P-Rex1 Links Mammalian Target of Rapamycin Signaling to Rac Activation and Cell Migration*§

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Polarized cell migration results from the transduction of extracellular cues promoting the activation of Rho GTPases with the intervention of multidomain proteins, including guanine exchange factors. P-Rex1 and P-Rex2 are Rac GEFs connecting Gβγ and phosphatidylinositol 3-kinase signaling to Rac activation. Their complex architecture suggests their regulation by protein-protein interactions. Novel mechanisms of activation of Rho GTPases are associated with mammalian target of rapamycin (mTOR), a serine/threonine kinase known as a central regulator of cell growth and proliferation. Recently, two independent multiprotein complexes containing mTOR have been described. mTORC1 links to the classical rapamycin-sensitive pathways relevant for cell growth and proliferation; mTORC2 links to the activation of Rho GTPases and cytoskeletal events via undefined mechanisms. Here we demonstrate that P-Rex1 and P-Rex2 establish, through their tandem DEP domains, interactions with mTOR, suggesting their potential as effectors in the signaling of mTOR to Rac activation and cell migration. This possibility was consistent with the effect of dominant-negative constructs and short hairpin RNA-mediated knockdown of P-Rex1, which decreased mTOR-dependent leucine-induced activation of Rac and cell migration. Rapamycin, a widely used inhibitor of mTOR signaling, did not inhibit Rac activity and cell migration induced by leucine, indicating that P-Rex1, which we found associated to both mTOR complexes, is only active when in the mTORC2 complex. mTORC2 has been described as the catalytic complex that phosphorylates AKT/PKB at Ser-473 and elicits activation of Rho GTPases and cytoskeletal reorganization. Thus, P-Rex1 links mTOR signaling to Rac activation and cell migration.

P-Rex1 and P-Rex2 are Rac guanine exchange factors connecting G protein-coupled receptors, through Gβγ and phosphatidylinositol 3-kinase, to Rac activation. In particular, P-Rex1 has been associated with the activation of Rac2, generating reactive oxygen species in neutrophils. P-Rex2 (showing two splice variants) is similarly regulated by Gβγ and phosphatidylinositol 3-kinase. Northern blot assays revealed a differential distribution of the two members of the P-Rex family, suggesting that they exert equivalent functions in different cellular populations (1–3, 7–11). The complex architecture of this family of proteins, constituted by a catalytic DH domain, followed by a phosphatidylinositol 3,4,5-trisphosphate-sensitive pleckstrin homology domain, two DEP and two PDZ domains in tandem, and a long carboxyl terminus (except for P-Rex2b, which is the short version, having a reduced carboxyl terminus), suggests that these Rac guanine exchange factors might be regulated by diverse protein-protein interactions modulating signal transduction pathways associated with the activation of Rac. In fact, in the developing brain, P-Rex1 is associated with neuronal migration in response to nerve growth factor (12, 13).

The mammalian target of rapamycin, mTOR, a highly conserved serine-threonine kinase activated in response to growth factors, amino acids and glucose, is a central regulator of cell growth, proliferation and, according to recent findings, is also critical for cell migration (14–25). mTOR has been found to be part of the multiprotein complex mTORC1. This is a rapamycin-sensitive complex, composed of mTOR, GβL/mLST8, and Raptor, which regulates cell growth by promoting mRNA translation, ribosome biogenesis, and autophagy (26–28).

Only recently, a second mTOR complex was described. mTORC2 is constituted by mTOR, GβL/mLST8, and Rictor/mAVO3; this complex phosphorylates AKT/PKB at Ser-473 and regulates the actin cytoskeleton via rapamycin-insensitive mechanisms involving Rho GTPases (4–6). The initial reports on the characterization of mTORC2 demonstrated the ability of this complex to activate Rho GTPases and cytoskeletal events and opened the possibility that mTORC2 would be responsible for cell movement in response to agonists activating mTOR pathways. Interestingly, a recent report indicated that both

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§ The abbreviations used are: P-Rex, phosphatidylinositol 3,4,5-trisphosphate- and Gβγ-regulated guanine-nucleotide exchange factor for Rac; GEF, guanine nucleotide exchange factor; mTOR, mammalian target of rapamycin; DH, Dbl homology; PDZ, PSD-95/Discs-large/ZO-1 homology; DEP, domain found in Dishevelled, Egl-10, and Pleckstrin; AKT, serine/threonine kinase with a pleckstrin homology domain; shRNA, short hairpin RNA; TIAM, T-lymphoma invasion and metastasis; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GST, glutathione S-transferase; HA, hemagglutinin 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GFP, green fluorescent protein; S6K, S6 kinase.
mTORC1 and mTORC2 are participants of type I insulin-like growth factor-stimulated motility (15). It is not yet clear if differential signals activate mTORC1 and mTORC2 complexes. Neither is known what effector systems are directly sensitive to mTORC2 activation. Although the participation of Rho guanine exchange factors in the activation of Rho GTPases and cytoskeletal dynamics elicited by mTORC2 is expected, their identity and mechanism of action remain to be revealed. Here we identified the Rac guanine exchange factors P-Rex1 and P-Rex2 as the putative catalytic components of mTORC2 critical for the activation of Rac and cell migration elicited by leucine through the rapamycin-insensitive mTOR complex 2. These results implicate that P-Rex family members link mTORC2 signaling to Rac activation and cell migration in response to the activation of the rapamycin-insensitive mTOR complex.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The DEP-DEP domains of P-Rex1 were used as a bait to screen a human brain cDNA library with the Matchmaker System III (Clontech) following both the manufacturer’s instructions and the ones indicated by Vazquez-Prado et al. (29). Clones were obtained under high stringency conditions using media lacking adenine, histidine, leucine, and tryptophan. The specificity of the interaction between the DEP-DEP P-Rex1 domain with the mTOR carboxyl-terminal domain was confirmed using the control bait and prey provided by the Matchmaker System III kit.

Constructs and Antibodies—prk5-myc-Rictor, prk5-myc-Raptor, and prk5-HA-G were obtained from D. Sabatini (see Ref. 4). The shRNA for P-Rex1 corresponds to RNA interference codex HP_187924 cloned into the pSM2 vector, which was generated based on the sequence of the hairpin TGCTGTTGACAGTGAGCGAGGACACAC-TGTGCTCCAGATTAGTGAAG.
**P-Rex1, Effector of mTOR-dependent Cell Migration**

CCACAGATGTAATCTGGAAGCACAGTGCTCCGTGCC- 
TACTGCTCGGA. mTOR shRNA was obtained from P. 
Amorphophilomthol. Anti-P-Rex1 rat monoclonal antibodies were 
obtained from M. Hoshino (see Ref. 13).

**Cell Culture and Transfection**—HEK 293T cells and HeLa 
cells were maintained in Dulbecco’s modified Eagle’s medium 
containing 10% fetal bovine serum. Cells were transfected using 
Polyfect (Qiagen) according to the manufacturer’s instructions 
and harvested after 48 h. When indicated cells were starved of 
leucine for 2 h in Dulbecco’s modified Eagle’s Speciality Media 
(Cell & Molecular Technologies) before stimulation.

**Cross-linking Assay and Immunoprecipitations**—HEK 293T 
cells in 10-cm dishes were placed on ice, rinsed once with 
phosphate-buffered saline, and lysed in 1 ml of ice-cold buffer (50 
mm Tris (pH 7.5), 5 mm EDTA, 150 mM NaCl, and either 1% 
Triton X-100 or 0.3% CHAPS, for immunoprecipitation of 
mTOR complexes) containing protease inhibitors (1 mM phenyl-
methanesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). For cross-linking assays 2.5 mg/ml dithiobis(succin-
imidypropionate) (Pierce) was added to the lysis buffer, and 
beads were added and rotated for 45 min at 4 °C. Beads were 
washed three times with 1 ml of lysis buffer and resuspended 
in 1X protein sample buffer. The interaction between endogenous P-Rex1 
and mTor was detected using lysates from HeLa cells processed 
as described for HEK 293T cells; in this case, mTor antibody 
was from Santa Cruz Biotechnology and PTEN antibody, also 
from Santa Cruz Biotechnology, was used as a control, and both 
were goat polyclonal antibodies; 10 μl of each antibody per 1 ml 
of lysate was used. Endogenous P-Rex1 was detected with Anti-
P-Rex1 rat monoclonal antibodies obtained from M. Hoshino 
(see Ref. 13).

**Immunoblotting**—Proteins were transferred to Immobilon 
membranes (Millipore), and the following antibodies were used 
for the immunoblots: mouse monoclonal antibodies against 
Rac1 (Transduction Laboratories), GFP (Covance), FLAG 
(Sigma), HA (Covance), Myc (Covance), rabbit monoclonal 
antibodies against mTOR (Cell Signaling), phospho-S6 riboso-
mal protein Ser-240/244 (Cell Signaling), S6 ribosomal protein 
(Cell Signaling), and GST (prepared in the laboratory).

**Rac-GFP Assays**—HeLa cells in 10-cm dishes were 
transfected with the indicated plasmids and shRNAs; 48 h later, cells 
were starved and stimulated as described above. When 
directed, cells were incubated with rapamycin (Calbiochem) for 30 
min before the assay. Cells were rinsed once with phosphate-
buffered saline and lysed in 1 ml of ice-cold buffer (50 mm Tris 
(pH 7.5), 5 mm EDTA, 150 mm NaCl, 10 mm MgCl2 and 1% 
Triton X-100) containing protease inhibitors (1 mM phenyl-
methanesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Extracts were cleared, and aliquots were saved to 
check for total protein expression. 50 μl of GST-PAK CRIB 
beads were added and rotated for 45 min at 4 °C. Beads were 
washed three times with 1 ml of lysis buffer and resuspended 
in 30 μl of protein sample buffer, boiled, and loaded onto a 
12% gel.

**Migration Assays**—Serum-free media containing either 
L-leucine or 10% FBS, which was used as a positive control, was 
placed in the bottom wells of a Boyden chamber; media con-
taining HeLa cells, starved for 2 h in serum-free media lacking 
leucine, were added to the top chamber. The two chambers 
were separated by a polycarbonate filter membrane (Neuro 
Probe, 8 μm-pore), coated with 10 μg/ml fibronectin (Calbio-
chem). After a 6-h incubation, membranes were stained with 
crystal violet, placed on a glass slide, and scanned. Densitomet-
ric quantitation was performed with ImageJ software. Where 
directed cells were previously transfected with plasmids and 
shRNAs and starved for 2 h before this assay as described above. 
For the rapamycin assay, cells were starved for 2 h and preincub-
cubated with rapamycin for 30 min. Rapamycin was added to 
the cell suspension placed on the top chamber wells and also

![FIGURE 2. Interaction of endogenous mTOR with P-Rex1.](Image)

![FIGURE 3. mTOR interacts with all the different P-Rex family members.](Image)
to the chemoattractant added to the bottom chamber wells in order to be present during the migration time.

**In Vitro Translation and in Vitro Interactions**—All *in vitro* translation reactions were carried out using the Expressway cell-free *Escherichia coli* expression system (catalog number K9901-00, Invitrogen) according to the manufacturer instructions. Briefly, for each reaction we mixed in an Eppendorf tube, the *E. coli* SlyD-Extract, 2.5× *in vitro* protein synthesis *E. coli* reaction buffer (−amino acids), 50 mM amino acids (−Met), 75 mM methionine, T7 Enzyme Mix. 3XFLAG·mTor prey cloned into pEF1/His (Invitrogen) was used as DNA template containing the T7 promoter. The reactions were incubated in a shaker at 3000 rpm at 37°C for 30 min. After 30 min of incubation, 50 μl of Feed Buffer (2× IVPS Feed Buffer, 50 mM amino acids (−Met), 75 mM methionine) was added, followed by an additional incubation for 6 h. The reactions were terminated by adding 1 ml of ice-cold buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100 containing protease inhibitors). *In vitro* translated 3XFLAG·mTor prey was incubated overnight with glutathione beads containing either P-Rex1 carboxyl-terminal domain or P-Rex1 DEP·DEP domains, both expressed as GST fusion proteins isolated from transfected HEK 293T cells. Before incubation with *in vitro* translated mTor prey, beads were washed three times with 0.5 M NaCl. The isolated complexes were denatured in SDS sample buffer under reducing conditions, resolved by 10% SDS-PAGE, and transferred to Immobilon membranes for Western blotting using FLAG and GST antibodies.

**RESULTS**

mTOR Interacts with P-Rex1 in Yeast and Mammalian Cells—In order to identify proteins that may regulate P-Rex1, we used the tandem DEP domains of P-Rex1 (Fig. 1A) as bait in a yeast two-hybrid screen using a human brain cDNA library. We found that in yeast P-Rex1 DEP·DEP interacted with the carboxyl-terminal region, including the kinase domain, of mTOR; this interaction was specific

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**FIGURE 4. mTOR signals to Rac via P-Rex1.** A, HeLa cells in serum-free media lacking leucine were stimulated with 52 μg/ml of i-leucine for the indicated times, and Rac-GTP was isolated with PAK-CRIB fused to GST. Lysates were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting for Rac, phosphorylated S6, and total S6. mTOR stimulation by leucine activated Rac after 1 min and remained for 10 min. Leucine also induced phosphorylation of the ribosomal protein S6 for the same period of time. Cells transfected with the Rac DEP·DEP domains of P-Rex1 were stimulated with leucine; Rac activity and S6 phosphorylation were determined as indicated above. Controls included cells transfected with empty shRNA vector (control) or TIAM with and without mTOR shRNA. A representative blot detecting the indicated proteins is shown. Rac activity as well as S6 phosphorylation decreased dramatically after leucine stimulation in cells transfected with mTOR shRNA. B, HeLa cells transfected with mTOR shRNA were stimulated with leucine; Rac activity and S6 phosphorylation were determined as indicated above. Controls included cells transfected with empty shRNA vector (control) or TIAM with and without mTOR shRNA. A representative blot detecting the indicated proteins is shown. Rac activity and S6 phosphorylation were determined as indicated above. Controls included cells transfected with EGFP or TIAM with and without dominant-negative P-Rex1. A representative blot detecting the indicated proteins is shown. A significant decrease in Rac activity was found in cells transfected with the dominant-negative P-Rex1. C, HeLa cells transfected with P-Rex1 shRNA were stimulated with leucine; Rac activity and S6 phosphorylation were determined as indicated above. Controls included cells transfected with empty shRNA vector (control) or TIAM with and without P-Rex1 shRNA. A representative blot detecting the indicated proteins is shown. A significant decrease in active Rac after leucine stimulation was found in cells transfected with P-Rex1 shRNA. E, HEK 293T cells were co-transfected with 3XFLAG-tagged P-Rex1, GST·mTOR prey, and either EGFP or EGFP-DEP·DEP. GST was affinity-purified with glutathione beads, and total cell lysates (TCL) and pull-downs were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting. The interaction of mTOR prey with 3XFLAG-tagged P-Rex1 was weakened by the transfection of the DEP·DEP domains of P-Rex1 compared with cells transfected with EGFP. GST was used as negative control. F, HeLa cells transfected with the DEP·DEP domains of P-Rex1 were stimulated with leucine; Rac activity was determined as indicated above. Controls included cells transfected with EGFP or TIAM. A representative blot detecting the indicated proteins is shown. Rac activity decreased dramatically after leucine stimulation in cells transfected with the DEP·DEP domains of P-Rex1. **WB:** Western blot.
were transfected into the human epithelial cell line HEK293T as GST fusion and 3XFLAG-tagged proteins, respectively. The corresponding cell extracts were incubated with glutathione Sepharose beads. The carboxy-terminal region of mTOR (mTOR prey) was detected associated with P-Rex1-DEP-DEP fused to GST but not with GST used as control (Fig. 1C). Endogenous mTOR also interacted with P-Rex1-DEP-DEP expressed as a GST fusion protein (Fig. 1D). To further analyze this interaction, HEK293T cells were co-transfected with the mTOR prey fused to GST and GFP-tagged P-Rex1-DEP-DEP domains (Fig. 1E) or HA-tagged mTOR prey and 3XFLAG-tagged P-Rex1 (Fig. 1F). In either case, both the bait (P-Rex1-DEP-DEP) and full-length P-Rex1 specifically interacted with mTOR prey (Fig. 1, E–F). To demonstrate a direct interaction between P-Rex1 and mTOR, an in vitro interaction assay was conducted using in vitro translated FLAG-tagged mTOR prey and recombinant P-Rex1-DEP-DEP domains or P-Rex1 carboxy-terminal domain (used as control), both expressed as GST fusion proteins. P-Rex1 domains were affinity-purified and washed with 0.5 M NaCl before the interaction assay. mTOR prey was detected associated with P-Rex1 DEP-DEP (Fig. 1G). The interaction of both endogenous proteins was also assessed by immunoprecipitating mTOR, and detecting P-Rex1, a control lysate from cells in which P-Rex1 was knocked down, was included in the Western blot to further support the identification of the band corresponding to endogenous P-Rex1 (Fig. 1F). We next wished to evaluate the possible interaction between mTOR and other members of the P-Rex family. HEK 293T cells were co-transfected with GST-tagged mTOR prey and GFP-tagged P-Rex1, P-Rex2a, or P-Rex2b (Fig. 3). The GST pulldown assays proved that mTOR not only interacts with P-Rex1 but also with P-Rex2a and P-Rex2b. No interaction was observed with the Rho GEF PDZ-RhoGEF (PRG) used as control (Fig. 3). These results, together with the two-hybrid screen, demonstrate that P-Rex family of Rac GEFs, including P-Rex1, P-Rex2a, and P-Rex2b, are able to interact directly with mTOR, opening the possibility that this group of GEFs could participate in the activation of Rac and cytoskeletal events downstream of mTORC2.

mTOR Activates Rac via P-Rex1—The rapamycin-insensitive mTOR-containing complex, mTORC2, has been found to control the actin cytoskeleton through Rho GTPases (4, 6). Thus, we wanted to determine whether the interaction of mTOR with P-Rex1 could be involved in the recently described ability of mTORC2 to promote the activation of Rac. Since amino acids activate mTOR, we decided to use L-leucine as a model to evaluate the role of this protein in Rac activation and cell migration. First, we evaluated whether mTOR stimulation by leucine would promote the formation of active, GTP-bound, Rac in HeLa cells (Fig. 4A). The amount of GTP-bound Rac increased with the addition of leucine after 1 min of stimulation and remained elevated for 10 min. These results correlated with the activation of S6K, a very well characterized mTOR effector, determined by an increase in the phosphorylation of its substrate, S6, in response to leucine, in samples from the same cell lysates. TIAM, a well characterized Rac GEF, used as positive control, was able to induce activation of Rac and promote the phosphorylation of the ribosomal protein S6. Next, considering that knocking down mTOR with specific short interfering RNA has been reported to decrease basal Rac-GTP in NIH 3T3 cells (6), we evaluated the participation of mTOR in leucine-induced Rac activation; mTOR shRNA was transfected, and the amount of GTP-bound Rac was measured after leucine stimulation (Fig. 4B). The ability of leucine to induce the activation of Rac was dramatically decreased in cells transfected with mTOR shRNA; as expected, the phosphorylation of S6, induced by leucine, was also prevented in cells in which mTOR was knocked down (Fig. 4B). These results correlated with the important decrease of mTOR detected by Western blot (Fig. 4B). To investigate the participation of P-Rex1 in the ability of mTORC2 to promote Rac activation in response to leucine, two independent approaches were used. First, a dominant-negative P-Rex1 (ΔDH-P-Rex1) was transfected, and the amount of GTP-bound Rac was measured after leucine stimulation (Fig. 4C). Second, P-Rex1 shRNA was used to knock down the expression of endogenous P-Rex1 (Fig. 4D). The activation of Rac induced by leucine was significantly decreased by both complementary approaches (Fig. 4, C and D). These results indicate a relevant participation of this GEF in the signaling pathway from mTOR to Rac.

It is well known that branched chain amino acids such as leucine regulate the mTORC1 complex by activation of mTOR and further phosphorylation of the S6 protein (27, 32). In these experiments we wished to address the state of phosphorylation of the S6 after leucine stimulation in nontransfected cells (Fig. 4A) and in cells transfected with the mTOR shRNA (Fig. 4B), the dominant-negative P-Rex1 (Fig. 4C), and the shRNA to P-Rex1 (Fig. 4D). The results show a significant decrease in the
mTOR promotes cell migration via P-Rex1. A, HeLa cells transfected with EGFP or dominant-negative P-Rex1 (EGFP ΔDH-P-Rex1) were subjected to chemotaxis assays 48 h post-transfection. Cells starved for 2 h in serum-free media lacking leucine were stimulated with L-leucine (520 μg/ml) for 6 h at 37 °C in a Boyden chamber. 10% FBS was used as positive control. Migrating cells were stained, scanned, and analyzed by densitometry. A remarkable increase in the number of control migrating cells after a leucine or FBS stimulation was found. Cells transfected with dominant-negative P-Rex1 showed a dramatic decrease in their migration when stimulated by leucine. Relative cell migration (% of FBS) was determined by comparing control cells with cells transfected with the dominant-negative. Three independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with control group. B, HeLa cells transfected with empty shRNA vector (control), P-Rex1 shRNA, or mTOR shRNA were subjected to chemotaxis assays, stained, and analyzed by densitometry as described in A. An increase in the number of control migrating cells was found after leucine or FBS stimulation. Cells transfected with P-Rex1 shRNA and mTOR shRNA showed a significant decrease in their migration when stimulated by leucine. Relative cell migration (% of FBS) was determined by comparing control cells with cells transfected with the dominant-negative. Three independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with control group. C, HeLa cells were starved for 2 h, preincubated with either vehicle (DMSO) or rapamycin (20 and 100 ng/ml, respectively) for 30 min, and placed in a Boyden chamber. Rapamycin or vehicle was also added to both the cell suspension and the chemoattractants (leucine and 10% FBS) to the same concentrations indicated above. Cells were thus left to migrate in the presence of rapamycin for 6 h at 37 °C. Migrating cells were stained, scanned, and analyzed by densitometry. Only a slight decrease in the number of migrating cells toward serum was found when rapamycin was present at the higher concentration. Relative cell migration (% of FBS) was determined by comparing vehicle-treated cells with rapamycin-treated cells. Four independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with vehicle group. D, HeLa cells were starved for 2 h, incubated with either vehicle (DMSO) or rapamycin (20 ng/ml), and stimulated with L-leucine (52 μg/ml) for the indicated times. Cells were lysed, and a Rac-GTP pulldown assay was carried out. Lysates were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting for GTP-bound Rac, total Rac, phosphorylated S6, and total S6. A representative blot is shown. No significant decrease in active Rac after leucine stimulation was found in cells treated with rapamycin compared with vehicle-treated cells. E, model depicting P-Rex1 as a part of both mTOR complexes and only active in mTORC2, in which this GEF is involved in Rac signaling and cell migration.

P-Rex1, Effector of mTOR-dependent Cell Migration

mTOR promotes cell migration via P-Rex1. A, HeLa cells transfected with EGFP or dominant-negative P-Rex1 (EGFP ΔDH-P-Rex1) were subjected to chemotaxis assays 48 h post-transfection. Cells starved for 2 h in serum-free media lacking leucine were stimulated with L-leucine (520 μg/ml) for 6 h at 37 °C in a Boyden chamber. 10% FBS was used as positive control. Migrating cells were stained, scanned, and analyzed by densitometry. A remarkable increase in the number of control migrating cells after a leucine or FBS stimulation was found. Cells transfected with dominant-negative P-Rex1 showed a dramatic decrease in their migration when stimulated by leucine. Relative cell migration (% of FBS) was determined by comparing control cells with cells transfected with the dominant-negative. Three independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with control group. B, HeLa cells transfected with empty shRNA vector (control), P-Rex1 shRNA, or mTOR shRNA were subjected to chemotaxis assays, stained, and analyzed by densitometry as described in A. An increase in the number of control migrating cells was found after leucine or FBS stimulation. Cells transfected with P-Rex1 shRNA and mTOR shRNA showed a significant decrease in their migration when stimulated by leucine. Relative cell migration (% of FBS) was determined by comparing control cells with cells transfected with the indicated shRNAs. Three independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with control group. C, HeLa cells were starved for 2 h, preincubated with either vehicle (DMSO) or rapamycin (20 and 100 ng/ml, respectively) for 30 min, and placed in a Boyden chamber. Rapamycin or vehicle was also added to both the cell suspension and the chemoattractants (leucine and 10% FBS) to the same concentrations indicated above. Cells were thus left to migrate in the presence of rapamycin for 6 h at 37 °C. Migrating cells were stained, scanned, and analyzed by densitometry. Only a slight decrease in the number of migrating cells toward serum was found when rapamycin was present at the higher concentration. Relative cell migration (% of FBS) was determined by comparing vehicle-treated cells with rapamycin-treated cells. Four independent experiments were averaged and plotted. Error bars represent S.E. *, p < 0.05 difference with vehicle group. D, HeLa cells were starved for 2 h, incubated with either vehicle (DMSO) or rapamycin (20 ng/ml), and stimulated with L-leucine (52 μg/ml) for the indicated times. Cells were lysed, and a Rac-GTP pulldown assay was carried out. Lysates were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting for GTP-bound Rac, total Rac, phosphorylated S6, and total S6. A representative blot is shown. No significant decrease in active Rac after leucine stimulation was found in cells treated with rapamycin compared with vehicle-treated cells. E, model depicting P-Rex1 as a part of both mTOR complexes and only active in mTORC2, in which this GEF is involved in Rac signaling and cell migration.
with the Src homology 3 domain of STAM) that was used as a negative control (Fig. 5B).

**mTOR Induces Cell Migration via P-Rex1**—Regulation of the actin cytoskeleton by mTORC2 has been described (4, 6). Moreover, recent findings in Dictyostelium show the involvement of the TORC2 complex in chemotaxis and cell polarity (33). So far, we have demonstrated that leucine activates Rac via mTOR and P-Rex1-dependent pathways; therefore, we wished to determine whether leucine could also induce cell migration dependent on P-Rex1. The ability of HeLa cells to migrate in the presence of leucine was demonstrated (supplemental Fig. 1). Then a migration assay was performed using HeLa cells transfected with the dominant-negative P-Rex1 or the shRNA to P-Rex1, and cells transfected with empty shRNA vector (pENTR) were also used as a control. Cells were either non-stimulated or stimulated with leucine or fetal bovine serum as a positive control and left to migrate for 6 h at 37 °C. The results show a remarkable increase in the number of migrating cells when stimulated with leucine or serum in control cells. On the contrary, cells transfected with either dominant-negative P-Rex1 or P-Rex1 shRNA showed a dramatic decrease in the number of migrating cells only when stimulated with leucine (Fig. 6, A and B). As expected, cells in which mTOR was knocked down with specific shRNA were unable to migrate in response to a leucine gradient (Fig. 6B). A transfection efficiency of at least 50% (monitored with GFP) was accomplished for these experiments. To discern if mTORC1 is relevant for the chemokinetic activity induced by leucine, we evaluated the migration of HeLa cells incubated with rapamycin, an inhibitor of mTORC1; the results, shown in Fig. 6C, indicated that only serum-induced migration of HeLa cells was only slightly decreased in presence of a high dose of rapamycin, whereas the one induced by leucine was not affected by it. A Rac activation assay was also carried out; the results (Fig. 6D) showed no decrease in Rac activity after leucine stimulation in cells treated with rapamycin compared with control cells. Overall, these results demonstrate that leucine induces cell migration and Rac activation in a P-Rex1-dependent manner involving the participation of the mTORC2 complex.

**DISCUSSION**

Our findings provide a novel mechanism by which mTORC2 can control the actin cytoskeleton through the activation of Rho GTPases (4, 5). Based on our results, we can postulate that by virtue of its ability to form molecular complexes with mTOR, P-Rex1 can link mTORC2 activation to the stimulation of Rac signaling, leading to the regulation of the actin cytoskeleton and cell migration. This molecular pathway involves proteins that have previously been identified as part of the mTORC2 complex, mLST8 and Rictor (4, 5) (Fig. 6E). Our data show that both P-Rex1 and mTOR are needed for Rac activation and cell migration induced by leucine, and whether they are part of the same signaling route or acting through independent pathways remains to be elucidated. However, as P-Rex1 is also a constituent of mTORC1, it may also participate in mTORC1-initiated Rac activation, as recently observed in cell migration in response to insulin-like growth factor (15); or alternatively, P-Rex1 may be a component of oligomeric complexes containing both mTORC1 and mTORC2 (16–18). Both possibilities are under current evaluation.

The fact that Tiam1 was able to activate both Rac and the ribosomal protein S6 drew our attention. These results are coincident with demonstrated ability of Rac to promote the activation of p70 S6K (30) and the ability of this Rac GEF, in a complex with spinophilin, to contribute to the activation of p70 S6K (31). The dominant-negative P-Rex1 and the shRNA to P-Rex1 had no effect on pS6 levels; this could be due to P-Rex1 participating only in Rac activation processes (15) even when it is also associated to complex 1 of mTOR.

Eukaryotic cells rely on the polarization of the actin cytoskeleton and chemotaxis to find nutrients in order to survive starvation conditions; the participation of TOR signaling in these events has been recognized in yeast and Dictyostelium discoideum (18, 19). In yeast, Rom2 is the RhoGEF acting downstream of TOR2 in the pathway leading to the reorganization of the actin cytoskeleton (20). Interestingly, ROM2 contains a DEP domain as part of its structure; thus the finding that P-Rex1 uses its DEP domains to interact with mTOR suggests that P-Rex1 and its homologs might represent the mammalian orthologs of ROM2. Indeed, our findings showing that HeLa cells can migrate in the presence of leucine, and the central role of P-Rex1 in this process, may reflect the existence of a mammalian counterpart of this ancestral survival mechanism. Furthermore, the identification of P-Rex1 as a participant in the signaling route by which mTOR controls Rac activation and cell migration may also provide a novel therapeutic target for pharmacological intervention in many pathological conditions that are characterized by the overactivity of mTOR, including human cancer (21).

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