Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton

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Supplemental Experimental Procedures

Materials

Reagents were obtained from the following sources: protein G-sepharose from Pierce; [γ-^{32}P]ATP from NEN; mTOR, S6K1, ATM, EGFR, and PKCα antibodies as well as HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; phospho-T389 S6K1, phospho-S657 PKCα, phospho-S916 PKCμ, PKCμ, phospho-S366 eEF2k, and eEF2K antibodies from Cell Signaling; phospho-S729 PKCε and PKCε antibodies from Upstate Biotechnology; HA monoclonal antibody from Covance; myc monoclonal antibody from Oncogene Research Products; *Drosophila* S6K antibody from Mary Stewart, North Dakota State University; Alexa Fluor 488-conjugated secondary anti-mouse antibody and Texas red-X-phalloidin from Molecular Probes; paxillin monoclonal antibody from BD Transduction Laboratories; DMEM, leucine, glucose, RPMI, and RPMI without leucine from Life Technologies; rapamycin, LY294002, and antimycin A from Calbiochem; phorbol myristate acetate from Sigma. The GβL antibody was described previously [S1], and the rictor and raptor antibodies were developed with the antibody service from Covance using the following peptides: rictor, RGRSLKNLRVRGRND, amino acid sequence 6–20; and raptor, MESEMLQSPLLGLGEEDEAD, amino acid sequence 1–20.

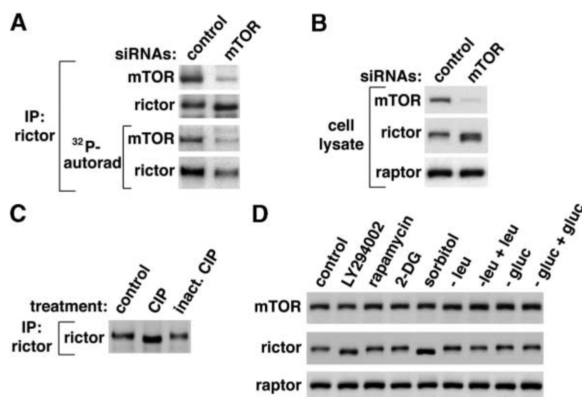

Figure S1. mTOR May Regulate the Rictor Phosphorylation State

(A) HeLa cells with reduced expression of mTOR or of a control protein were metabolically labeled with ^{32}P and the level of phosphorylated rictor determined by immunoprecipitation followed by autoradiography and immunoblotting for the indicated proteins.

(B) The mobility of rictor in SDS-PAGE is affected by mTOR. HeLa cells with siRNA-mediated reductions in mTOR or controls were analyzed by immunoblotting for mTOR, rictor, and raptor.

(C) The phosphorylation state of rictor affects its mobility in SDS-PAGE. Rictor immunoprecipitates were incubated with or without calf intestinal phosphatase (CIP) or heat-inactivated CIP and analyzed by SDS-PAGE and immunoblotting for rictor.

(D) Osmotic stress increases the mobility of rictor in SDS-PAGE. Lysates of HeLa cells exposed for 1 hr to 20 nM rapamycin, 20 μM LY294002, 100 mM 2 deoxyglucose (2-DG), medium without leucine or glucose, or medium without leucine or glucose followed by the readdition of the missing component for 10 min were analyzed by immunoblotting for rictor.

Purification and Identification of Rictor

mTOR immunoprecipitates prepared from 60 million HeLa cells were resolved by SDS-PAGE, and proteins were visualized by Coomassie blue or silver staining. The ~200 kDa band corresponding to rictor was digested with trypsin, the mixtures fractionated on a Poros 50 R2 RP micro-tip, and resulting peptide pools analyzed by matrix-assisted laser-desorption/ionization reflectron time-of-flight (MALDI-reTOF) MS using a BRUKER UltraFlex TOF/TOF instrument (Bruker Daltonics; Bremen, Germany), as described [S2]. Selected experimental masses (*m/z*) were taken to search the human segment of a nonredundant protein database (NR; ~108,000 entries; National Center for Biotechnology Information; Bethesda, MD) utilizing the PeptideSearch (Matthias Mann, Southern Denmark University, Odense, Denmark) algorithm, with a mass accuracy restriction better than 40 ppm, and maximum one missed cleavage site allowed per peptide. Mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in LIFT mode. Fragment ion spectra were taken to search NR using the MASCOT MS/MS Ion Search program (Matrix Science Ltd.; London, UK). Any identification thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

Cloning of the Full-Length Human Rictor cDNA and Its Sequence Analysis

Human and mouse cDNA and EST sequences obtained from public databases were used to electronically assemble a putative full-length cDNA. The human KIAA1999 cDNA was the largest fragment available but is missing ~1.3 kb of 5′ coding sequence. To prepare the full-length rictor cDNA, three DNA fragments were combined: a human EST (BG623200), a human cDNA (KIAA1999), and a PCR product spanning the gap between the EST and cDNA that was made from first strand cDNA derived from HeLa cell total RNA. The PCR product was prepared using a forward primer corresponding to a sequence 5′ of the BamH1 site at position 950 of the rictor ORF and a reverse primer corresponding to a sequence 3′ of the PacI site at position 1616 and was added to the 3′ end of the BG623200 EST. The SalI/PacI and PacI/XmaI segments of the extended BG623200 and KIAA1999, respectively, were subcloned into the prk5 expression vector in a three-way ligation. Prior to use, a corrupted section between the SpeI sites at 2682 and 3196 of the KIAA1999 cDNA was replaced with a wild-type fragment obtained by RT-PCR. In order to make these SpeI sites unique for the repair, the third SpeI site at 9135 of KIA1999 was removed by excising the noncoding fragment between SwaI sites at 6934 and 9367. All rictor fragments generated by PCR were confirmed by DNA sequencing.

Rictor sequences from several species were analyzed using the MEME Motif Discovery Tool [S3] to identify regions of sequence conservation and internal repeats. A motif length range of 20–50 amino acids was imposed on the algorithms. One internal repeat was found among all of the analyzed sequences. The repeats (except for *S. cerevisiae*) were aligned to each other using Clustalx v.1.81 [S4].

Immunoprecipitations, Kinase Assays, and Metabolic Labeling

3×10^6 HeLa or HEK293T cells growing in 10 cm dishes were rinsed once with cold PBS and lysed on ice for 20 min in 1 ml of ice-cold

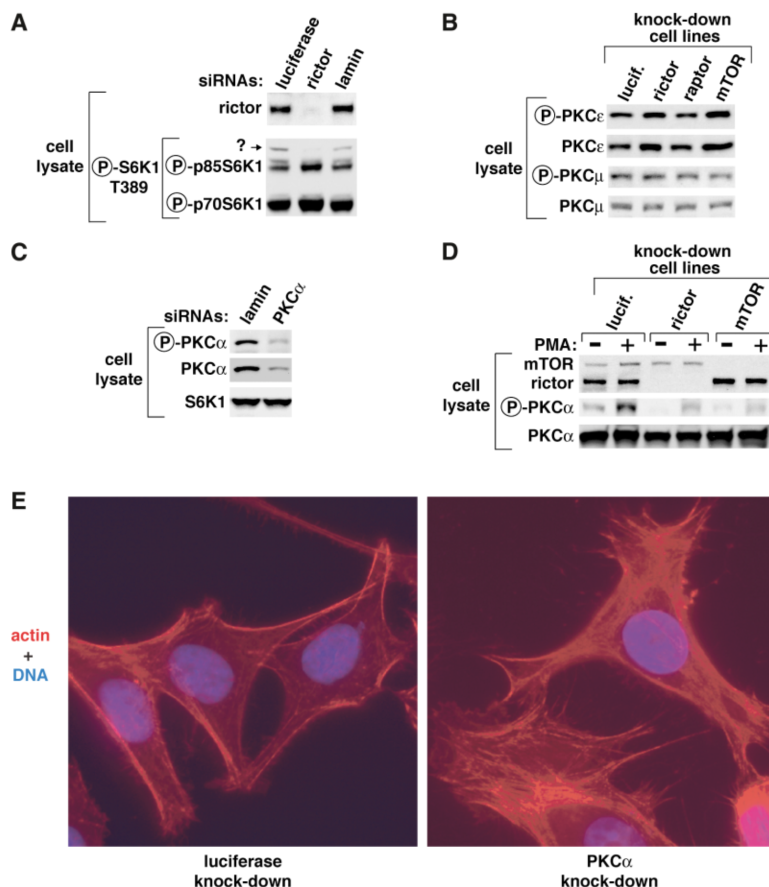

Figure S2. The Expression of Rictor and mTOR Regulate the Phosphorylation of PKCα

(A) Phospho-T389 S6K1 immunoblot shows that the intensity of a faint background band (arrow) is reduced in HeLa cells infected with an siRNA-expressing lentivirus that targets rictor but not luciferase or lamin.

(B) Levels of phospho-S729 PKCε and phospho-S916 PKCμ do not decrease in immunoblots prepared from knockdown cells described in Figure 4A.

(C) siRNA-mediated reduction in the expression of total PKCα in HeLa cells also reduces the immunoblot signal from a phosphospecific antibody recognizing phospho-S657 of PKCα but does not affect the levels of S6K1.

(D) Treatment of HeLa cells with 200 nM phorbol myristate acetate (PMA) for 15 min increases the phosphorylation of PKCα on S657, and this increase is suppressed in cells with reduced expression of rictor and mTOR. Control cells were infected with an siRNA-expressing lentivirus targeting luciferase (lucif.).

(E) Like cells with reduced rictor expression, cells with reduced expression of PKCα have an altered actin cytoskeleton.

lysis buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, and EDTA-free protease inhibitors [Roche]) containing either 0.3% CHAPS or 1% Triton X-100. After centrifugation at 13,000 × g for 10 min, 4 μg of the indicated antibodies were added to the cleared supernatant and incubated with rotation for 90 min. 20 μl of a 50% slurry of protein G-sepharose was then added and the incubation continued for 1 hr. Captured immunoprecipitates were washed four times with lysis buffer and once with wash buffer (50 mM HEPES [pH 7.5], 40 mM NaCl, and 2 mM EDTA). Samples were resolved by SDS-PAGE and proteins transferred to PVDF and visualized by immunoblotting as described [S1]. In vitro mTOR kinase assays were also as described [S1].

Two million HeLa cells in 6 cm dishes and transfected with the lentiviral plasmid encoding siRNAs targeting luciferase or mTOR were metabolically labeled by the addition of 0.5 mCi of $[^{32}P]$orthophosphate (NEN) for 2 hr. Cells were rinsed twice with cold PBS and lysed in 300 μl of ice-cold CHAPS lysis buffer. After centrifugation, the cleared supernatants were collected, rictor immunoprecipitates prepared as above using with the rictor antibody, immunoprecipitates washed four times in lysis buffer, proteins resolved by SDS-PAGE, and radioactivity incorporation visualized by autoradiography. PKCα kinase assays were performed as described with a commercial PKC assay kit (Upstate Biotechnology) using PKCα immunoprecipitated with a PKCα antibody (Santa Cruz).

Plasmid and siRNA Transfections

Effectene (Qiagen) was used to transfect 1.2 million HEK293T cells in 6 cm dishes with up to 1 μg of the expression plasmids indicated in the figure legends. 48 hr after DNA addition, the cells were rinsed once with PBS and lysed in 800 μl of ice-cold lysis buffer containing either CHAPS or Triton X-100 and analyzed by immunoprecipitation and immunoblotting as above. Sequences and transfection conditions for synthetic siRNAs targeting lamin, mTOR, and raptor have been described [S1] and are available at http://web.wi.mit.edu/sabatini/pub/siRNA_sequences.html. The sequences of the sense and antisense strands of the siRNA targeting rictor are ACUUGUGAAGAAUCGUAUCdTdT and GAUACGAUUCUUCACAAGUdTdT, respectively. Those for PKCα are UCCUUGUCCAAGGAGGCUGdTdT and CAGCCUCCUUGGACAAGGAdTdT.

Lentiviral shRNA Cloning, Production, and Infection

Desalted oligonucleotides (IDT) were cloned into LKO.1 [S5] with the AgeI/EcoRI sites at the 3′ end of the human U6 promoter. The sequences of the oligonucleotides are as follows: mTOR 609 sense, CCGGTTCAGCGTCCCTACCTTCTTCTCTTCCTGTCAAGAAGAAGGTAGGGACGCTGATTTTTG; mTOR 609 antisense, AATTCAAAAATCAGCGTCCCTACCTTCTTCTTGACAGGAAGAGAAGAAGGTAGGGACGCTGAA; Raptor 4145 sense, CCGGAGGGCCCTGCTACTCGCTTTTCTCGAGAAAAGCGAGTAGCAGGGCCCTTTTTTG; Raptor 4145 antisense, AATTCAAAAAAGGGCCCTGCTACTCGCTTTTCTCGAGAAAAGCGAGTAGCAGGGCCCT; Rictor 3274 sense, CCGGTACTTGTGAAGAATCGTATCTTCTCGAGAAGATACGATTCTTCACAAGTTTTTTG; Rictor 3274 antisense, AATTCAAAAAACTTGTGAAGAATCGTATCTTCTCGAGAAGATACGATTCTTCACAAGTA. The numbers indicate the nucleotide positions in the transcripts (with position 1 set at the start codon) at which the 21 bp stem of the shRNA begins.

Plasmids were propagated in and purified from Stbl2 bacterial cells (Invitrogen) and cotransfected together with the Delta VPR CMV VSVG plasmids into actively growing HEK293T using Fugene (Roche) as described [S5]. Virus-containing supernatants were collected at 36 and 60 hr after transfection and concentrated by ultracentrifugation for 1.5 hr at 23,000 rpm in an SW28 rotor at 4°C. Pellets were resuspended overnight at 4°C in $1/600^{th}$ of the original volume. Cells were infected twice in the presence of 6 μg/ml protamine sulfate, selected for puromycin resistance, and analyzed on the fifth day after infection.

Generation and Application of dsRNA for RNAi in *Drosophila* S2 Cells

Primers were designed within the coding sequence of each respective gene to amplify a 700–800 bp cDNA fragment. The following primers were used: EGFP forward, ATGGTGAGCAAGGGCGAGGA GCTGT; EGFP reverse, TTACTTGTACAGCTCGTCCATGCCG; dTOR (CG5092) forward, CAGGAGTTATTTTAAATGTGCTTCG; dTOR reverse, CCAAAATTCTTTGATCAGCTTAAAA; dRaptor (CG4320) forward, TGTCTGACAACACCCATTAACATAG; dRaptor reverse, GTAC TTGTATTCCTTGACCAGATCC; dRictor (CG8002) forward, GCTTAT TCCTAGACAGCATTATCCA; dRictor reverse, TTTTGAGTACTTCG ATGCCTTTTAC; dS6K (CG10539) forward, CCTTCATAGTGGAGCT AGTTTATGC; dS6K reverse, CTTAGCGTTGTATCATCAGGTGAAT.

Each primer included a GAA and T7 promoter sequence (GAAT TAATACGACTCACTATAGGGAGA) at its 5′ end. Primers were used in a one-step RT PCR reaction (Qiagen) to amplify a cDNA fragment using total *Drosophila* S2 cell RNA as template. The total RT-PCR reaction was purified using a PCR purification column (Qiagen) in a final volume of 40 μl. 8 μl of the RT-PCR product was then used as a template in a 20 μl in vitro transcription reaction using the Megascript kit (Ambion) to generate the corresponding dsRNA fragments. The GFP template was amplified from an EGFP expression plasmid (Stratagene). *Drosophila* S2 cells actively growing in Schneider medium (Life Technologies) were washed and resuspended in *Drosophila* SFM (Life Technologies) to a final density of 1×10^6 cells in 1 ml volume. 30 μg of dsRNA was added to the 1 ml of cells in SFM and incubated for 45 min at 25°C. 2 ml of Schneider medium with 10% serum was then added back to the cells. After 24 hr, the cells were starved again and an additional 30 μg of dsRNA was added. After 4 days cells were harvested, washed once with cold PBS, lysed in the 1% Triton X-100 lysis buffer, and analyzed by immunoblotting as above. Antibodies developed against mammalian phospho-S6K1, phospho-PKCα, and PKCα were used to detect the *Drosophila* homologs of these proteins/modifications.

Immunofluorescence

HeLa cells transduced with the siRNA-expressing lentiviruses or transfected with synthetic siRNAs were cultured overnight on fibronectin-coated glass coverslips. Cells were fixed for 15 min with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeablized for 10 min in 0.5% NP40 in PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (PBS+), and washed twice in PBS+. Nonspecific binding sites were blocked for 30 min by incubating in PBS+ containing 10% fetal bovine serum followed by a 1 hr incubation in the same blocking buffer containing a 1:1000 dilution of the paxillin antibody. After washing in PBS+, blocking buffer containing 1:800 of the Alexa Fluor 488-conjugated secondary anti-mouse antibody, 1:800 of Texas red-X-phalloidin, and 1:1000 of Hoechst dye was added to the coverslips for 1 hr. The coverslips were then washed twice with PBS+, mounted in glycerol containing 0.1% p-phenylenediamine on glass slides, and visualized with fluorescence microscopy.

Supplemental References

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