively, and described previously (40, 43). All human and murine Rictor mutants were generated using the QuikChange methodology (Stratagene, La Jolla, CA). The vectors encoding HA-tagged wild-type, constitutively activated (F5A-T389E-R3A), and kinase-inactive (K100R) S6K1 and Flag-tagged Rheb were provided by John Blenis (Harvard Medical School, MA) and have been described previously (32, 71). The vector encoding glutathione *S*-transferase (GST) in fusion with kinase-inactive Akt for mammalian cell expression was kindly provided by Dario Alessi (University of Dundee, United Kingdom). To subclone murine Rictor in pBabe-puro and produce retroviral particles used in the generation of stable cell lines, HA-tagged wild-type and mutant Rictor were amplified by PCR using primers 5′-AAG AAT TAC GTA ATG GCT TCT AGC TAT CCT TAT GAC GTG CCT and 5′-AAG AAT TAC GTA TCA GGA TTC AGC AGA CTC ATC AAC TAT AGG. To subclone human Rictor in pGEX-2T, myc-tagged Rictor was amplified by PCR using primers 5′-TTG GAT CCG ATC CAA AAG GAG GAA AAC TGT CAT CTG AAA AT and 5′-TTG AAT TCT CAA GGC TCT AGC TGT AAT GAT TTC TGT GCT GAG GA. Recombinant fragments of human wild-type Rictor or the T1135A mutant in frame with GST, corresponding to amino acids (aa) 1115 to 1160, were expressed in BL-21 cells and purified using glutathione-Sepharose beads (GE Healthcare, Piscataway, NJ).

Antibodies. Antibodies targeted against RXRXXpS/T consensus sequences, Rictor (for immunoblotting), S6, phospho-S6 (S240/244), Akt, phospho-Akt (S473), S6K1 and phospho-S6K1 (T389), phospho-glycogen synthase kinase 3α/β (phospho-GSK3α/β) (S21/9), FoxO1/3a, and phospho-FoxO1/3a (T24/T32) were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies targeted against phospho-NDRG1 (phospho-N-myc downstream-regulated gene 1) (Thr346/356/366) were purchased from Kinasource (Dundee, United Kingdom). The Rictor antibodies used for immunoprecipitation were purchased from Bethyl Laboratories (Montgomery, TX). Anti-HA, -myc, -Flag, and phospho-ERK1/2 monoclonal antibodies were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The phospho-RSK (Ser380) antibody was purchased from R&D Systems (Minneapolis, MN). The rabbit phospho-Rictor (T1135) polyclonal antibody was generated and affinity purified in collaboration with Genscript (Piscataway, NJ). All secondary horseradish peroxidase (HRP)-conjugated antibodies used for immunoblotting were purchased from Chemicon (Temecula, CA).

Cell culture and transfection. HEK293, HeLa, and MEF cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose supplemented with 10% fetal bovine serum (FBS) and antibiotics. The $p53^{-/-}$ and $p53^{-/-}$ $TSC2^{-/-}$ MEFs were a generous gift from David Kwiatkowski (Brigham and Women's Hospital, Boston, MA). HEK293 stable cell lines were generated using pBabe-puro-derived retroviral particles, and expressing cells were selected using puromycin (2 μg/ml). HEK293 cells were transfected by calcium phosphate precipitation as previously described (64). Cells were grown for 24 h after transfection and serum starved using serum-free DMEM where indicated for 16 to 18 h. Starved cells were pretreated with wortmannin (100 nM), U0126 (10 μM), rapamycin (100 nM), or PI-103 (1 μM) (Biomol, Plymouth Meeting, PA) and stimulated with FBS (10%), insulin (25 to 100 nM), epidermal growth factor (EGF) (25 to 50 ng/ml), or phorbol 13-myristate 12-acetate (PMA) (100 ng/ml) for 5 to 20 min before being harvested. Unless indicated otherwise, all drugs and growth factors were purchased from Invitrogen (Burlington, Ontario, Canada).

Flow cytometry. For analysis of Akt phosphorylation using fluorescence-activated cell sorting (FACS), 2.5×10^6 cells stably expressing HA-tagged wild-type Rictor or the T1135A and T1135D mutants were seeded in 100-mm dishes, grown in 10% FBS for 24 h, serum-starved overnight, and stimulated with EGF (25 ng/ml) for 5 min. Cells were harvested in phosphate-buffered saline (PBS)-EDTA (1 mM), rinsed by centrifugation, and fixed in 3.7% formaldehyde for 10 min at 37°C. The fixative was removed by centrifugation and cells permeabilized in 90% methanol for 30 min at 4°C. Cells were washed thrice and blocked in washing buffer (PBS-bovine serum albumin [BSA] [0.5%]) for 10 min at room temperature. Cells were incubated for 1 h with anti-HA (HA.11) (Covance) and anti-phospho-Akt (S473) (Cell Signaling) antibodies diluted in washing buffer according to the manufacturer's recommendation. Cells were washed thrice and incubated for 30 min with R-phycoerythrin (PE)-conjugated anti-rabbit (Invitrogen) and fluorescein isothiocyanate (FITC)-conjugated AffiniPure anti-mouse (Jackson ImmunoResearch Laboratories, Baltimore, MD) secondary antibodies diluted in washing buffer according to the manufacturer's recommendation. Cells were washed thrice, resuspended in PBS, and processed on a BD FACS Canto II cytometer, and data were analyzed using BD FACSDiva software (BD Bioscience). Briefly, single HA-expressing cells were sorted and analyzed for phospho-Akt (S473) immunoreactivity. The percentage of cells positive for phospho-Akt (S473) was quantified for each sample and divided by the percentage of positive cells in stimulated HA-tagged Rictor T1135A-expressing cells. All values were divided by unstimulated empty-vector-transfected cells so that the value for unstimulated empty-vector-transfected cells was 1. Results are representative of at least three independent experiments and are expressed as the mean ± standard deviation (SD) for duplicates.

RNA interference (RNAi) and viral infections. For small interfering RNA (siRNA)-mediated knockdown of S6K1 and Raptor, validated 21-nucleotide cRNAs with symmetrical two nucleotide overhangs were obtained from Qiagen (Valencia, CA). Predesigned Dicer-substrate siRNA duplexes against the 3′ untranslated region (3′UTR) of human Rictor (HSC.RNAI.N152756.10.4) were purchased from Integrated DNA Technologies (Coralville, IA). HEK293 cells were transfected using calcium phosphate and 50 nM siRNA per 35-mm dish. Transfection efficiency was determined to be greater than 90% using a fluorescently labeled mock siRNA. At 24 hours following transfection, cells were serum

starved for 16 to 18 h and stimulated with growth factors. For short hairpin RNA (shRNA)-mediated knockdown of endogenous S6K1, lentiviruses were produced using the pLKO.1 vector system from the MISSION TRC shRNA library. Cells were infected in the presence of 4 μg/ml Polybrene, and 3 days after viral infection, cells were treated and selected with 2 μg/ml puromycin. shRNA constructs were obtained from Sigma-Aldrich (shRNA1, TRCN0000003158; shRNA2, TRCN0000003169). Retroviruses were produced using the pBabe-puro vector system to overexpress ectopic murine wild-type Rictor or the T1135A and T1135D mutants. Two days after viral infection, positive pools were selected in 2 μg/ml puromycin.

Immunoprecipitations and immunoblotting. Cell lysates were prepared as previously described (63). Briefly, cells were washed with ice-cold PBS and lysed in 10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride, and a Complete protease inhibitor cocktail tablet (Roche). For immunoprecipitations, cell lysates were incubated with the indicated antibody or control IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h, followed by 1 h of incubation with protein A–Sepharose CL-4B beads (GE Healthcare). Unless used for kinase assays, immunoprecipitates were washed thrice in lysis buffer and beads were eluted and boiled in 2× reducing sample buffer (5× is 60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue). Eluates and total cell lysates were subjected to 10 to 12% SDS-PAGE, and resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for immunoblotting. For mTOR coimmunoprecipitation assays, the same procedure was followed, except cells were lysed in CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} buffer (40 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.3% CHAPS). Beads from immunoprecipitations were washed thrice in CHAPS buffer and eluted in reducing sample buffer.

Protein phosphotransferase assays. For S6K1 assays, beads from immunoprecipitations were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 5 mM β-glycerophosphate). Kinase assays were performed with bacterially purified recombinant GST-Rictor fusion proteins (3 μg per assay) or immunopurified full-length HA-tagged Rictor as substrates, under linear assay conditions. Assays were performed for 10 min at 30°C in kinase buffer supplemented with 5 μCi [γ-^{32}P]ATP. For mTORC2 kinase assays, endogenous or transfected HA-tagged Rictor was immunoprecipitated from cells lysed in CHAPS buffer containing 120 mM NaCl, as previously described (23). Immunoprecipitates were washed thrice in lysis buffer and incubated for 30 min at 30°C with 500 ng purified GST-Akt (kinase inactive) in mTORC2 kinase buffer (10 mM HEPES [pH 7.4], 100 μM ATP, 25 mM β-glycerophosphate, 10 mM $MgCl_2$). All samples were subjected to SDS-PAGE, and incorporation of cold or radioactive [^{32}P]phosphate was determined by immunoblotting or autoradiography using a Fuji PhosphorImager with Multi-Gauge V3.0 software, respectively. The data presented are representative of at least three independent experiments.

Proliferation assays. For proliferation assays, 3×10^3 cells expressing wild-type Rictor or the T1135A or T1135D mutant were seeded in 96-well plates. Cells were grown at 37°C in DMEM with 4.5 g/liter glucose supplemented with 5% FBS and antibiotics. The relative number of viable cells was measured every 24 h during five consecutive days using a nonradioactive cell proliferation assay from Promega (Madison, WI). Briefly, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling reagent (phenazine methosulfate) were added to each well for a 4-h incubation according to the manufacturer's recommendation. Absorbance was measured at 490 nm using a Tecan GENios Plus microplate reader, and results displayed represent the mean of triplicates ± standard error (SE).

Confocal microscopy. For immunofluorescence analyses, 3.5×10^5 cells were seeded in six-well plates containing coverslips. Cells were stimulated as indicated, washed in PBS, and fixed in 3.7% formaldehyde for 20 min at room temperature. Cells were washed for 5 min in PBS and permeabilized for 1 h in PBS containing 0.2% Triton X-100 and 2 mg/ml BSA. Cells were washed thrice in washing buffer (0.04% Triton X-100 and 2 mg/ml BSA in PBS) and incubated overnight with anti-HA (HA.11) antibodies diluted in washing buffer. Cells were washed thrice and incubated 2 h with Alexa 488-conjugated anti-mouse (Invitrogen) secondary antibodies diluted in washing buffer. Cells were washed thrice and stained 2 min with DAPI (4′,6′-diamidino-2-phenyindole, dilactate) at 1 μg/ml. Coverslips were mounted on slides, and images from single confocal sections (0.7 μm) were acquired with a Zeiss LSM 510 Meta laser scanning confocal microscope using a Plan Apochromat 100× objective. All images were acquired using identical parameters.

MS. To identify phosphorylation sites in Rictor, endogenous and exogenous Rictor were immunoprecipitated from approximately 5×10^7 HEK293 cells stimulated with insulin or PMA. Following SDS-PAGE, Coomassie blue-stained gel bands corresponding to immunoprecipitated HA-tagged or endogenous Rictor were excised and subjected to in-gel trypsin digestion, as described previously (10). The resultant peptides were extracted, desalted with a C_{18} StageTip, and subjected to microcapillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LTQ-orbitrap high-resolution hybrid mass spectrometer (ThermoScientific). All candidate MS/MS spectra were manually inspected.

RESULTS

Identification of Rictor as a target of mTORC1 signaling. To analyze the possible regulation of Rictor through phosphorylation, we used an antibody that recognizes the phosphorylated consensus motif Arg/Lys-X-Arg/Lys-X-X-pSer/Thr (RXRXXpS/T, where X is any amino acid), which is often found in substrates of AGC kinases, including RSK, Akt, and S6K1 (3). This approach was successfully used by several groups to identify new substrates for these basophilic kinases (1, 10, 42, 53, 63). HEK293 cells transfected with HA-tagged Rictor were stimulated with different agonists and inhibitors (Fig. 1A), and immunoprecipitated Rictor was analyzed for phosphorylation by immunoblotting with the anti-RXRXXpS/T antibody. Using this method, we found that treatment of serum-starved cells with the phorbol ester PMA, epidermal growth factor (EGF), serum, and insulin strongly stimulated Rictor phosphorylation on RXRXXpS/T consensus sites (Fig. 1B). We observed phosphorylation of Rictor irrespective of whether the Ras/ERK or PI3K/Akt pathway was stimulated, as shown by the phosphorylation of ERK1/2 (Thr202/Tyr204) or Akt (Ser473), suggesting that these pathways may converge on the same downstream Rictor kinase. Both Ras/ERK and PI3K/Akt pathways promote mTORC1 activation through inactivation of the TSC1/2 tumor suppressor complex (11, 37, 48, 53, 63), indicating that Rictor phosphorylation may occur in an mTORC1-dependent manner.

To test this, we used wortmannin and U0126 to specifically inhibit PI3K- and MEK1/2-dependent signaling, respectively. While treatment of cells with wortmannin completely inhibited Rictor phosphorylation induced by insulin stimulation (Fig. 1C), we found that U0126 treatment strongly inhibited Rictor phosphorylation induced by PMA (Fig. 1D). Importantly, Rictor phosphorylation at RXRXXpS/T consensus sites was completely abrogated when cells were treated with the mTORC1 inhibitor rapamycin prior to insulin (Fig. 1C) or PMA (Fig. 1D) stimulation, suggesting the involvement of a basophilic kinase downstream of mTORC1. These results were confirmed in two different cell lines (HEK293 and HeLa) by looking at the phosphorylation status of endogenous Rictor (Fig. 1E and F). Together, these findings demonstrate that Rictor is targeted for phosphorylation on RXRXXpS/T consensus sites in an mTORC1-dependent manner, suggesting the involvement of the AGC family kinase S6K1 (Fig. 1A).

S6K1 is required for Rictor phosphorylation in cells. To determine the role of S6K1 in Rictor phosphorylation, we used small interfering RNA (siRNA)-mediated RNAi to specifically reduce expression of S6K1 in HEK293 cells. We found that siRNA-mediated knockdown of S6K1 resulted in complete inhibition of Rictor phosphorylation on RXRXXpS/T consensus sites (Fig. 2A), demonstrating that endogenous S6K1 me-

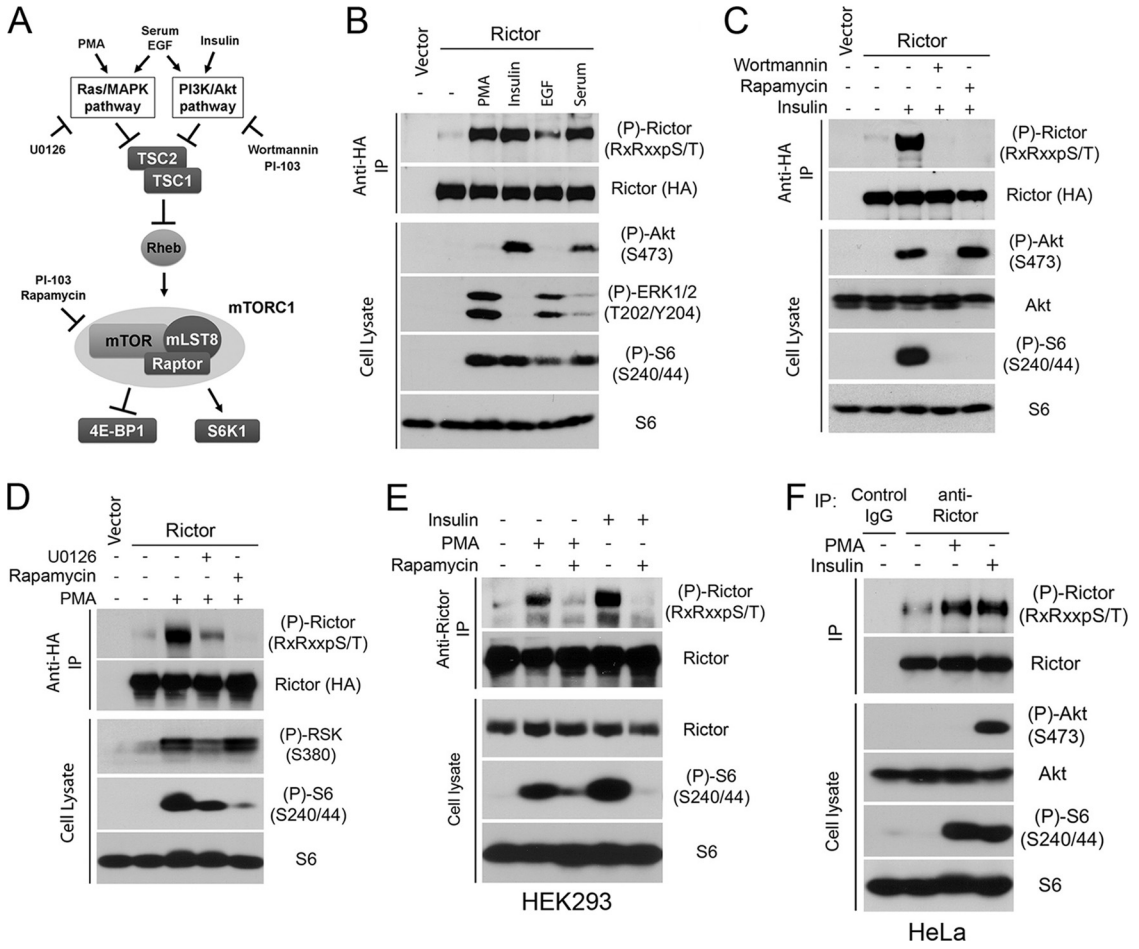

FIG. 1. Identification of Rictor as a target of mTORC1 signaling. (A) Schematic representation of the agonists and pharmacological inhibitors used in this study. (B) HEK293 cells were transfected with empty vector or HA-tagged Rictor; serum starved overnight; and stimulated for 10 or 20 min with PMA (50 ng/ml), insulin (100 nM), EGF (50 ng/ml), or fetal bovine serum (10%). Immunoprecipitated (IP) Rictor was then assayed for phosphorylation with a phospho-motif antibody that recognizes the RXRXXpS/T consensus motif. (C and D) As for panel B, except cells were pretreated with U0126 (20 μM), rapamycin (100 nM), or wortmannin (100 nM) for 30 min prior to PMA or insulin stimulation. (E and F) Endogenous Rictor was immunoprecipitated from HEK293 (E) or HeLa (F) cells and assayed as for panel B.

diates Rictor phosphorylation in response to insulin and PMA stimulation. The role of endogenous S6K1 was also determined by generating stable cell lines expressing either a short hairpin RNA (shRNA) against a different target sequence of S6K1 or a scrambled shRNA control (Fig. 2B). Using this approach, we found that stable knockdown of S6K1 expression resulted in a robust inhibition of Rictor phosphorylation upon insulin stimulation, indicating that S6K1 is essential for Rictor phosphorylation following mTORC1 activation.

To further assess the role of S6K1 in Rictor phosphorylation in cells, Rictor was transfected with wild-type S6K1 or constitutively activated (F5A-T389E-R3A) and kinase-inactive (K100R) mutants of S6K1 (Fig. 3A). Compared to control vector, expression of wild-type S6K1 robustly increased Rictor phosphorylation in response to insulin stimulation (Fig. 3B), suggesting that Rictor is an S6K1 substrate in cells. Expression of constitutively activated S6K1 (CA) resulted in robust Rictor phosphorylation even in the absence of serum and insulin stimulation. This increase was insensitive to rapamycin treatment (Fig. 3C), consistent with the idea that S6K1 is sufficient to stimulate Rictor phosphorylation in cells. S6K1 phosphotransferase activity was found to be required for Rictor phosphorylation, as kinase-inactive S6K1 (KD) did not increase Rictor phosphorylation above the level already stimulated by endogenous S6K1 activity (Fig. 3B). In fact, kinase-inactive S6K1 was found to partly reduce Rictor phosphorylation in cells (to the same extent as the observed reduction in S6 phosphorylation), suggesting that this inactive form of S6K1 partly acts as a dominant negative allele. Together, these findings strongly support a role for S6K1 in the phosphorylation of Rictor *in vivo*.

To determine whether Rheb-mediated activation of mTORC1 was sufficient to stimulate Rictor phosphorylation in the absence of growth factors and serum, HEK293 cells were transfected with the small GTPase Rheb and serum starved overnight. Similar to the case for cells expressing constitutively activated S6K1, we found that transient overexpression of Rheb strongly stimulated both S6 and Rictor phosphorylation in the absence of serum and growth factors (Fig. 3D). Furthermore, Rheb-mediated Rictor phosphorylation was completely inhibited by rapamycin treat-

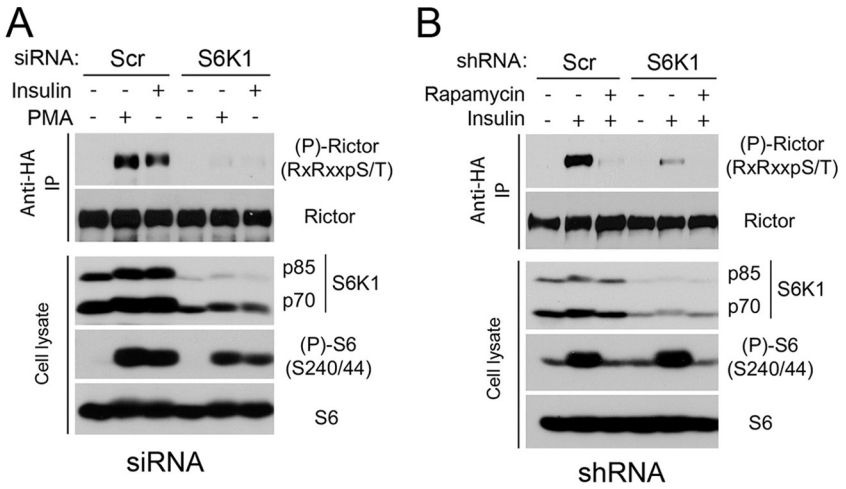

FIG. 2. S6K1 is required for Rictor phosphorylation in cells. (A) HEK293 cells were cotransfected with HA-tagged Rictor and siRNA duplexes targeted against a scrambled sequence (Scr) or human S6K1. Cells were serum starved overnight and stimulated with PMA (50 ng/ml) or insulin (100 nM) for 20 min. Immunoprecipitated Rictor was then assayed for phosphorylation with the anti-RXRXXpS/T antibody. (B) HEK293 cells stably expressing an shRNA targeting a scrambled sequence (Scr) or S6K1 were transfected with HA-tagged Rictor, serum starved overnight, and treated with rapamycin (100 nM) for 30 min prior to insulin (100 nM) stimulation for 20 min. Immunoprecipitated Rictor was assayed as for panel A.

ment, indicating that mTORC1 activation was both necessary and sufficient to promote Rictor phosphorylation in cells.

S6K1 phosphorylates Rictor on Thr1135 *in vivo* and *in vitro*. Next, we analyzed the sequence surrounding Ser/Thr residues within Rictor for similarities to phosphorylation sites in known substrates of S6K1 (Fig. 4A). Four Ser/Thr residues (Ser21, Ser1113, Thr1135, and Ser1219) were found to fit the R/KXRXXpS/T consensus sequence, and two of these sites (Ser21 and Ser1219) were previously identified as phosphorylated in four large-scale mass spectrometry (MS) studies (9, 15, 16, 57). To further examine the phosphorylation status of Rictor in cells, we performed MS/MS analyses of immunoprecipitated endogenous and exogenous Rictor from cells that were stimulated with insulin or PMA. Interestingly, we identified a total of 13 phosphorylation sites in Rictor, corresponding to Ser21, Ser265, Ser1035, Ser1037, Thr1135, Ser1217, Ser1219,

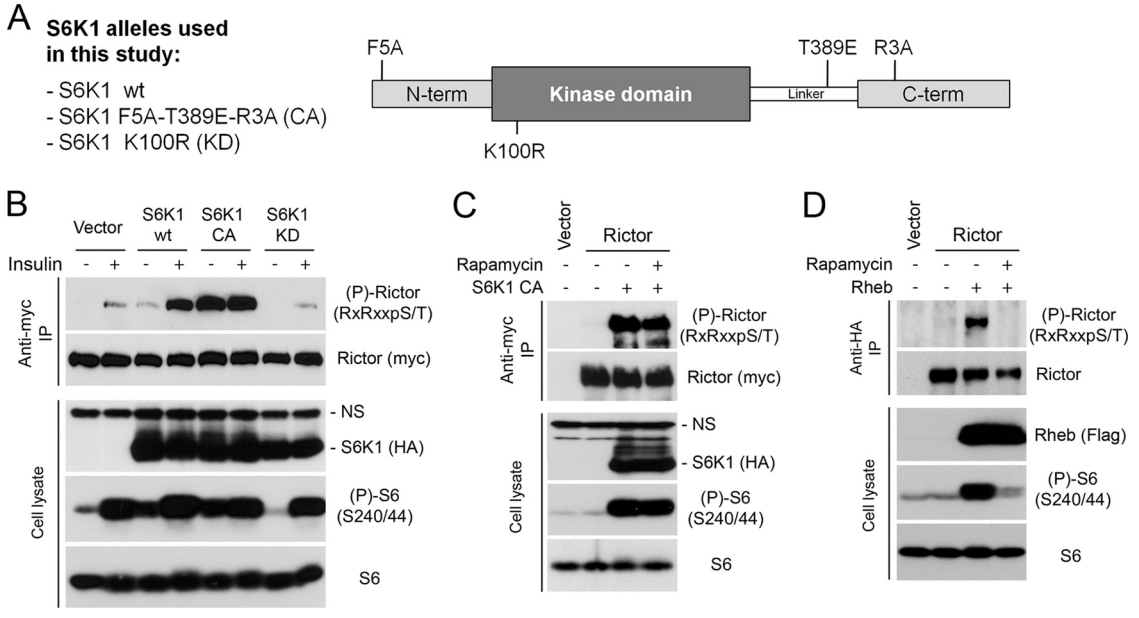

FIG. 3. S6K1 is sufficient to promote Rictor phosphorylation in cells. (A) Schematic representation of S6K1 constructs used in this study. Constitutively activated S6K1 (CA) consists of F5A, T389E, and R410/413/414A (R3A) mutations. The kinase-inactive allele of S6K1 (KD) consists of a K100R mutation in subdomain II of the kinase domain. (B) HEK293 cells were cotransfected with myc-tagged Rictor and constructs expressing HA-tagged wild-type (wt), constitutively activated (CA), or kinase-inactive (KD) S6K1. Cells were serum starved overnight and treated with insulin (100 nM) for 20 min prior to harvesting. The phosphorylation of Rictor was assayed in anti-myc immunoprecipitates. (C and D) As for panel B, except S6K1 CA- and Rheb-expressing cells were pretreated with rapamycin (100 nM) for 30 min prior to harvesting.

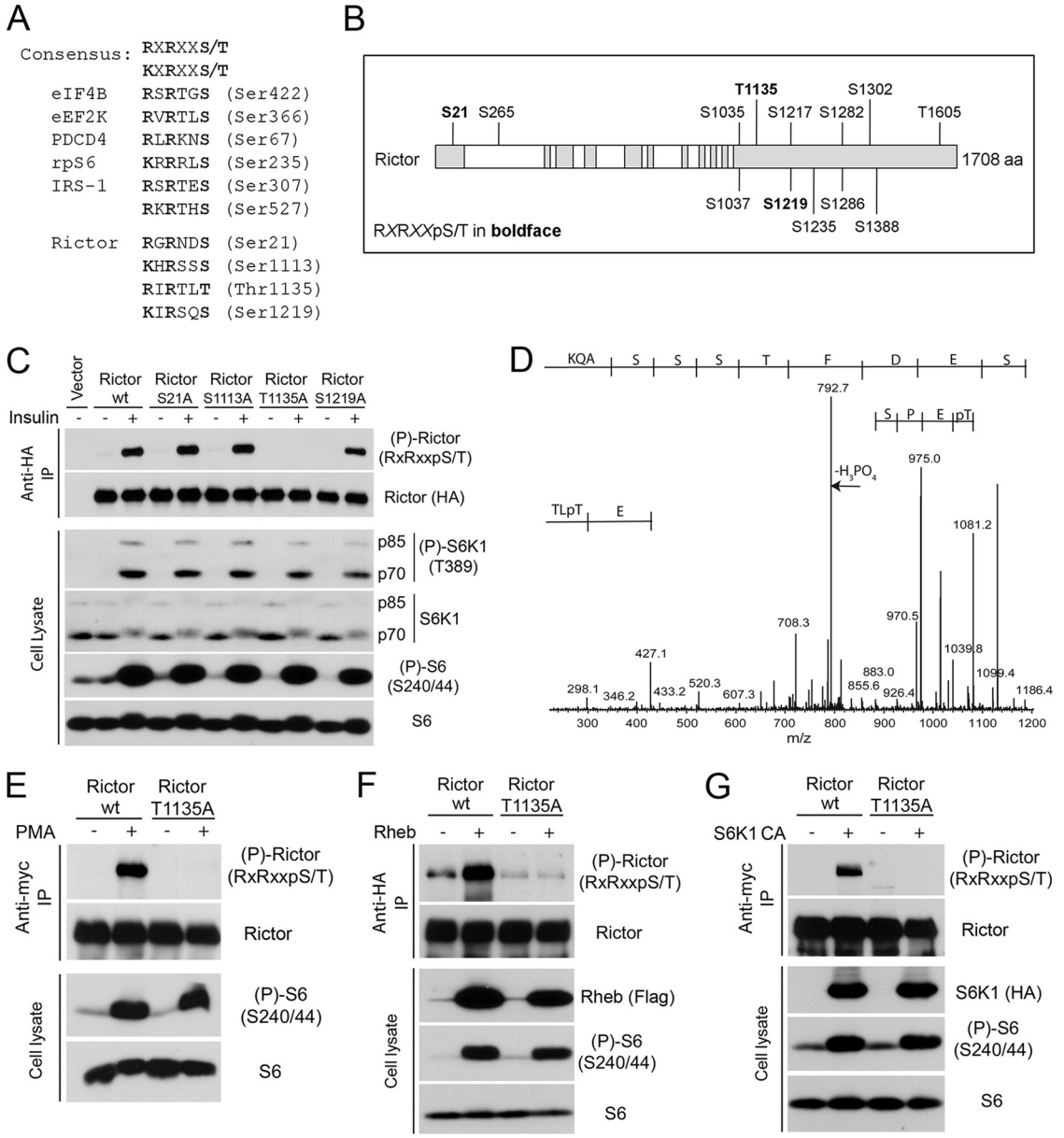

FIG. 4. Identification of Thr1135 as a rapamycin-sensitive phosphorylation site in Rictor. (A) Phosphorylation sites in known S6K1 substrates fit the K/RXRXXpS/T consensus. Rictor contains four residues fitting this consensus, corresponding to Ser21, Ser1113, Thr1135, and Ser1219. (B) MS/MS analyses of immunoprecipitated endogenous and exogenous Rictor from HEK293 cells revealed 13 phosphorylation sites, three of which are located within the K/RXRXXpS/T consensus motif (Ser21, Thr1135, and Ser1219). The white area identifies region of highest conservation between Rictor orthologs. (C) HEK293 cells were transfected with wild-type Rictor or potential S6K1 phosphorylation site mutants S21A, S1113A, T1135A, and S1219A; serum starved overnight; and stimulated with insulin (100 nM) for 20 min. Immunoprecipitated Rictor was then immunoblotted for phosphorylation at RXRXXpS/T sites. (D) Tandem mass spectrum of the precursor at *m/z* 825.03+, corresponding to the phosphopeptide TL(pT)EPSVDLNHSEDFTSSSAQK from murine Rictor. (E) As for panel C, except cells were stimulated with PMA (50 ng/ml) and assayed for Rictor phosphorylation by immunoblotting. (F and G) HEK293 cells were transfected with wild-type Rictor and wild-type Rheb (F) or constitutively activated S6K1 (G). Cells were serum starved overnight, and immunoprecipitated Rictor was immunoblotted for phosphorylation at RXRXXpS/T sites.

Ser1235, Ser1282, Ser1286, Ser1302, Ser1388, and Thr1605. Of these, three phosphopeptides contained R/KXRXXpS/T consensus sequences, corresponding to phosphorylated Ser21, Thr1135, and Ser1219 (shown in boldface in Fig. 4B). To identify the residue(s) phosphorylated by S6K1, we mutated Ser21, Ser1113, Thr1135, and Ser1219 to unphosphorylatable alanine residues and transfected these mutants in HEK293 cells. While mutation of Ser21, Ser1113, and Ser1219 did not significantly alter Rictor phosphorylation, we found that mutation of Thr1135 completely prevented the phosphorylation of Rictor induced by insulin treatment (Fig. 4C). Identification of phosphorylated Thr1135 was obtained by MS/MS sequencing, and the assignment is shown for this residue in the corresponding tryptic peptide (Fig. 4D). We also determined that Thr1135 is

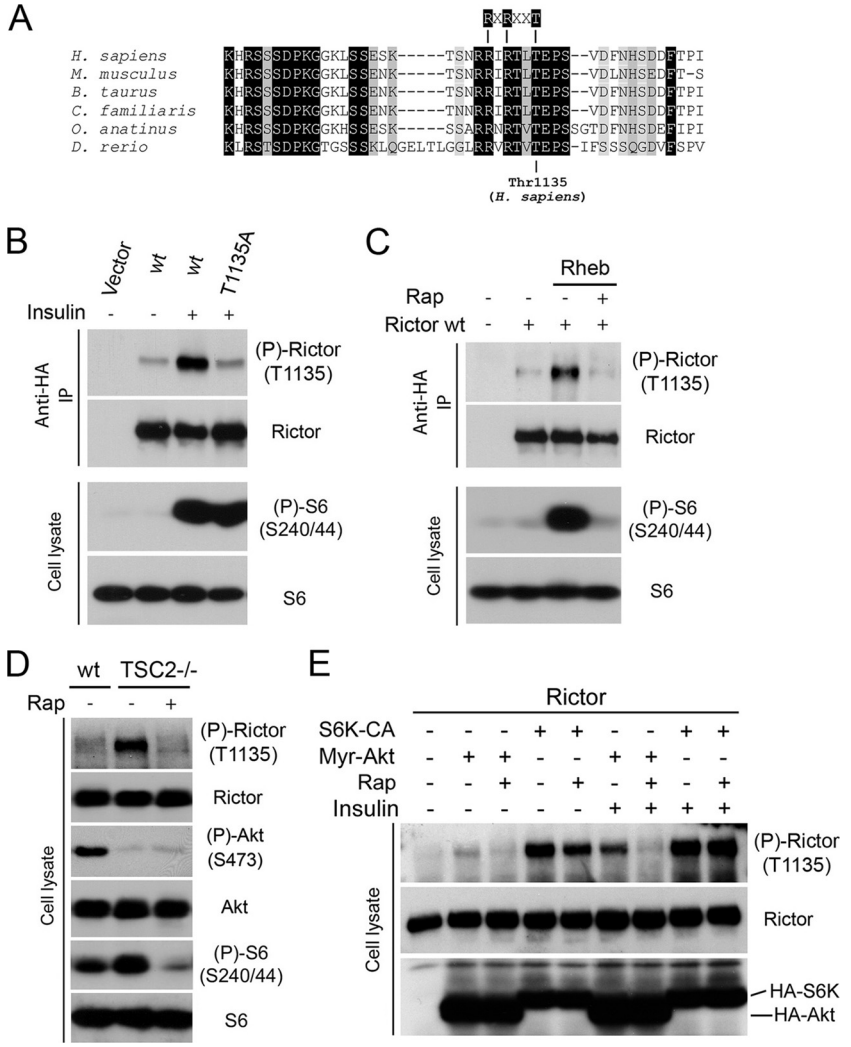

FIG. 5. Detection of Rictor phosphorylation at Thr1135 using a novel phosphospecific antibody. (A) Primary sequence alignment showing conservation of Thr1135 as well as −3 and −5 basic residues within Rictor from different vertebrate species. (B) A phosphospecific antibody against Thr1135 was generated and tested on immunoprecipitated Rictor. HEK293 cells were transfected with wild-type Rictor or a phosphorylation site mutant, serum starved overnight and stimulated with insulin (100 nM) for 20 min. Rictor immunoprecipitates were assayed using the phospho-Thr1135 antibody. (C) HEK293 cells were transfected with wild-type Rictor and Rheb, serum starved overnight, and treated with rapamycin (20 nM) for 30 min where indicated. Rictor phosphorylation was assayed using the phospho-T1135 antibody. (D) $TSC2^{+/+}$ or $TCS2^{-/-}$ MEFs were grown in the absence of serum and treated with rapamycin (100 nM) for 30 min where indicated. Endogenous Rictor was assayed for phosphorylation using the phospho-Thr1135 antibody. (E) HEK293 cells stably expressing HA-tagged wild-type Rictor were transfected with either constitutively activated Akt (Myr-Akt) or constitutively activated S6K1 (S6K-CA). Cells were serum starved overnight, treated with rapamycin (100 nM), and stimulated with insulin (100 nM) where indicated. Rictor phosphorylation was assayed using the phospho-T1135 antibody.

the main potential S6K1 site within Rictor that is phosphorylated in cells treated with PMA (Fig. 4E), as well as cells overexpressing Rheb (Fig. 4F) or constitutively activated S6K1 (Fig. 4G).

Thr1135, and the −3 and −5 basic residues, are conserved in Rictor from vertebrate species (Fig. 5A), suggesting that phosphorylation of this residue by a basophilic kinase plays an important function. Thr1135 does not appear to be conserved in invertebrate Rictor orthologs; however, the C-terminal half of Rictor is very poorly conserved overall (Fig. 4B). To confirm the results obtained using the RXRXXpS/T phospho-motif antibody, we generated a phosphospecific antibody directed against phosphorylated Thr1135. First, the specificity of the antibody was tested on HA-tagged wild-type Rictor and the unphosphorylatable T1135A mutant immunoprecipitated from HEK293 cells. We found that the phosphospecific antibody had high specificity toward wild-type Rictor over the Rictor mutant. Indeed, insulin treatment (Fig. 5B) and Rheb overexpression (Fig. 5C) robustly increased immunoreactivity of the antibody against wild-type Rictor but not the T1135A mutant (Fig. 5B). Its recognition of wild-type Rictor was inhibited by short-term rapamycin treatment (Fig. 5C), demonstrating that Thr1135 is an mTORC1-regulated site.

We also determined the status of Rictor phosphorylation in MEFs with a constitutive activation in mTORC1. $TSC2^{-/-}$ MEFs were assayed for Rictor phosphorylation using the phos-

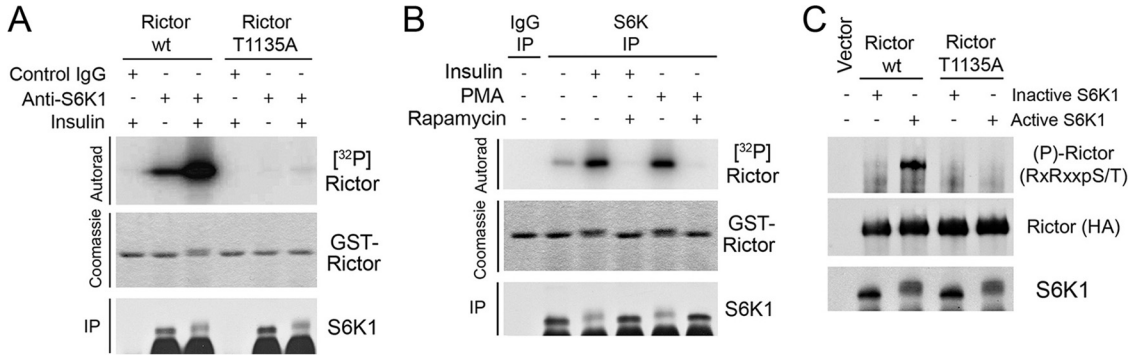

FIG. 6. S6K1 phosphorylates Rictor on Thr1135 *in vitro*. (A) S6K1 was immunoprecipitated from HEK293 cells stimulated with insulin (100 nM) and incubated in a kinase reaction with [γ-^{32}P]ATP with GST-Rictor fusion proteins containing wild-type and T1135A sequences. (B) As for panel A, except cells were pretreated with rapamycin (100 nM) for 30 min and stimulated with insulin or PMA for 20 min prior to immunoprecipitation of S6K1. (C) Immunoprecipitated wild-type or kinase-inactive S6K1 from insulin-stimulated cells was incubated with immunopurified full-length wild-type or T1135A Rictor in a kinase reaction without radioactivity. The resulting samples were subjected to SDS-PAGE and immunoblotted for Rictor phosphorylation on RXRXXpS/T consensus sites.

phospecific antibody and compared to TSC2$^{+/+}$ cells. We found that Rictor phosphorylation at Thr1135 was constitutively high in TSC2$^{-/-}$ cells and that short-term rapamycin treatment completely inhibited this phosphorylation event (Fig. 5D). The high level of Rictor phosphorylation correlated with high S6K1 activity, as shown by S6 phosphorylation at Ser240/244. Consistent with previous reports (34), Akt phosphorylation was found to be strongly decreased in TSC2$^{-/-}$ MEFs, consistent with S6K1-dependent phosphorylation of Rictor playing an inhibitory role in mTORC2.

To rule out any direct involvement of Akt in Thr1135 phosphorylation, we overexpressed constitutively activated Akt (Myr-Akt) or S6K1 (S6K1-CA) in HEK293 cells and determined the status of Rictor phosphorylation on Thr1135. Cells were serum starved overnight, treated or not with rapamycin, and stimulated with insulin. Consistent with a role for S6K1, we found that expression of activated S6K1 increased Rictor Thr1135 phosphorylation by more than 10-fold compared to that in cells expressing activated Akt in the absence or presence of insulin stimulation (Fig. 5E). Expression of activated Akt did increase Rictor phosphorylation compared to that in empty-vector-transfected cells, but rapamycin treatment completely inhibited this effect in the presence or absence of insulin. These results strongly suggest that Akt does not directly stimulate Rictor phosphorylation but promotes mTORC1-dependent phosphorylation of Rictor.

The specificity of S6K1 toward Rictor was also verified *in vitro*. Incubation of insulin-activated S6K1 immunoprecipitated from cells was found to efficiently promote ^{32}P incorporation in recombinant wild-type Rictor (Fig. 6A). Phosphorylation of Rictor was specific for Thr1135, as mutation of this residue completely inhibited S6K1-mediated ^{32}P incorporation in Rictor T1135A. Under these conditions, both insulin and PMA treatment were found to promote S6K1-mediated phosphorylation of recombinant Rictor *in vitro*, while rapamycin treatment completely blocked this effect (Fig. 6B). Full-length wild-type and T1135A mutant Rictor immunopurified from cells were also tested in a similar *in vitro* experiment without radioactivity. Using these substrates, we found that mutation of Thr1135 completely abrogated Rictor phosphorylation at RXRXXpS/T consensus sites *in vitro* (Fig. 6C). Together, these data demonstrate that S6K1 directly phosphorylates Rictor at Thr1135 both *in vivo* and *in vitro*.

A Rictor mutant that cannot be phosphorylated on Thr1135 promotes mTORC2-directed phosphorylation of Akt. The phosphorylation of Rictor by S6K1 suggests the possibility that mTORC2-directed Akt phosphorylation is negatively regulated by mTORC1 signaling. To test this, we first verified whether Raptor knockdown could trigger Akt phosphorylation at Ser473, an mTORC2-dependent site (26, 39, 68, 70). HEK293 cells were transfected with siRNA duplexes targeted against Raptor or a scramble sequence, grown in the presence of serum or serum starved overnight, and stimulated with either insulin or EGF. As reported by others (68), we found that Raptor knockdown increased Akt Ser473 phosphorylation in response to insulin but also in cells growing with serum and in response to EGF stimulation (Fig. 7A). Because acute rapamycin treatment of cells completely inhibits Rictor phosphorylation, we also determined whether Akt Ser473 phosphorylation was modulated by short-term rapamycin treatment. HEK293 cells were serum starved overnight and pretreated with rapamycin for 30 or 60 min prior to stimulation with growth factors. Consistent with a potential mTORC1-dependent negative regulation of mTORC2, we found that pretreatment of cells with rapamycin for as short a time as 30 min considerably increased Akt Ser473 phosphorylation in response to insulin, EGF (Fig. 7B), and serum (data not shown). Together, these findings support the idea that mTORC1 rapidly elicits an inhibitory signal that negatively regulates mTORC2 activity.

To address the direct physiological relevance of S6K1-mediated phosphorylation of Rictor, we determined whether mutation of Thr1135 affects Akt phosphorylation at Ser473 or SGK1-dependent NDRG1 phosphorylation at Thr346/56/66. HEK293 cells stably expressing wild-type Rictor or the T1135A and T1135D mutants were serum starved overnight, stimulated with EGF for 5 or 10 min, and assayed for Akt and NDRG1 phosphorylation by immunoblotting. While expression of wild-type Rictor elevated Akt Ser473 phosphorylation levels, we found that expression of the Rictor T1135A mutant further

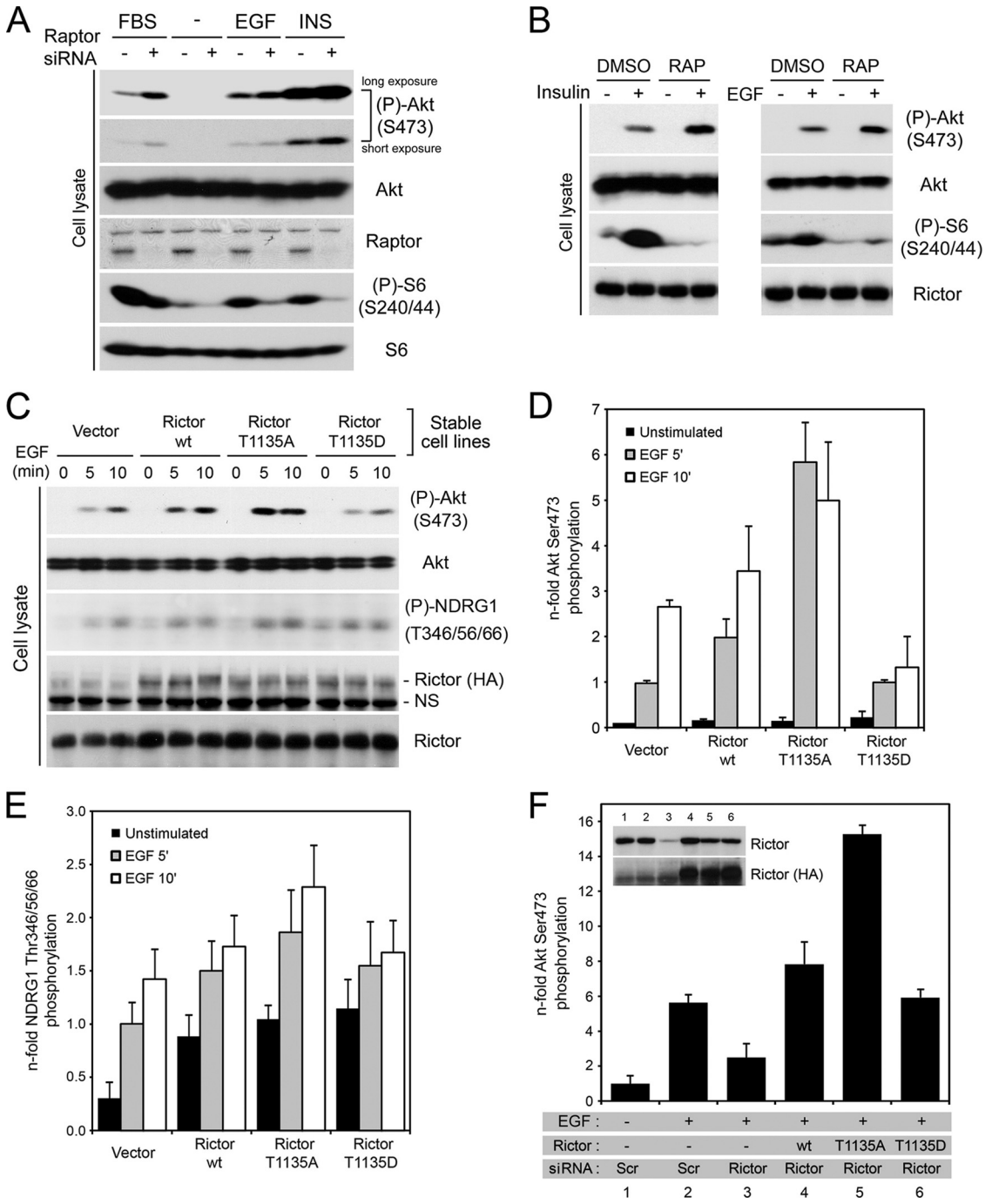

FIG. 7. A Rictor mutant that cannot be phosphorylated on Thr1135 promotes mTORC2-directed phosphorylation of Akt. (A) HEK293 cells were transfected with siRNA duplexes targeted against a scrambled sequence or human Raptor. Cells were grown in the presence of serum or serum starved overnight and stimulated with EGF (25 ng/ml) or insulin (100 nM). Akt phosphorylation was assayed by immunoblotting from cell lysates using Akt phospho-Ser473 antibodies. (B) HEK293 cells were serum starved overnight and treated with rapamycin for 30 min prior to stimulation with insulin (25 nM) (left panel) or EGF (25 ng/ml) (right panel). Akt phosphorylation was assayed as for panel A. DMSO, dimethyl sulfoxide. (C) HEK293 cells stably expressing an empty vector, Rictor wild-type Rictor, or the T1135A or T1135D mutant were seeded at similar densities and serum starved overnight. Cells were stimulated with EGF (25 ng/ml) for 5 or 10 min and assayed for Akt and NDRG1 phosphorylation using NDRG1 phospho-Thr346/Thr356/Thr366 antibodies. (D and E) Results from three independent experiments as for panel C were quantified, and the mean (± SD) fold stimulation in Akt (D) and NDRG1 (E) phosphorylation was calculated compared to an empty-vector control. (F) siRNA duplexes targeted against a scrambled sequence or human Rictor were transfected in HEK293 cells stably expressing an empty vector or siRNA-resistant wild-type Rictor or the T1135A or T1135D mutant. Cells were serum starved overnight, stimulated with EGF (25 ng/ml), and harvested for FACS analysis of Ser473 phosphorylation (see Materials and Methods for details). Levels of phosphorylated Akt were determined in HA-Rictor positive cells. Data are expressed as fold stimulation in Akt phosphorylation compared to unstimulated empty-vector-transfected cells.

increased Akt phosphorylation induced by EGF stimulation (Fig. 7C). Interestingly, expression of the Rictor T1135D mutant did not significantly promote Akt phosphorylation, suggesting that aspartic acid mutation of Thr1135 acts as a phosphomimetic. These changes were quantified from three independent experiments, and expression of the Rictor T1135A mutant was found to stimulate Akt phosphorylation by approximately 2-fold compared to wild-type Rictor (Fig. 7D). Similar quantifications were performed for NDRG1 phosphorylation at Thr346/56/66, and although there appeared to be a similar trend (Fig. 7E), NDRG1 phosphorylation was not found to be significantly increased in cells expressing the Rictor T1135A mutant.

To confirm this result, we performed RNAi to remove endogenous Rictor from HEK293 cells and rescued its expression with wild-type Rictor or the T1135A and T1135D mutants. Using fluorescence-activated cell sorter (FACS) analysis to quantify Akt phosphorylation at the single-cell level, we found that reducing endogenous Rictor expression strongly inhibited Akt phosphorylation at Ser473 stimulated by EGF treatment (Fig. 7F). Expression of exogenous wild-type Rictor to a near-endogenous level (Fig. 7F, inset) completely rescued Akt phosphorylation in Rictor knockdown cells, indicating the validity of this assay to test Rictor phosphorylation site mutants. FACS analysis of cells expressing the Rictor T1135A mutant revealed increased Akt phosphorylation compared to that with wild-type Rictor, indicating that phosphorylation of Thr1135 negatively regulates Akt phosphorylation at Ser473 (Fig. 7F). Consistent with the Rictor T1135D mutant playing a phosphomimetic role, we found that cells expressing this mutant did not have increased Akt phosphorylation compared to wild-type Rictor-expressing cells. Together, these data support the idea that Thr1135 phosphorylation negatively regulates mTORC2-directed Akt phosphorylation.

Rictor phosphorylation at Thr1135 inhibits Akt signaling and cell proliferation. Prolonged rapamycin treatment was shown to correlate with mTORC2 disassembly and hypophosphorylation of Rictor (21, 39, 79). Thus, we determined the impact of Rictor phosphorylation at Thr1135 on mTORC2 assembly by coimmunoprecipitation in CHAPS-containing lysis buffer, as previously described (66). HEK293 cells were transfected with HA-tagged Rictor and Flag-tagged mTOR, serum starved overnight, and coimmunoprecipitated following insulin treatment to trigger Rictor phosphorylation at Thr1135. Under these conditions, we did not find major differences in mTOR binding to wild-type Rictor or the Thr1135phosphorylation site mutants (Fig. 8A), suggesting that the rapid S6K1-dependent phosphorylation of Rictor does not regulate mTORC2 assembly. Similar data were also obtained for the association of endogenous mTOR with HA-tagged Rictor (data not shown).

We also determined whether mutation of Thr1135 could affect mTORC2 kinase activity. Using endogenous Rictor immunoprecipitates, we first assayed associated mTOR activity toward kinase-inactive GST-Akt purified from mammalian cells. We found that EGF stimulation increased mTORC2 activity by about 2.5-fold, which was completely inhibited by pretreatment of cells with the dual PI3K/mTOR inhibitor, PI-103 (Fig. 8B). Using these conditions, we measured mTORC2 kinase activity in HA-tagged Rictor immunoprecipitates from unstimulated and EGF-stimulated cells. While EGF treatment increased mTORC2 activity by approximately 2-fold, we did not find differences between cells expressing wild-type Rictor or the T1135A and T1135D mutants (Fig. 8B). These findings suggest that phosphorylation of Rictor at Thr1135 does not regulate the kinase activity of mTORC2.

Long-term, but not short-term, rapamycin treatment has also been shown to trigger dephosphorylation and cytoplasmic accumulation of Rictor (62). Although our findings indicate that short-term treatments with rapamycin are sufficient to inhibit Thr1135 phosphorylation (Fig. 1C to E, 2B, 3D, and 5C to E), we assessed the subcellular localization of wild-type Rictor and the T1135A and T1135D mutants in HEK293 cells stably expressing the different alleles. Using confocal microscopy, we found Rictor to be cytoplasmic and excluded from the nucleus. However, we did not observe major differences between the subcellular localizations of wild-type Rictor and the Thr1135 phosphorylation site mutants (Fig. 8C). We also examined Rictor localization following growth factor stimulation and short-term rapamycin treatment but did not observe changes in Rictor subcellular localization under these conditions (data not shown).

We found that cells expressing the Rictor T1135A mutant have increased mTORC2-mediated Akt phosphorylation compared to cells expressing wild-type Rictor or the T1135D mutant. To verify whether Akt signaling was increased in Rictor T1135A-expressing cells, we verified the phosphorylation status of well-established Akt substrates, such as FoxO1, FoxO3a (8, 61), and GSK3α/β (14). HEK293 cells stably expressing wild-type Rictor or Thr1135 phosphorylation site mutants were serum starved overnight and stimulated with EGF. We found that cells expressing the Rictor T1135A mutant had increased GSK3α (Ser21) and GSK3β (Ser9) phosphorylation, as well as increased FoxO1/3a (Thr24/Thr32) phosphorylation, compared to cells expressing wild-type Rictor or the Rictor T1135D mutant (Fig. 8D). These findings support the notion that Rictor phosphorylation at Thr1135 negatively regulates mTORC2-directed Akt activation and signaling. To determine whether this increased Akt signaling could promote cell proliferation, we performed proliferation assays by comparing the different stable cell lines. Equal numbers of cells stably expressing wild-type, T1135A, or T1135D Rictor were grown in culture medium containing 5% FBS, and the relative number of viable cells was measured during five consecutive days by MTS assay. Using this method, we found that cells expressing the Rictor T1135A mutant had a significant proliferative advantage over cells expressing wild-type Rictor or the T1135D mutant (Fig. 8E). These data are consistent with the known roles of Akt in cell growth, survival, and proliferation and suggest that Rictor phosphorylation by S6K1 restrains these functions.

Taken together, our results demonstrate that Rictor integrates mTORC1-dependent signals to modulate mTORC2-directed Akt phosphorylation. Specifically, we show that mTORC1-activated S6K1 mediates Thr1135 phosphorylation of Rictor and inhibits mTORC2 and Akt signaling (Fig. 8F).

DISCUSSION

In this study, we have uncovered a new regulatory link between the two mTOR complexes. We show that growth factors promote the mTORC1-dependent and rapamycin-sensitive phosphorylation of Rictor (Fig. 1). Overexpression of Rheb

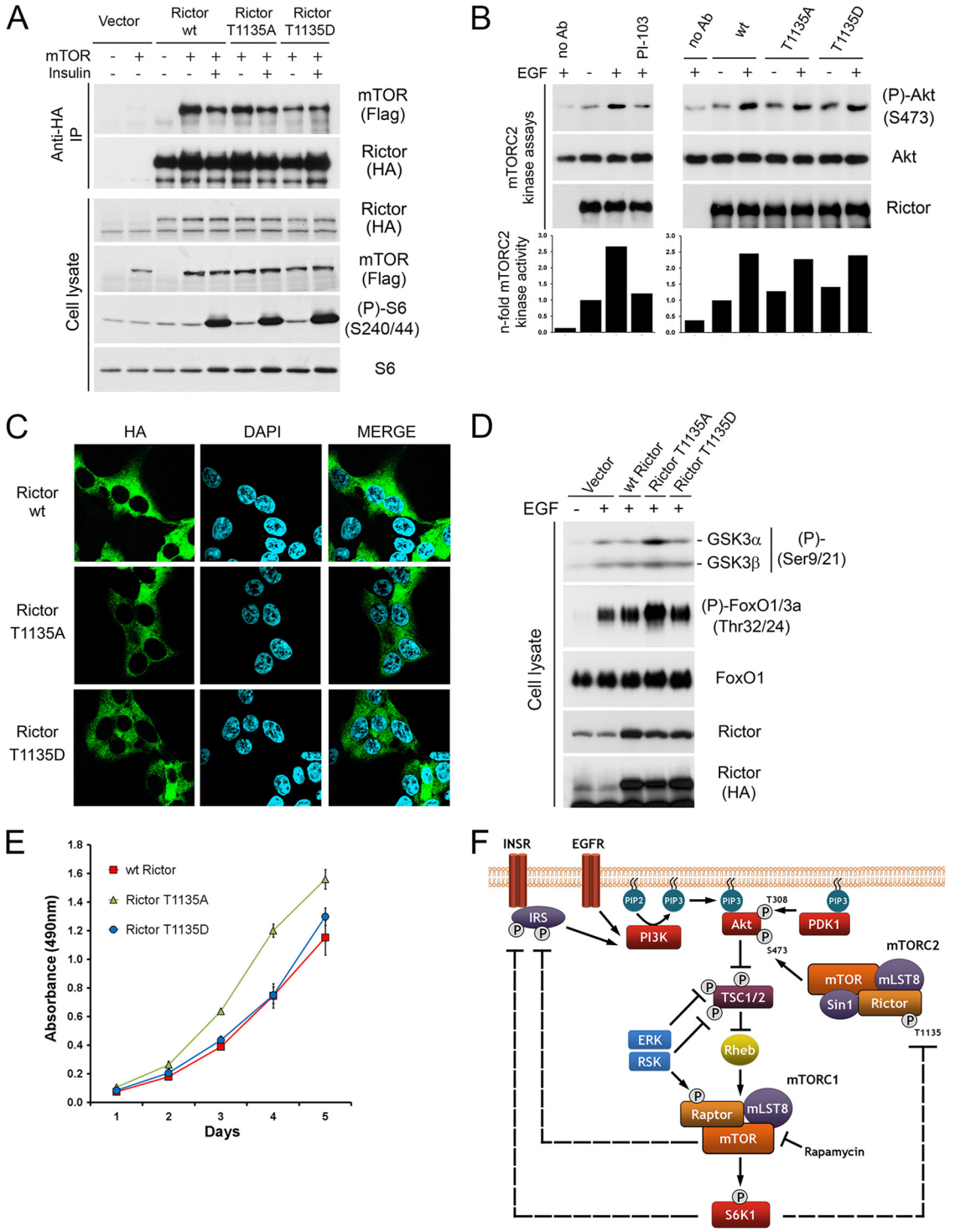

FIG. 8. Rictor phosphorylation at Thr1135 inhibits Akt signaling and cell proliferation. (A) HEK293 cells were transfected with Flag-tagged mTOR and wild-type HA-tagged Rictor or Thr1135 mutants. Associated mTOR was determined in anti-HA immunoprecipitates using anti-Flag antibodies. (B) mTORC2 kinase assays were optimized by immunoprecipitating endogenous Rictor from HEK293 cells stimulated with EGF (25 ng/ml) and treated with the dual PI3K/mTOR inhibitor PI-103 where indicated (left panel). Using these assay conditions, mTORC2 activity was measured in HA-tagged Rictor wild-type or mutant immunoprecipitates from unstimulated or EGF-stimulated cells (right panel). mTORC2 activity toward kinase-inactive GST-Akt purified from mammalian cells was assayed. (C) Subcellular localization of wild-type Rictor and the Rictor Thr1135 mutants. Confocal images of serum-growing HEK293 cells stably expressing HA-tagged wild-type Rictor or Thr1135 mutants are shown. (D) HEK293 cells stably expressing wild-type Rictor or Thr1135 mutants were serum starved overnight and stimulated with EGF (25 ng/ml). Phosphorylation of Akt substrates FoxO1/3a and GSK3α/β was assayed by immunoblotting. (E) HEK293 cells stably expressing wild-type Rictor or the Thr1135 mutants were grown in culture medium containing 5% FBS. The relative number of viable cells was measured during five consecutive days using an MTS assay. (F) Upon growth factor stimulation, PI3K is recruited to the plasma membrane using IRS-dependent and

and loss of TSC2, leading to the constitutive activation of mTORC1, were found to promote Rictor phosphorylation in serum-starved HEK293 cells and in MEFs (Fig. 3, 4, and 5), suggesting that activation of mTORC1 is sufficient to regulate Rictor. We demonstrate that S6K1 is both required (Fig. 2) and sufficient (Fig. 3) to promote Rictor phosphorylation, and we identify Thr1135 as the primary site regulated by S6K1 *in vivo* (Fig. 4 and 5) and *in vitro* (Fig. 6). MS/MS analyses revealed additional phosphorylation sites in Rictor, most of them within the less conserved, carboxyl-terminal half of the protein (Fig. 4B). While our work focused on S6K1-dependent phosphorylation events, subsequent studies will be required to determine the identity of the protein kinase(s) involved and the molecular function associated with these additional phosphorylation sites. Biochemical and genetic evidence has demonstrated that mTORC2 phosphorylates Akt at Ser473 (26, 39, 68, 70). Using Akt phosphorylation as output, we found that expression of the Rictor T1135A mutant increases mTORC2-directed Akt phosphorylation and signaling (Fig. 7 and 8), indicating that mTORC1 negatively regulates mTORC2 via the phosphorylation of Rictor on Thr1135 (Fig. 8F).

The upstream mechanisms regulating mTORC2 are currently poorly known. Both mTORC2 and PI3K are required for proper phosphorylation of Akt at Ser473, but the mechanisms regulating their functional interaction remain elusive (34). It has been recognized for some time that rapamycin treatment of cells increases the sensitivity of the PI3K/Akt pathway to insulin, suggesting that mTORC1 normally inhibits insulin signaling. At present, mTORC1-mediated inhibition of Akt phosphorylation has generally been attributed to the phosphorylation of IRS-1 by mTORC1 and S6K1 (29, 69, 72), which reduces IRS-1 protein stability and PI3K signaling. However, mounting evidence suggests the presence of a more general mechanism for this negative regulation (6, 31). Indeed, while insulin and IGF-1 do not stimulate Akt phosphorylation in TSC2-deficient MEFs, this is also the case with growth factors that do not require IRS-1 to stimulate PI3K activity, such as EGF, PDGF, and, to some degree, serum (80). Short-term rapamycin treatment of cells also increases Akt phosphorylation in response to EGF, PDGF, and serum (Fig. 7A and data not shown), suggesting the presence of additional mTORC1-dependent mechanisms that do not involve IRS-1. Our findings demonstrate that S6K1 directly phosphorylates a component of mTORC2 and provide a potential IRS-1-independent mechanism for the negative regulation of Akt phosphorylation by mTORC1/S6K1 signaling.

While expression of the Rictor T1135A mutant was found to increase mTORC2-directed Akt phosphorylation and signaling, we did not observe that phosphorylation of Thr1135 modified mTORC2 kinase activity *in vitro* (Fig. 8B). These results indicate that Rictor phosphorylation may reduce mTORC2's affinity toward Akt by a mechanism independent of mTOR kinase activity. Many potential mechanisms could explain these findings. Based on studies with yeast, Rictor appears to serve as a scaffolding protein that is important for maintaining mTORC2 integrity (78). As shown by others (67), we found that growth factor stimulation and short-term rapamycin treatment of cells do not disrupt or increase Rictor binding to mTOR (Fig. 8A and data not shown). In addition, mutation of Thr1135 did not affect the interaction between mTOR and Rictor, suggesting that Rictor phosphorylation at Thr1135 does not regulate mTORC2 assembly. Prolonged rapamycin treatment was shown to disrupt mTORC2 assembly and to correlate with the hypophosphorylation of Rictor (21, 39, 67, 79), but based on our results, it is unlikely that the complete inhibition of Rictor Thr1135 phosphorylation following acute rapamycin treatment is directly responsible for these effects.

Rictor phosphorylation at Thr1135 may also regulate the interaction of an unknown mTORC2 binding partner. A recent study aimed at identifying novel Rictor-interacting proteins using a TAP-tagging strategy revealed that three 14-3-3 isoforms (ε, α, and β) may interact with Rictor (58). However, the same group did not find 14-3-3 isoforms in endogenous Rictor immunoprecipitates, nor did large-scale studies aimed at identifying 14-3-3 substrates ever identify Rictor as a 14-3-3 binding protein (41, 54). Nonetheless, analysis of potential 14-3-3 binding sites in Rictor using Scansite (56) revealed Thr1135 as a potential 14-3-3 binding site. By coimmunoprecipitation assays, we have found that Rictor and 14-3-3 can interact in cells (data not shown), suggesting that 14-3-3 may play some roles in regulating mTORC2 function. Interestingly, a recent article with similar findings on Rictor phosphorylation demonstrated that Rictor interacts with 14-3-3 in a Thr1135-dependent manner (17). These results potentially explain the molecular mechanisms triggered by Thr1135 phosphorylation, but the regulation of 14-3-3 binding to Rictor and its impact on mTORC2 signaling remain elusive. Because Thr1135 phosphorylation does not affect Rictor subcellular localization (Fig. 8C), one interesting possibility is that 14-3-3 binding regulates Rictor protein turnover rates. Although we (Fig. 5D) and others (17, 33) have found that Rictor expression levels do not appear to be reduced in $TSC2^{-/-}$ MEFs, which have constitutively high mTORC1 activity and Rictor phosphorylation, a more thorough analysis of Rictor protein stability will be required to definitely test this hypothesis. Acidic amino acid (Asp or Glu) substitution of phosphorylation sites to mimic phosphorylation has been suggested to generate weak 14-3-3 binding sites (24). Our results demonstrate that the Rictor T1135D mutant acts as a phosphomimetic, indicating that phosphorylation of Thr1135 may not solely regulate 14-3-3 binding. The potential role for Rictor phosphorylation in the recruitment or regulation of a Ser/Thr phosphatase could be such a possibility. Evidently, more experimentation will be required to determine the exact molecular mechanisms regulated by Thr1135 phosphorylation.

One caveat when using rapamycin analogs as a single agent

-independent mechanisms. PIP3-mediated recruitment of Akt to the plasma membrane promotes PDK1- and mTORC2-dependent phosphorylation of Akt at Thr308 and Ser473, respectively. Upon activation, Akt phosphorylates TSC2, which releases the inhibitory function of the TSC complex on Rheb. GTP-loaded Rheb then activates mTORC1, which phosphorylates and activates S6K1. Activated S6K1 phosphorylates several substrates, including IRS-1 and Rictor, which play roles in the negative regulation of mTORC2 signaling.

for cancer therapy is that in many cases it is cytostatic and does not induce cell death. Furthermore, as demonstrated by our results, mTORC1 inhibition potentiates growth factor activation of Akt by reducing Rictor phosphorylation at Thr1135, which may increase cell survival and enable acquisition of additional lesions that may contribute to resistance to therapy. Our findings suggest that specific inhibitors of mTOR catalytic activity, which would target both mTORC1 and mTORC2, may be more suitable than rapalogs alone for the treatment of cancer, as they would abrogate any mTORC2 activation resulting from inhibition of mTORC1.

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