PRAS40 Regulates mTORC1 Kinase Activity by Functioning as a Direct Inhibitor of Substrate Binding

Mammalian target of rapamycin (mTOR), a highly conserved Ser-Thr phosphatidylinositol 3-kinase-related protein kinase, is involved in the control of cell growth, survival, proliferation, and metabolism (1, 2). It is present in two structurally and functionally separate complexes, mTORC1 and mTORC2. mTORC1 is rapamycin-sensitive and contains mTOR, rictor, and mLST8 (also known as GβL), whereas mTORC2 is rapamycin-insensitive and contains mTOR, rictor, mLST8, and hSIN (2). mTORC1 catalyzes the phosphorylation of eIF4E binding protein-1 (eIF4E-BP1, also known as PHAS-I) and p70 S6 kinase 1 (p70 S6K1), whereas mTORC2 phosphorylates Ser-473 in the hydrophobic motif of Akt/PKB (3). Raptor associates with mTOR in the mTORC1 complex by multiple binding regions and has a positive role in mTOR kinase activity as evidenced by experiments that deletion or knockdown of raptor abolished mTORC1 activity (4–6). Raptor is thought to act as a scaffold to bind and present substrates to mTORC1, mediated by a putative TOR signaling motif (TOS motif) (4, 7–9). mLST8, a 36-kDa protein with 7 WD40 repeats, associates with the mTOR kinase domain and is present in both mTORC1 and -2 (10, 11). The TORC1 and TORC2 form multimeric complexes in yeast, flies, and mammalian cells (12–14).

Because of the critical role of the TOR kinase activity in controlling cell size and growth, many inputs, such as growth factors, amino acids, energy sufficiency, and environmental stress such as hypoxia, can regulate its activity (2). Extensive studies have been performed to understand the mechanisms whereby these inputs control mTOR activity. For example, regulation of the mTOR pathway by growth factors such as insulin appears to be mediated via the phosphatidylinositol-3′OH kinase-Akt pathway. The stimulation of eIF4E phosphorylation by insulin depends on activation of Akt, and Akt can directly phosphorylate mTOR (15–17). In addition Akt has been proposed to phosphorylate TSC2, a component of the tuberous sclerosis protein complex, TSC1/TSC2, and thereby affect its GTPase-activating protein activity toward the GTP-binding protein Rheb, a activator of mTOR (18). Regulation of mTORC1 by amino acids has been proposed to occur through hVPS34, a class III phosphatidylinositol-3′OH kinase, and by energy insufficiency through the AMP-activated protein kinase to tuberous sclerosis protein (19, 20).

However, the mechanism of mTOR activation remains controversial (21). We have recently demonstrated that insulin produced a stable increase of mTORC1 kinase activity, and this response was associated with a marked increase in substrate binding to raptor (14). In the present study we identify proline-rich Akt substrate (PRAS40, also known as AKT1S) as a novel partner of mTORC1. PRAS40 was previously isolated as a sub-}

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The abbreviations used are: mTOR, mammalian target of rapamycin; raptor, regulatory-associated protein of mTOR, and mLST8, is activated and phosphorylates eukaryotic initiation factor 4E-binding protein (4E-BP) and p70 S6 kinase to promote protein synthesis and cell size. Previously we found that activation of mTOR kinase in response to insulin was associated with increased 4E-BP1 binding to raptor. We therefore propose that PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

The abbreviations used are: mTOR, mammalian target of rapamycin; raptor, regulatory-associated protein of mTOR, 56k (aka 56 kinase); rictor, rapamycin-insensitive companion of mTOR; eIF4E, eukaryotic initiation factors 4E; FKBP12, FK506-binding protein of M, 12,000; GST, glutathione S-transferase; TOS, TOR signaling; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; HA, hemagglutinin; shRNA, short hairpin RNA; wt, wild type.
sequent activity. We, therefore, propose that another mechanism whereby insulin and potentially other growth factors regulate the mTORC1 activity is via an induced release of PRAS40 from the mTORC1 substrate binding site.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to mTOR (23), raptor (14), mLST8 (24), PRAS40 (22), eIF4E (25), and HA (14) have been described previously. Phosphospecific antibodies to the Thr-389 site of mTOR were from Cell Signaling Technology, Inc. FLAG and AU1 antibodies were from Sigma-Aldrich. Myc and GST epitope antibodies were from Santa Cruz Biotechnology, Inc. Recombinant human insulin (Novolin R) was from Novo Nordisk. Rapamycin, U0126, and FK506 were from Calbiochem-Novabiochem. TWEEN 20 was from Fischer. CHAPS was from Roche Applied Science. Nonidet P-40, Triton X-100, and wortmannin were from Sigma-Aldrich.

Cell Culture and Extract Preparation—3T3-L1 fibroblasts were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% newborn calf (Invitrogen) serum. Fibroblasts were converted to adipocytes by using differentiation medium as described previously (26). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) for 10–12 days after adding the differentiation medium. HEK293T, HEK293E, NIH-3T3, and CHO-IR cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. For experiments, the culture medium was replaced with Dulbecco’s modified Eagle’s medium overnight and then incubated at 37 °C without or with insulin and/or other additions. To terminate the incubation, the cells were rinsed once with chilled phosphate-buffered saline (145 mM NaCl, 5.4 mM KCl, and 10 mM sodium phosphate, pH 7.4) and then homogenized (0.8 ml of buffer/10-cm-diameter dish) in a syringe with a 20-gauge needle. Homogenization buffer was composed of buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 0.5 μM microcystin Lr. Buffer A contained 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% TWEEN 20 (unless otherwise indicated), 10 mM sodium phosphate, and 50 mM β-glycerophosphate, pH 7.4. Homogenates were centrifuged at 12,000 × g for 10 min, and the supernatants were retained for analyses.

Mutagenesis and Transfection—PRAS40 cDNAs having point mutations were generated by oligonucleotide-directed mutagenesis of HA-tagged PRAS40 in pcDNA3 by using a QuikChange II kit (Stratagene) according to the manual instructions. All mutations were confirmed by sequencing. 1 × 10⁶ of HEK293T or HEK293E cells were seeded in a 6-cm dish, and 24 h later plasmids were transfected using Lipofectamine 2000 (Invitrogen) at 1:1 ratio (w/v). The cells were harvested and analyzed at 36 h after transfection.

Lentivirus-mediated shRNA Silencing—Oligonucleotides encoding the short-hairpin RNA expression cassette targeting mouse PRAS40 from base 753–773 (GI:124376717) were annealed and subcloned into pLKO.1 (#8453, Addgene). The shRNA construct targeting green fluorescent protein from base 53,777 to 53,797 (GI:114145879) was used as the control. The shRNA constructs targeting human mTOR (#1855), raptor (#1857), rictor (#1853), and scramble (#1864) control were obtained from Addgene, and mLST8 was designed targeting base 307–329 (GI: 84626577) as reported before (11). HEK293T cells (10-cm dish) were co-transfected with 7.5 μg of plasmids expressing shRNA, 5 μg of pCMV-dR8.2dvpr, and 2.5 μg pCMV-VSV-G plasmids using Lipofectamine 2000. Virus-containing supernatants were collected at 36 and 60 h after transfection and used to infect NIH-3T3, CHO-IR, and HEK293T cells in the presence of 8 μg/ml Polybrene (Sigma). Infected cells were selected with 2.5 μg/ml puromycin (Sigma) and analyzed on the 7–10th day after infection.

Purification of Recombinant Proteins—His-tagged forms of wild type 4EBP1 and 4E-BP1 point mutations of Phe-113 to Ala (F113A) were expressed in bacteria and purified as described previously (27). The pGEX-4T-1 constructs encoding NH2-terminal GST-tagged PRAS40 and PRAS point mutations of Phe-129 to Ala (F129A) and GST-FKBPI2 were expressed in bacteria and purified as described previously (28). To assess purity and confirm concentrations of the recombinant proteins, samples were subjected to SDS-PAGE and stained with Coomassie Blue.

Immunoprecipitation and Kinase Assay—Cell extracts (800 μl for 10-cm dish) were incubated with antibodies (2 μg) bound to protein A-agarose beads (15 μl, for rabbit antibodies) or protein G-agarose beads (15 μl, for mouse antibodies) at 4 °C for 2 h with constant mixing. The beads were then washed 3 times with 1 ml of buffer A. For co-immunoprecipitation of endogenous PRAS40, antibodies were cross-linked on protein A-agarose beads by dimethyl pimelimidate (1 mg/ml) in 0.2 M sodium borate, pH 9.0, for 20 min at room temperature and then quenched by 0.1 M Tris-HCl, pH 8.0, at 4 °C for 2 h. Cross-linking assays in cells were performed as previously described (29). The mTOR kinase assay with immune complex was conducted as described before (14).

Binding of Raptor to 4EBP1 Affinity Resins and GST Pulldown Assays—4E-BP1 affinity resin was prepared as described previously (14). 800 μl of cell extracts were incubated with 15 μl (Amersham Biosciences) of beads coupled with His-tagged 4E-BP1 at 4 °C for 2 h with constant mixing and washed as described for immunoprecipitations.

GST-FKBPI2, GST-PRAS40, and mutants F129A (2 μg) were incubated with 15 μl of GSH-Sepharose beads in 1× phosphate-buffered saline for 1 h and then washed with 1 ml of buffer A 3 times. Before pulldown assays, the GST-FKBPI2-coupled beads were incubated with 1 μM either rapamycin, FK506, or Me3SO for 30 min and washed with buffer A. Cell extracts were added to the beads and incubated at 4 °C for 2 h with constant mixing. The beads were washed as described for immunoprecipitation.

Gel Filtration—Performed as previously described (14).

RESULTS

Identification of PRAS40 as an Interacting Partner for mTORC1—mTORC1 was immunoprecipitated from extracts of HEK293T cells using an antibody to the COOH-terminal region of raptor, and proteins were visualized by staining with Coomassie Brilliant Blue (Fig. 1A). To determine which proteins were specifically immunoprecipitated by virtue of their
immunoblots were performed to identify coimmunoprecipitated proteins. FLAG-raptor. mTORC1 and mTORC2 were isolated with FLAG antibodies, and with plasmids expressing AU1-mTOR, HA-PRAS40, and either FLAG-rictor or mTOR, rictor, raptor, mLST8, and PRAS40.

control from lysates of HEK293T cells. Immunoblots were performed for stably overexpressed FLAG-rictor (Fig. 2A). This revealed the identity of this protein as the proline-rich Akt substra (PRAS40) (22).

FIGURE 1. Identification of PRAS40 as an interacting partner for mTORC1. A, purification of mTORC1 with raptor antibodies (Ab). HEK293T cells infected with lentivirus expressing shRNA targeted to mTOR or rictor were lysed and immunoprecipitated (IP) with raptor antibodies (RAP) and non-immune IgG (NI) as control. After SDS-PAGE electrophoresis, the gel was stained with Coomassie Brilliant Blue. The bands representing mTOR, raptor, and mLST8 are indicated as well as a 40-kDa protein identified by mass spectrometry as PRAS40. B, PRAS40 interacts with mTORC1 but not mTORC2. mTOR complexes were isolated with antibodies to raptor (RAP), mTOR (TOR), FLAG (for stably overexpressed FLAG-rictor (FLAG-RCI)) and non-immune IgG (NI) as control from lysates of HEK293T cells. Immunoblots were performed for mTOR, rictor, raptor, mLST8, and PRAS40. C, HEK293T cells were transfected with plasmids expressing AU1-mTOR, HA-PRAS40, and either FLAG-rictor or FLAG-raptor. mTORC1 and mTORC2 were isolated with FLAG antibodies, and immunoblots were performed to identify coimmunoprecipitated proteins.

presence in mTORC1, mTOR or rictor levels were depressed by infecting the HEK293T cells with shRNA-expressing lentiviruses targeting the respective proteins. Raptor, mTOR, and mLST8 were detected in mTORC1 complexes in rictor knockdown cells, whereas mTOR and mLST8 recovery were substantially reduced in mTOR knockdown cells. A 40-kDa band appeared to be specifically precipitated in the mTORC1 complex by virtue of the fact that its presence was decreased in the mTOR knockdown cells in comparison to the rictor knockdown cells. Mass spectrometric analysis of the 40-kDa band revealed the identity of this protein as the proline-rich Akt substrate (PRAS40) (22).

To verify the interaction of PRAS40 with the mTOR complex, mTORC1 immune complexes were isolated from HEK293T cells with antibodies to either raptor or mTOR, whereas mTORC2 complexes were immunoprecipitated by FLAG antibodies from HEK293T cells infected with lentivirus encoding FLAG-rictor. PRAS40 was co-immunoprecipitated with mTORC1 by antibodies to either mTOR or raptor (Fig. 1B). Intact mTORC2 containing mTOR, rictor, and mLST8 were recovered by immunoprecipitation of FLAG-rictor. mTORC2 complexes did not contain PRAS40. We next examined the interaction between PRAS40 and mTOR complexes by transfecting AU1-mTOR and HA-PRAS40 into HEK293T cells with either FLAG-raptor or FLAG-rictor. Antibodies specific for FLAG were used to isolate mTORC1 or mTORC2 immune complexes. PRAS40 was only immunoprecipitated with FLAG-raptor (Fig. 1C). Based on our analyses of both endogenous and recombinantly expressed proteins, we conclude that PRAS40 is associated with mTORC1 but not mTORC2.

The Endogenous mTOR Complex Is Essential for PRAS40 Association, whereas Overexpressed Raptor Can Directly Interact with PRAS40—To further investigate the role of the individual components of mTORC1 in the interaction with PRAS40, we utilized lentiviral shRNA for mTOR, raptor, rictor, and mLST8 in HEK293T cells. Immunoblotting of total cell lysates demonstrated the effectiveness of the shRNA, with each of the targeted mTOR complex components exhibiting a decreased expression with their respective shRNA (Fig. 2A). In contrast, total endogenous PRAS40 levels were not decreased with any of these lentiviral shRNAs (Fig. 2A). However, either mTOR or raptor knockdown resulted in a substantial decrease of PRAS40 co-immunoprecipitation in mTORC1 isolated by either mTOR or raptor antibodies (Fig. 2A). In contrast, decreasing protein levels of either rictor or mLST8 were without effect on the amount of PRAS40 present in mTORC1. Because the total levels of mTOR remain largely unaffected by shRNA for raptor and the total levels of raptor remain largely unaffected by shRNA for mTOR, the decrease in PRAS40 in the precipitates of these two proteins indicates that the presence of either endogenous mTOR or raptor alone is insufficient to form a stable complex with endogenous PRAS40. Consistent with this model, the overexpression of mTOR alone was not sufficient to cause expressed PRAS40 to co-immunoprecipitate with this protein (Fig. 2B). However, when raptor was co-expressed with mTOR there was an association detected between mTOR and expressed PRAS40. Thus, raptor is likely necessary for mTORC1 association with PRAS40.

To determine the component of mTORC1 associated with PRAS40, extracts prepared with 0.2% of Tween 20, CHAPS, Nonidet P-40, and Triton X-100 were used for the immunoprecipitations of mTOR and raptor. Consistent with previous findings (4, 5, 11), intact mTORC1 was maintained in Tween 20 and CHAPS, whereas with Nonidet P-40 and Triton X-100, the mTOR, and raptor interaction was disrupted, but the mTOR and mLST8 interaction was preserved (supplemental Fig. S1A). Endogenous PRAS40 was detected in mTOR or raptor immunoprecipitates when Tween 20 or CHAPS was present. However, endogenous PRAS40 was not detectable in mTOR or raptor immunoprecipitates when Nonidet P-40 or Triton X-100 was present (supplemental Fig. S1A). Interestingly, when cells overexpressing mTOR and raptor were lysed in the presence of Triton X-100, expressed PRAS40 co-immunoprecipitated with FLAG-raptor but not FLAG-mTOR (Fig. 2C). In addition, purified recombinant PRAS40 coupled to glutathione beads retained only raptor when Triton X-100 was present, whereas both mTOR and raptor were retained on such beads when Tween 20 was utilized (supplemental Fig. S1B). The interaction
between expressed PRAS40 and expressed raptor in the presence of Triton X-100 demonstrates that PRAS40 can associate with raptor independent of mTOR under certain conditions. To better understand the nature of the interaction between raptor and PRAS40, Tween 20 or Triton X-100 extracts prepared from cells with or without raptor overexpression were size-fractionated on a Superose 6 HR 10/30 column. Raptor was immunoprecipitated from the various fractions and analyzed for mTOR, raptor, and PRAS40. As previously reported, mTORC1 exists as a large multimeric complex in cells (14). Multimeric complexes of expressed raptor without associated mTOR were also detected since co-expression of two differently tagged raptor plasmids resulted in co-immunoprecipitation of both tagged molecules with antibodies to a single tag even in the presence of Triton X-100 (supplemental Fig. S1C). In the presence of Tween 20 and in cells expressing only endogenous mTORC1, most of the PRAS40 immunoprecipitated with raptor was present in a complex that contained mTOR (Fig. 2D). Overexpression of FLAG-raptor in HEK293T cells resulted in the formation of multimeric raptor complexes without associated mTOR but containing PRAS40. In the presence of Triton X-100, PRAS40 association with the raptor-raptor multimeric complex was stable, whereas PRAS40 association with mTOR-raptor complex was dissociated (Fig. 2D). Thus, overexpressed raptor forms multimeric complexes that interact with PRAS40 in the presence of Triton X-100.

**Insulin Promotes a Marked Decrease of PRAS40 Association with mTORC1**—To investigate the role of PRAS40 on the activation of mTORC1 by insulin, PRAS40-mTORC1 interaction was examined in 3T3-L1 adipocytes. Insulin promoted a rapid decrease of PRAS40 association with mTORC1, which was essentially complete within 5 min (Fig. 3A). Concurrently insulin promoted the phosphorylation of Thr-389 in S6K (Fig. 3A). Significant effects of insulin on reducing PRAS40 and mTORC1 interaction were observed at concentrations as low as 0.6 nM, which coincided with insulin effects on the phosphorylation of S6K (Fig. 3B). The insulin-induced decrease of PRAS40 and mTORC1 association could be partially blocked by addition of the cross-linker, dithiobis(succinimidyl propionate), to the cells after

**FIGURE 2.** Both mTOR and raptor are required for association of endogenous mTORC1 with PRAS40, whereas overexpressed raptor directly associates with PRAS40. A, the interaction of endogenous mTORC1 and PRAS40 requires both mTOR and raptor. HEK293T cells were infected with shRNA lentiviruses targeting mTOR, raptor, mLST8, and scrambled shRNA. Endogenous mTOR complexes were immunoprecipitated (IP) with antibodies (Ab) to mTOR or raptor, and immunoblots were prepared to identify the levels of the complex components in cell lysates and co-immunoprecipitated proteins. B, overexpression of raptor is required for interaction between mTOR and PRAS40. FLAG-mTOR was co-transfected into HEK293T cells with HA-PRAS40 and either MYC-raptor, FLAG-rictor, or MYC-mLST8 as indicated. Immunoprecipitations using FLAG antibodies were conducted, and immunoblots were prepared to detect different epitope tags. C, PRAS40 interacts with overexpressed proteins of raptor but not mTOR in the presence of detergent Triton-X 100 (TX). HA-PRAS40 was co-expressed with FLAG-mTOR, Myc-raptor, FLAG-rictor, and AU1-mTOR in HEK293T cells. Extracts were prepared with 0.2% of either Tween 20 (TW) or Triton-X 100 as indicated and incubated with FLAG antibodies for immunoprecipitations. D, PRAS40 interacted with raptor multimeric complexes in the presence of Triton X-100. HA-PRAS40 with or without FLAG-raptor was transfected into HEK293T cells. Cell extracts prepared with 0.2% of Tween 20 or Triton X-100 were size-fractionated using a Superose 6 column with the same detergents as in the extracts. Each fraction was analyzed by immunoprecipitation with raptor antibody-coupled beads, and the precipitates were analyzed for mTOR, raptor, and HA-PRAS40 by immunoblotting. Elution of volumes and molecular weight standards are indicated at the top of the panel.
insulin treatment (Fig. 3B). To investigate which signaling pathway contributed to the insulin effect on PRAS40 association with mTORC1, adipocytes were incubated with inhibitors of several kinases. The insulin-induced decrease of PRAS40 and mTORC1 association was abolished by treating adipocytes with wortmannin at a concentration (100 nM) sufficient to block Akt activation but too low to inhibit mTOR directly (23). Inhibiting activation of mitogen-activated protein kinase with U0126 did not attenuate the effect of insulin. Rapamycin reduced PRAS40 association with mTORC1 in the absence of insulin signaling, but the decreased association from mTORC1 stimulated by insulin was still observed (Fig. 3C).

**Rapamycin Reduces Association of PRAS40 and mTORC1**

Rapamycin forms a complex with FKBP12 that directly binds to mTOR through an FKBP12 binding region known as the FRB domain (1). As seen in Fig. 3C, rapamycin markedly decreased the amount of mTORC1-associated PRAS40 in the absence of insulin treatment. To further investigate this, HEK293E cells were treated with either 20 nM rapamycin, 20 nM FK506, or vehicle (Me₂SO). Rapamycin decreased the amount of mTORC1-associated PRAS40, whereas FK506 had no effect (Fig. 3D). To determine whether the rapamycin effect was a direct effect, we utilized an mTOR mutant (S2035W), which does not bind rapamycin-FKBP12 (supplemental Fig. S2B) and is resistant to rapamycin treatment (30). The ability of rapamycin to reduce the association of PRAS40 from mTORC1 was abolished with this mutant mTOR, confirming that the PRAS40-mTOR sensitivity to rapamycin requires rapamycin-FKBP12 binding to mTOR (supplemental Fig. S2A). In vitro, rapamycin-FKBP12 bound to glutathione beads recovered mTOR and raptor, but not PRAS40, whereas control beads containing antibodies to the FLAG epitope did capture PRAS40 as well as raptor from the lysates of the cells expressing either FLAG-tagged wild type or mutant mTOR (supplemental Fig. S2B).

**Two Conserved Motifs Were Identified to Be Critical for PRAS40 Binding to mTORC1**—By aligning the PRAS40 amino acid sequence among different species including human, rat, Danio rerio, and Xenopus laevis, we found two conserved motifs that were potentially interesting. Amino acid residues 129–134 (human), Phe-Val-Met-Asp-Glu (FVMDE), are similar to the TOS motif in S6K1 and 4EBP1, namely FIDL and FEMA1, respectively. In addition, residues 182–185, Lys-Ser-Leu-Pro (KSLP), have similarity with the RAIP motif found in 4EBP1 (Fig. 4A). The TOS motif is required for raptor to bind S6K1 and 4EBP1 and the subsequent mTOR-mediated phosphorylation of S6K1 and 4EBP1 in vivo and in vitro (7–9). The RAIP motif regulates phosphorylation of 4EBP1 by mTOR; however, its role in 4EBP1 binding to raptor is controversial (31, 27). To test the role of these two putative motifs in PRAS40, Phe-129 to Ala (P129A) and Pro-185 to Ala (P185A) reduced the recovery of raptor with little change of the amount of mTOR by PRAS40 immunoprecipitation in the presence of Tween 20 (Fig. 4B and S3A), whereas the effect of
the two mutations on raptor-PRAS40 interaction was more complete in the presence of Triton X-100 (Fig. 4C and supplemental S3B). As shown in Fig. 2D, when overexpressing raptor, PRAS40 is associated with raptor-mTOR and raptor-raptor complex in the presence of Tween 20 but only with the raptor-raptor complex in the presence of Triton X-100. The S183A and P185A mutations, thus, appear to reduce PRAS40-raptor interaction in the absence of mTOR, but the complex containing both mTOR and raptor are little changed with these PRAS40 mutations. This suggests that mTOR might participate in association with PRAS40 and contribute to stabilize the interaction between raptor and these residues in PRAS40. In contrast, mutations of a number of other residues (including Pro-77, Pro-78, Pro-84, Pro-86, Pro-93, Pro-94, Thr-160, Pro-161, Phe-193, Ser-202, Ser-203, Thr-246, and Phe-249) had little effect.

PRAS40 Functions as a Negative Regulator of mTORC1 Activity—Because insulin reduces the association of PRAS40 from mTORC1, this could function in the insulin-induced activation of mTORC1 kinase activity. To investigate this hypothesis, we utilized the ability of mTORC1 to phosphorylate S6K1 in vivo and 4EBP1 in vitro. To measure the in vivo kinase activity of the expressed mTOR complex without interference with the activity of the endogenous mTOR, the mTOR rapamycin-resistant mutant (S2035W) was utilized, and endogenous mTORC1 activity was inhibited by rapamycin pretreatment. The mutant mTOR S2035W, raptor, and S6K1 were expressed together with either wt PRAS40 or a mutant PRAS40 in HEK293E cells, treated with rapamycin to block endogenous mTORC1 kinase activity, and then stimulated with insulin. Overexpression of wt PRAS40 resulted in an inhibition of phosphorylation of S6K1 at Thr-389 (Fig. 5A). In contrast, the PRAS40 mutant, F129A, which does not bind raptor, did not attenuate phosphorylation of S6K1, suggesting that this inhibitory effect of PRAS40 on mTORC1 depends on the binding of PRAS40 to mTORC1. To determine whether the inhibition by the expressed PRAS40 is due to a direct effect on mTORC1 itself, recombinant wt PRAS40 and F129A were expressed, purified from bacteria, and incubated in vitro with mTORC1.
isolated by raptor antibodies from insulin-treated 3T3-L1 adipocytes. Whereas wt PRAS40 abolished phosphorylation of 4EBP1 by mTORC1, the mutant F129A did not (Fig. 5B).

To examine the ability of the PRAS40 mutant to bind to mTORC1 in vitro, GST-tagged recombinant wt PRAS40 or the mutant F129A were first bound to glutathione beads and then tested for their ability to bind to mTORC1. In comparison to wt PRAS40, mutant F129A bound little mTORC1 (supplemental Fig. S3C). These data are in good agreement with the observed ability of these mutant molecules to inhibit in vitro mTORC1 kinase activity (Fig. 5B) and the effect of these mutations on the in vivo association with mTORC1 (Fig. 4B). Interestingly, prior treatment of the cells with insulin markedly increased both the amounts of mTOR and raptor bound by recombinant PRAS40 (supplemental Fig. S3C), presumably due to decreased levels of endogenous PRAS40 associating with raptor.

**Evidence That PRAS40 Prevents Substrate Binding to Raptor**—Because PRAS40 and mTOR substrates such as 4EBP1 and S6K1 bind raptor via their TOS motif, PRAS40 could occupy the site for substrate binding in raptor. To investigate whether PRAS40 has an effect on substrate binding, PRAS40 was knocked down in CHO-IR and NIH-3T3 cells, and the ability of mTORC1 to bind 4EBP1 was examined *in vitro*. Recombinant 4EBP1 coupled to CNBr-activated Sepharose beads retained both mTOR and raptor, but not PRAS40, from control lysates, indicating that the binding of 4EBP1 to mTORC1 is mutually exclusive with that of PRAS40 (Fig. 6A). Insulin enhanced the amounts of both mTOR and raptor retained by 4EBP1 beads, consistent with the previously demonstrated insulin-induced dissociation of PRAS40 from mTORC1. In contrast, in PRAS40 knockdown cells, the amounts of both mTOR and raptor recovered by 4EBP1 beads were increased, and insulin was without any further effect (Fig. 6A). Thus, PRAS40 knockdown has a similar effect on 4EBP1 binding to mTORC1 as insulin treatment. We next measured the mTOR kinase activity in mTORC1 immune complexes isolated from PRAS40 knockdown cells (Fig. 6B). When the kinase activity was normalized for the amount of mTOR present in the precipitates, 32P incorporation into 4EBP1 was elevated with PRAS40 knockdown, and the insulin stimulation was eliminated (Fig. 6C). These data are consistent with a negative role for PRAS40 in regulation of mTOR kinase activity by inhibiting access to substrates.

As shown previously (14), recombinant 4EBP1-coupled resin retains increased the amounts of mTORC1 and elf4E in response to insulin. This is because of the insulin-stimulated dissociation of the endogenous 4EBP1-elf4E complex. We previously demonstrated that the binding of raptor to 4EBP1 resin could be inhibited by supplementing extracts with free wild type 4EBP1, but not the 4EBP1 TOS motif mutant F114A (14). Similarly, supplementing extracts with recombinant wild type PRAS40, but not the TOS motif mutant F129A, inhibited raptor binding to the 4EBP1 resin (Fig. 6D). The elf4E binding motif in 4EBP1 is not found in PRAS40. This can be used as a measure of specificity for the competition reaction, as demonstrated by wild type or TOS mutant 4EBP1 competing with the 4EBP1 affinity resin for binding to elf4E, whereas PRAS40 does not (Fig. 6D). These results show that PRAS40 prevents 4EBP1 from binding raptor.

**DISCUSSION**

It is currently unclear how the mTOR complex is activated to phosphorylate 4EBP1 and S6K1 in response to insulin and nutrient stimulation. One hypothesis is that nutrients regulate the mTOR kinase activity via regulating its interaction with raptor (5). However, insulin does not appear to regulate mTOR and raptor interaction. It seems clear that a major function of raptor is to bind substrates such as 4EBP1 and S6K1 through a conserved TOS motif (4, 7–9). We have previously shown that insulin treatment enhanced 4EBP1 binding to raptor via the TOS motif, but the molecular mechanism for this increased substrate binding was unknown (14). In this study we have identified PRAS40 as an mTORC1 partner that interacts with raptor through a TOS motif. Moreover, we have found that insulin treatment reduced the amount of PRAS40 associated with mTORC1, and PRAS40 inhibits 4EBP1 binding to raptor.
In summary, we have found in the present study that PRAS40 is an insulin-regulated component of the mTORC1 complex. This interaction appears to be mediated via a TOS-like motif in PRAS40, and this interaction directly inhibits substrate binding. These findings directly explain the prior observations that insulin induces an increase in 4EBP1 binding and kinase activity in mTOR and provide a workable model for how insulin regulates mTORC1 enzymatic activity.

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