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

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Summary

The mammalian TOR (mTOR) pathway integrates nutrient- and growth factor-derived signals to regulate growth, the process whereby cells accumulate mass and increase in size. mTOR is a large protein kinase and the target of rapamycin, an immunosuppressant that also blocks vessel restenosis and has potential anticancer applications. mTOR interacts with the raptor and GβL proteins [1–3] to form a complex that is the target of rapamycin. Here, we demonstrate that mTOR is also part of a distinct complex defined by the novel protein rictor (rapamycin-insensitive companion of mTOR). Rictor shares homology with the previously described pianissimo from *D. discoidieum* [4], STE20p from *S. pombe* [5], and AVO3p from *S. cerevisiae* [6, 7]. Interestingly, AVO3p is part of a rapamycin-insensitive TOR complex that does not contain the yeast homolog of raptor and signals to the actin cytoskeleton through PKC1 [6]. Consistent with this finding, the rictor-containing mTOR complex contains GβL but not raptor and it neither regulates the mTOR effector S6K1 nor is it bound by FKBP12-rapamycin. We find that the rictor-mTOR complex modulates the phosphorylation of Protein Kinase C α (PKCα) and the actin cytoskeleton, suggesting that this aspect of TOR signaling is conserved between yeast and mammals.

Results and Discussion

To identify novel components of the mTOR signaling complex, we purified mTOR with methods that preserve the raptor-mTOR interaction [1]. We had previously noticed that the mTOR complex immunopurified from HEK293T cells contains a low-abundance 200 kDa protein (data not shown), but only when purified from HeLa cells did the complex contain enough of this protein for its identification (Figure 1A). Like the raptor-mTOR interaction, the p200-mTOR interaction is sensitive to Triton X-100 but stable in CHAPS-containing buffers (Figure 1A).

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Peptide mass fingerprinting analysis using mass spectrometry and mass spectrometric sequencing revealed that p200 is novel and not in the databases of full-length human proteins. We named the 200 kDa protein rictor for rapamycin-insensitive companion of mTOR. Starting from a truncated cDNA that encodes part of rictor (accession # KIAA1999), we used EST mining and RT-PCR to assemble a full-length open reading frame that predicts a protein of 1708 amino acids and 192 kDa. We could not identify any domains of known function in rictor and, compared to mTOR, raptor, and GβL, the protein is not well conserved among eukaryotes. Rictor shares regions of homology with several poorly characterized proteins, including pianissimo from *D. discoidieum* [4], STE20p from *S. pombe* [5], and AVO3p from *S. cerevisiae* [6, 7]. Proteins of similar domain structure and conservation are also encoded in the *A. gambiae* and *D. melanogaster* genomes, suggesting that other eukaryotes may have rictor-like proteins. These proteins share a region of about 200 amino acids in length (Figure 1B, box 1) of 44% similarity (8% identity) as well as several smaller conserved regions, including a repeated block of 20 amino acids (Figure 1B, box 5). Despite these regions of similarity, both rictor and dRictor (rictor's likely *Drosophila* homolog) have long C-terminal extensions without apparent conservation or similarity to other proteins. Pianissimo is implicated in cAMP-induced cell migration [4], and recent work from the Hall group indicates that in *S. cerevisiae*, AVO3p is part of a rapamycin-insensitive TOR2p-containing complex that regulates the actin cytoskeleton [6]. In *S. pombe*, STE20p functions in the cell cycle arrest induced by nutrient deprivation [5].

Using a rictor antibody, we confirmed that rictor is part of the endogenous mTOR complex and does not co-immunoprecipitate with control proteins (Figure 1C). Leucine levels and mitochondrial inhibition with antimycin regulate the activity of S6K1 and 4E-BP1 [1, 8–10] as well as the stability of the raptor-mTOR interaction [1], but these conditions do not affect the mTOR-rictor interaction (Figure 1C). Similarly, under the appropriate cell lysis conditions, rapamycin treatment of cells eliminates the binding of mTOR to raptor [1] without affecting the interaction of rictor with mTOR (Figure 1C). Like raptor but not the control hGCP3 (human γ-tubulin component protein 3), recombinant myc-rictor enters the endogenous mTOR complex and can be used to isolate endogenous mTOR (Figure 1D). The interaction between recombinant rictor and mTOR has similar detergent sensitivities as that between the endogenous proteins (Figure 1D).

Raptor and mTOR are in a nearly stoichiometric ratio (0.8–0.9 raptor to 1.0 of mTOR) in mTOR complexes in HEK293T cells [1]. During the purification of rictor, it was apparent that in HeLa cells this is not true and that complexes in these cells have less raptor but more rictor. When comparing mTOR complexes across mammalian cell types, we observe an inverse correlation between the relative amounts of raptor and rictor (Figure

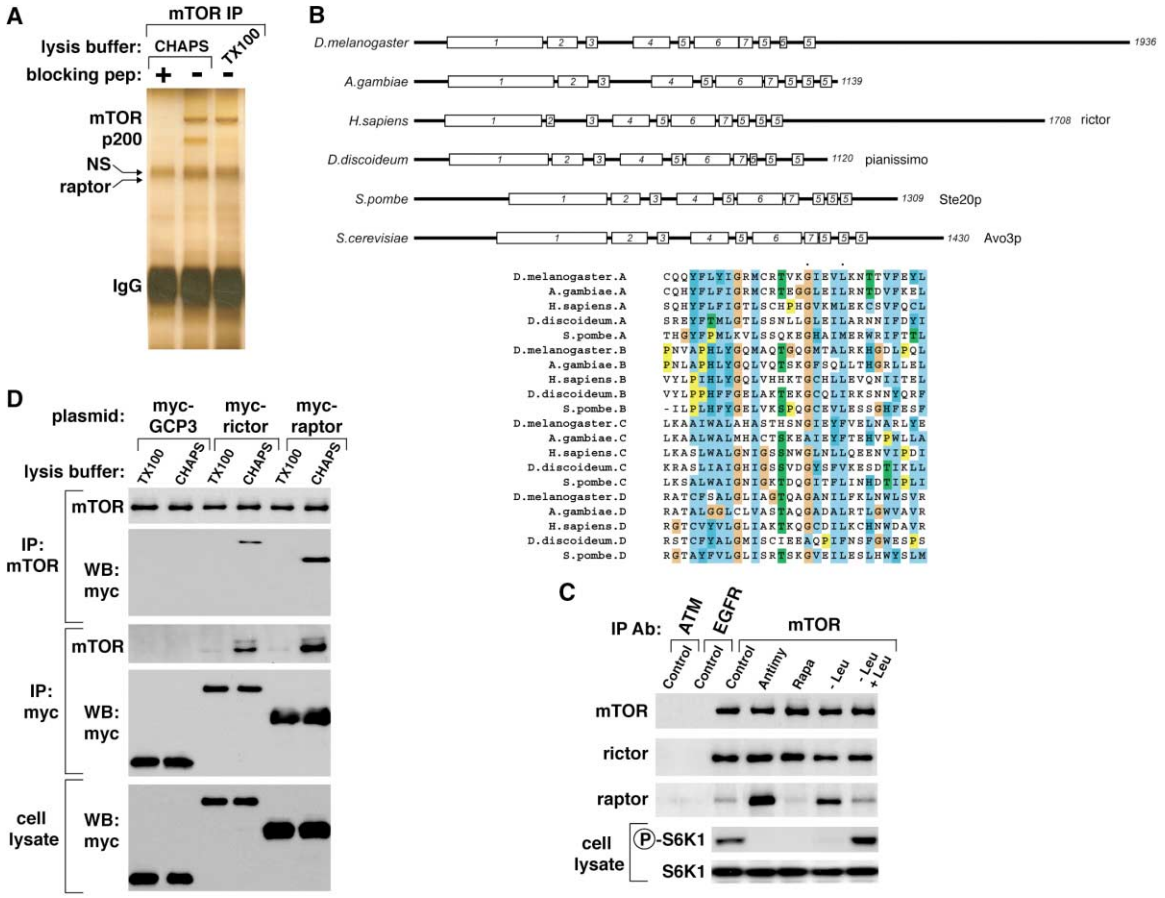

Figure 1. Rictor Is a Novel mTOR-Associated Protein

(A) Silver stain of SDS-PAGE analysis of mTOR immunoprecipitates prepared from HeLa cells lysed in a CHAPS- or Triton X-100-containing buffer. Plus sign indicates inclusion of the blocking peptide for the mTOR antibody during the immunoprecipitation. The ~200 kDa band corresponds to rictor and a nonspecific band (NS) obscures raptor.

(B) Putative rictor homologs share common domain architectures. Indicated proteins have seven domains with sequence conservation and similar relative locations within each protein and are shown schematically as boxes. Domain five is repeated four times within each of the homologs and the multiple sequence alignment shows the sequence pattern of this repeat. Sequences with the following accession numbers were used to create the alignment: *D. melanogaster*, AAQ22398.1; *A. gambiae*, XP_309233.1; *H. sapiens*, AY515854; *D. discoidieum*, AAC35553.1; *S. pombe*, NP_596021.1; *S. cerevisiae*; NP_011018.1.

(C) Specific interaction between endogenous mTOR and rictor. Immunoprecipitates prepared from HEK293T cells with the indicated antibodies were analyzed by immunoblotting for mTOR, rictor, and raptor. Prior to use, cells were treated with 5 μM Antimycin A for 15 min (Antimy), 20 nM rapamycin for 15 min (Rapa), deprived of leucine for 90 min (−Leu), or deprived of leucine and stimulated with 52 μg/ml leucine for 10 min (−Leu+Leu). Immunoblots for phospho-T389 S6K1 and S6K1 show that cell treatments had the intended effects. Abbreviations: EGFR, epidermal growth factor receptor; ATM, ataxia telangiectasia mutated.

(D) Endogenous mTOR interacts with recombinant rictor and raptor. Cellular lysates and mTOR immunoprecipitates prepared from HEK293T cells expressing myc-rictor, myc-raptor, or myc-GCP3 were analyzed by immunoblotting for myc-tagged proteins. In parallel, anti-myc immunoprecipitates were analyzed by immunoblotting for mTOR.

2A and data not shown). Although mTOR complexes in HeLa, HEK293T, and DU145 cells contain about the same amount of mTOR, those in HeLa and DU145 cells have more rictor than those in HEK293T cells. The opposite is true for raptor, as mTOR complexes in HEK293T cells have more raptor than complexes in HeLa and DU145 cells (Figure 2A). Budding yeast has two distinct TOR complexes defined by proteins with similarity to raptor and rictor [6, 7]. To determine if this is true in mammalian cells, we isolated mTOR complexes using antibodies recognizing mTOR, raptor, or rictor and then determined the composition of the isolated complexes. As expected, mTOR isolated with the mTOR antibody associates with raptor, rictor, and GβL in a detergent-sensitive manner (Figure 2B). In contrast, complexes isolated with the raptor antibody contain mTOR and GβL but not rictor, while those isolated with the rictor antibody contain mTOR and GβL but not raptor (Figure 2B). Thus, raptor and rictor independently associate with mTOR and GβL and define two distinct mTOR complexes. Consistent with this, the expression of wild-type raptor, but not of a mutant that cannot bind mTOR [1], suppresses the interaction of co-expressed rictor with mTOR (Figure 2C).

As rapamycin does not affect the interaction between rictor and mTOR (Figure 1C) and in yeast the AVO3p-

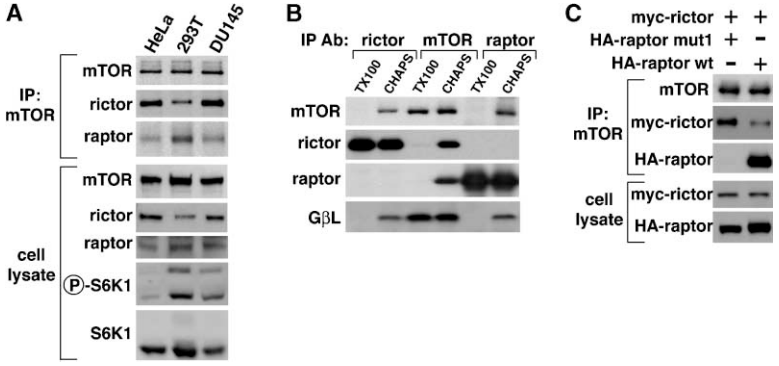

Figure 2. Rictor and Raptor Define Two Distinct mTOR-Containing Complexes

(A) Immunoblot analyses for indicated proteins of mTOR immunoprecipitates and cell lysates prepared from HeLa, HEK293T, and DU145 cells. Equal amounts of total protein were analyzed from each cell type.

(B) Immunoblot analyses for the presence of the indicated components of the mTOR signaling complex in immunoprecipitates prepared from HEK293T cell lysates with antibodies against rictor, mTOR, or raptor.

(C) Recombinant wild-type raptor, but not a mutant raptor, suppresses the binding of rictor to mTOR. mTOR immunoprecipitates prepared from HEK293T cells expressing the indicated tagged proteins were analyzed by immunoblotting with anti-myc and anti-HA antibodies.

containing TOR complex is rapamycin insensitive [6], we asked if the rictor-mTOR complex interacts with FKBP12-rapamycin. Our previous work shows that FKBP12-rapamycin destabilizes the raptor-mTOR interaction so that no raptor remains bound to mTOR [1]. As others have not observed this [3, 6], we suspected that a component of our buffer system might be critical for the destabilizing effect of rapamycin on the mTOR-raptor interaction. This turns out to be the case—we find that for rapamycin to affect the interaction, the cell lysis buffer must contain a molecule with a phosphate group (sodium pyrophosphate and β-glycerophosphate in our buffer) (Figure 3A). Using a phosphate-free buffer, we were able to show that in the presence of rapamycin, HA-FKBP12 expressed in HEK293T cells binds to the raptor- but not the rictor-containing mTOR complexes (Figure 3B). Thus, rictor, analogous to yeast AVO3p, is unlikely to participate in rapamycin-sensitive functions of mammalian TOR.

To confirm this, we determined whether rictor regulates S6 Kinase 1 (S6K1), a controller of cell size and an mTOR substrate [11, 12] whose phosphorylation state is rapamycin sensitive [13, 14]. Unlike reductions in raptor or mTOR expression [1], an siRNA-mediated knockdown of rictor does not decrease the phosphorylation of S6K1 in HEK293T or HeLa cells (Figure 3C). In contrast, we observe a slight increase in phospho-S6K1 that correlates with a small increase in the amount of raptor in mTOR complexes from cells with reduced rictor expression. Similarly, in *Drosophila* S2 cells, dsRNA-induced RNAi against dS6K, dTOR, or dRaptor eliminates the phosphorylation of dS6K while a dsRNA targeting dRictor causes an increase in dS6K phosphorylation (Figure 3D). As might be expected, the activation of S6K caused by reductions in rictor levels leads to a small increase in the mean sizes of human and *Drosophila* cells (data not shown). Thus, rictor is a positive regulator neither of cell size nor of S6K phosphorylation, and intracellularly the composition of the mTOR complex is dynamic so that decreases in rictor levels lead to increases in the amount of the raptor-containing mTOR complex.

Consistent with these findings, rictor-containing mTOR complexes purified from HEK293T or HeLa cells do not phosphorylate S6K1 in vitro (Figure 3E). In contrast, raptor-containing mTOR complexes—isolated with antibodies recognizing mTOR or raptor—phosphorylate S6K1 in a rapamycin-sensitive fashion. A potential explanation for the inability of the rictor-mTOR complex to phosphorylate S6K1 might be that when bound to rictor, mTOR is inactive. This is unlikely because mTOR still appears to autophosphorylate in rictor-containing complexes from HeLa or HEK293T cells (see arrows in Figure 3E) and the rictor complex phosphorylates the nonphysiological substrate myelin basic protein (data not shown). In kinase assays, a protein of the same apparent molecular weight as rictor becomes phosphorylated (see arrows in Figure 3E), suggesting that rictor may be a substrate for mTOR. This may be true because in cells metabolically labeled with radioactive phosphate, a reduction in mTOR expression decreases the amount of radioactivity in rictor without affecting its expression (Supplemental Figure S1A at http://www.current-biology.com/cgi/content/full/14/14/1296/DC1). In cells with reduced mTOR expression, rictor appears as a doublet in SDS-PAGE analyses, suggesting that dephosphorylated rictor migrates more quickly than the phosphorylated protein (Supplemental Figure S1B), a result we confirmed using in vitro phosphatase treatment of immunoprecipitated rictor (Supplemental Figure S1C). Using this shift in rictor migration, we searched for conditions that affect rictor phosphorylation within cells. Treatment of cells with LY294002, a PI 3-Kinase and mTOR kinase inhibitor [15], increased rictor mobility while rapamycin had no effect. Of many different stress conditions tested, only a sorbitol-induced osmotic stress increased rictor mobility (Supplemental Figure S1D).

Only recently are we beginning to understand the biochemical composition and regulation of the raptor-containing mTOR complex that is the target of rapamycin. Even before its characterization, many molecular (e.g., S6K1 phosphorylation) and cellular (e.g., cell size control) functions were ascribed to it because of their sensitivity to rapamycin. The rictor-containing mTOR complex does not appear to participate in rapamycin-sensitive processes and for us, an important insight into a function for rictor began with a fortuitous observation. In immunoblots prepared from cells with reduced rictor expression, we noticed a decrease in the intensity of a faint

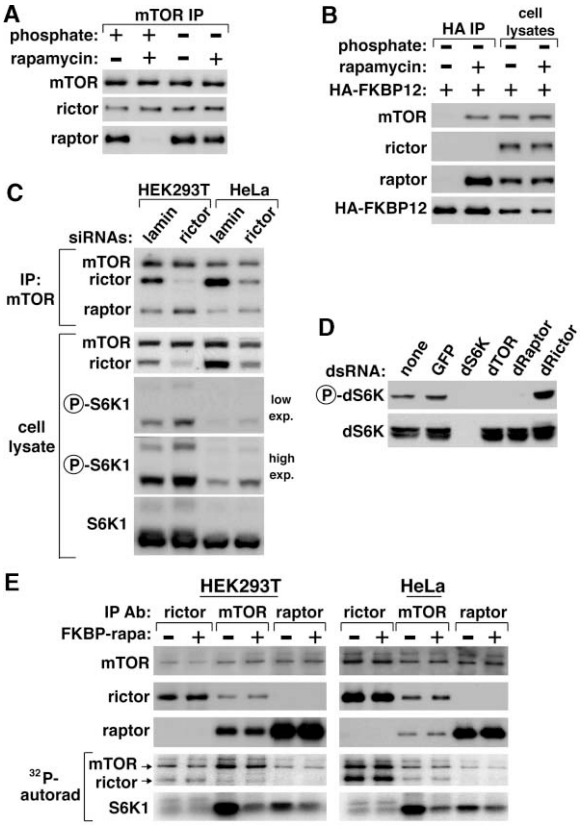

Figure 3. Rictor Does Not Participate in Rapamycin-Sensitive mTOR Functions

(A) The sensitivity of the raptor-mTOR interaction to rapamycin depends on the presence of phosphate-containing molecules in the lysis buffer. mTOR immunoprecipitates prepared from cells treated with or without 20 nM rapamycin for 10 min and lysed in a phosphate-containing or phosphate-free buffer were analyzed by immunoblotting for the indicated proteins.

(B) Raptor and mTOR, but not rictor, copurify with FKBP12-rapamycin. Anti-HA immunoprecipitates prepared from HEK293T cells expressing HA-FKBP12 and treated with or without 20 nM rapamycin for 15 min were analyzed by immunoblotting for the indicated proteins.

(C) Suppression of rictor expression slightly increases the amount of raptor in the mTOR complex and S6K1 activity. mTOR immunoprecipitates and cell lysates prepared from HEK293T or HeLa cells transfected with siRNAs targeting lamin or rictor were analyzed by immunoblotting for the indicated proteins.

(D) Suppression of *Drosophila* rictor expression increases the phosphorylation state of dS6K. The indicated dsRNAs were applied to *Drosophila* S2 cells and cell lysates were analyzed by immunoblotting with the mammalian phosphospecific S6K1 and *Drosophila* S6K antibodies.

(E) The rictor-containing mTOR complex does not phosphorylate S6K1. Immunoprecipitates prepared with the indicated antibodies were used in mTOR kinase assays using S6K1 as a substrate [1]. Where indicated, immunoprecipitates were treated with 100 nM FKBP12-rapamycin for 40 min before the start of the assays. Immunoblotting was used to monitor the levels of rictor, mTOR, and raptor in the kinase reactions.

background band recognized by the phospho-T389 S6K1 antibody (Supplemental Figure S2A). We reasoned that the antibody was cross-reacting with a protein containing a similar phosphorylation site, so we asked if the cross-reacting protein could be one of the Protein Kinase C (PKC) isoforms that are known to have phosphorylation sites homologous to T389 of S6K1. Using phosphospecific antibodies, we discovered that a reduction in rictor expression decreases the phosphorylation of S657 of PKCα (Figure 4A) but not of homologous sites in PKCϵ and PKCμ (Supplemental Figure S2B). The band recognized in immunoblots by the S657 PKCα phosphospecific antibody represents PKCα because siRNAs targeting PKCα reduces its intensity (Supplemental Figure S2C). That rictor expression affects PKCα phosphorylation was particularly interesting because in yeast, the rapamycin-insensitive TOR complex signals through PKC1 to regulate the actin cytoskeleton [6], although the role of PKC1 phosphorylation is unknown. Using lentiviral-mediated expression of siRNAs [16], we generated a set of HeLa cell lines with substantially reduced levels of rictor, raptor, or mTOR (Figure 4A). As expected, reductions in raptor or mTOR expression decreased the phosphorylation of S6K1 and a known substrate, eEF2K [17]. On the other hand, a reduction in rictor expression slightly increased S6K1 and eEF2K phosphorylation while, like a reduction in mTOR expression, decreasing PKCα phosphorylation. Neither rapamycin treatment nor a decrease in raptor expression affected the phosphorylation of PKCα (Figure 4A). PKCα has less kinase activity in the rictor and mTOR knockdown cells (Figure 4B), consistent with the importance of S657 phosphorylation for PKCα kinase activity [18]. Rictor and mTOR also regulate PKCα phosphorylation in *Drosophila* because dsRNAs targeting dRictor and dTOR, but not dRaptor, reduced the phosphorylation of a protein with the predicted molecular weight of dPKCα (Figure 4C). We have not found environmental stimuli that affect S657 phosphorylation (data not shown), but the classic PKC activator, phorbol myristate acetate (PMA), increases S657 phosphorylation in a manner sensitive to rictor and mTOR expression (Supplemental Figure S2D).

In mammalian cells, PKCα is ubiquitously expressed and has been implicated in varied cellular processes, including apoptosis, growth, cell cycle control, and the regulation of cell shape and mobility [19]. HeLa cells with constitutively reduced levels of rictor do not have apparent defects in cell proliferation, but we noticed that these cells are flatter and have a more square-like shape than controls (data not shown). Because in yeast AVO3p regulates actin organization through PKC1 in a rapamycin-insensitive manner [20], we reasoned that an altered actin cytoskeleton might account for the perturbed morphology of the rictor knockdown cells. In control cells and the smaller raptor knockdown cells, actin localizes to the cell cortex as well as diffusely throughout the cell cytoplasm (Figures 4D and 4E). In contrast, in rictor knockdown cells, thick actin fibers are present throughout much of the cytoplasm and cortical actin is less prominent. Many cells have cytoplasmic bundles of thick actin fibers that look like stress fibers and do not have clear connections to the remainder of the actin cytoskeleton (arrow in Figure 4D, rictor actin panel). In cells with reduced mTOR expression, the pattern of the actin staining is similar to that in rictor knockdown cells (arrow in Figure 4D, mTOR actin panel), al-

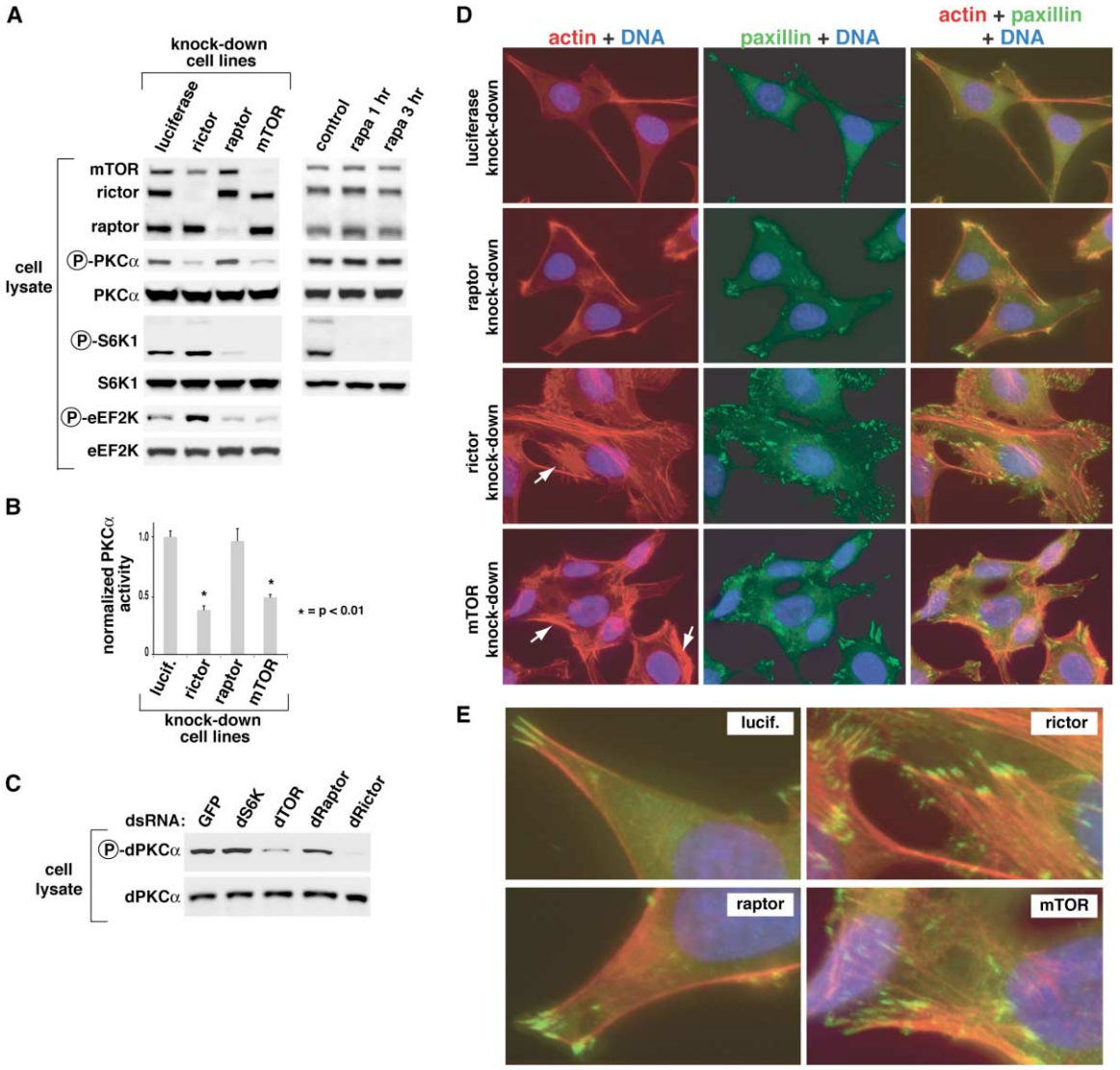

Figure 4. Rictor and mTOR, but not Raptor, Regulate the PKCα Phosphorylation State and the Actin Cytoskeleton

(A) Immunoblotting was used to analyze the phosphorylation states of PKCα (S657), S6K1 (T389), and eEF2K (S366) in HeLa cells with reduced expression of rictor, raptor, or mTOR. Lentiviruses were used to express siRNAs targeting rictor, raptor, mTOR, or luciferase.

(B) Kinase activity of PKCα isolated from samples like those in (A). Assay was performed in triplicate and activity was normalized to the luciferase (lucif.) control.

(C) dsRNAs corresponding to the genes for the indicated proteins were transfected into S2 *Drosophila* cells. After 4 days, lysates were prepared and analyzed by immunoblotting for dPKCα and phospho-dPKCα levels.

(D) Staining for actin (red), paxillin (green), and DNA (blue) reveals the organization of the actin cytoskeleton in HeLa cells infected with the siRNA-expressing lentiviruses described in (A). Arrows point to bundles of actin fibers. Images captured with a 60× objective are shown.

(E) Higher magnification of portions of the merged images from (D).

though more difficult to appreciate because of the reduced size of mTOR knockdown cells. The localization of paxillin, an adaptor protein present at the junction between the actin cytoskeleton and the plasma membrane [21], is also altered in the rictor and mTOR knockdown cells. These cells have many cytoplasmic paxillin patches that colocalize to the ends of the thick actin fibers while in the control and the raptor knockdown cells, the paxillin patches are present mainly at the cell periphery within cellular extensions (Figures 4D and 4E). As PKCα activity is reduced in rictor and mTOR knockdown cells, we asked if a PKCα knockdown affects the actin cytoskeleton of HeLa cells (Supplemental Figure S2E). The morphology of the actin cytoskeleton in cells with siRNA-mediated reductions in PKCα is similar but not a mimic of that in the rictor knockdown cells. Both have thick cytoplasmic actin fibers and less cortical actin staining than controls, but in the PKCα knockdown cells the thick actin fibers appear more numerous, better organized, and connected to the remainder of the cytoskeleton. Thus, similar to AVO3p and TOR in yeast, our findings indicate that rictor and mTOR regulate the

organization of the actin cytoskeleton and that PKCα is a mediator of this function. As the raptor-mTOR complex has several effectors, it is likely that the rictor-mTOR complex will have more than one effector involved in controlling the actin cytoskeleton. In yeast, the AVO3p-containing TOR complex regulates a GDP/GTP exchange factor (ROM2 GEF) that controls the capacity of RHO1 to bind and activate PKC1 [20]. Mammals have numerous RHO GEFs, and whether rictor-mTOR regulates PKCα through a similar mechanism remains to be tested. In our hands, rictor-mTOR neither binds nor phosphorylates PKCα, but given the complex mechanisms involved in the phosphorylation of S6K1 by raptor-mTOR, these negative findings must be taken with caution because the phosphorylation of substrates by rictor-mTOR might be equally complicated.

We can only speculate as to what the rictor branch of the mTOR pathway senses as signals that regulate the raptor branch of the pathway, such as nutrients and growth factors, do not regulate PKCα phosphorylation or the mTOR-rictor association. As the rictor-mTOR complex is not a target of rapamycin, it is unlikely to participate in cellular functions ascribed to mTOR based on their sensitivity to rapamycin. However, small molecules that directly inhibit the mTOR kinase, such as the well-known PI 3-kinase inhibitor, LY294002, may also suppress signaling by rictor-mTOR. Thus, the rictor complex may mediate functions assigned to PI 3-Kinase because of their sensitivity to LY294002 and insensitivity to rapamycin. It is also possible that with long-term rapamycin treatment of cells, FKBP12-rapamycin may bind to free mTOR and eventually inhibit the rictor-mTOR complex by perturbing its assembly. Now that two distinct mTOR complexes are known, with different downstream targets, it may be possible to isolate small molecules that selectively inhibit the rictor branch of the pathway and which are likely to have different pharmacological effects than rapamycin and direct mTOR inhibitors.

Experimental Procedures

For Experimental Procedures and additional figures, see Supplemental Data online at http://www.current-biology.com/cgi/content/full/14/14/1296/DC1.

Acknowledgments

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