PRAS40 Is a Target for Mammalian Target of Rapamycin Complex 1 and Is Required for Signaling Downstream of This Complex*

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Signaling through the mammalian target of rapamycin complex 1 (mTORC1) is positively regulated by amino acids and insulin. PRAS40 associates with mTORC1 (which contains raptor) but not mTORC2. PRAS40 interacts with raptor, and this requires an intact TOR-signaling (TOS) motif in PRAS40. Like TOS motif-containing proteins such as eIF4E-binding protein 1 (4E-BP1), PRAS40 is a substrate for phosphorylation by mTORC1. Consistent with this, starvation of cells of amino acids or treatment with rapamycin alters the phosphorylation of PRAS40. PRAS40 binds 14-3-3 proteins, and this requires both amino acids and insulin. Binding of PRAS40 to 14-3-3 proteins is inhibited by TSC1/2 (negative regulators of mTORC1) and stimulated by Rheb in a rapamycin-sensitive manner. This confirms that PRAS40 is a target for regulation by mTORC1. Small interfering RNA-mediated knockdown of PRAS40 impairs both the amino acid- and insulin-stimulated phosphorylation of 4E-BP1 and the phosphorylation of S6. However, this has no effect on the phosphorylation of Akt or TSC2 (an Akt substrate). These data place PRAS40 downstream of mTORC1 but upstream of its effectors, such as S6K1 and 4E-BP1.

Signaling through the mammalian target of rapamycin complex 1 (mTORC1) plays key roles in cellular regulation and in human disease (1). mTORC1 contains several known proteins, including raptor (2, 3). mTORC1 regulates the phosphorylation and function of proteins linked to the control of mRNA translation, including the S6 kinases and the eukaryotic initiation factor 4E-binding proteins (4E-BPs). Each of these proteins contains a TOR-signaling (TOS) motif that is thought to bind raptor (4, 5) and confer regulation by mTORC1 (5, 6), which controls multiple phosphorylation sites in each of these proteins. The regulation of targets for mTORC1 signaling requires inputs from amino acids and agents, such as insulin. The latter involves the Akt-catalyzed phosphorylation of TSC2, which inhibits its ability to promote hydrolysis of GTP bound to the G-protein Rheb (Ras homologue enriched in the brain), a positive regulator of mTORC1 (7, 8). However, it is still not fully understood how these inputs activate mTORC1 or how this complex signals downstream, raising the possibility that additional components are involved in regulating mTORC1 or in its downstream signaling.

PRAS40 (proline-rich Akt (acutely transforming retrovirus AKT8 in rodent T cell lymphoma) substrate of 40 kDa; also termed Akt1S1 (Akt1 substrate 1) (9) or p39 (10)) binds 14-3-3 proteins. 14-3-3 proteins are dimeric and interact with a range of phosphoproteins (11). Such binding often requires two phosphoserine or threonine residues in the partner. 14-3-3 proteins are thought to play multiple roles in cellular regulation (11). The binding of PRAS40 to 14-3-3 proteins requires both insulin and amino acids to be present and is partially inhibited by rapamycin (10). These features suggested that PRAS40 might be a target for control by mTORC1.

EXPERIMENTAL PROCEDURES

Chemicals, Biochemicals, and Other Reagents—General laboratory chemicals were purchased from Sigma, Fisher, or Calbiochem. Rapamycin and MG-132 were from Calbiochem. Recombinant human insulin was purchased from Sigma. Digoxigenin was bought from Roche Applied Science. Tissue culture reagents were purchased from Invitrogen.

Sources of Antisera—Anti-Myc and -FLAG were from Sigma.

Anti-HA high affinity antibody was from Roche Applied Science.

mTOR (mTAb1) antisera were from Upstate Biotechnology, Inc. (Cell Signaling) and from Professor R. Denton (Bristol, UK). Raptor and rictor antisera were kind gifts of Dr. E. Jacinto (Piscataway, NJ). Phosphospecific and other antisera to TSC2 (C-20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific antisera for 4E-BP1, S6, Akt, glycogen synthase kinase 3α/β, and extracellular signal-regulated kinase
were purchased from Cell Signaling, as were antisera for total S6 and total Akt. Phospho-PRAS40 antibody was from Calbiochem. Anti-4E-BP1, previously described (12), was prepared by Dr. J. Parra. Anti-PRAS40 was raised in sheep using the peptide immunogen reported in Ref. 9 and was obtained from the Division of Signal Transduction Therapy (Dundee). Peroxidase-conjugated anti-digoxigenin antibody was purchased from Roche Applied Science.

Raptor and 14-3-3 Far Western Blotting Procedures—The raptor probe used in the raptor far Western blot was prepared by transfecting a 10-cm dish of HEK293 cells with 5 μg of Myc-raptor. Twenty-four hours following transfection, the medium was replaced with fresh complete medium. Sixteen hours following replacement of medium, cells were lysed in 500 μl of extraction buffer (13), and the lysate was preclarified by centrifugation at 16,000 × g for 10 min at 4 °C. The supernatant was collected and diluted 1:10 in extraction buffer (13) containing 5% (w/v) fat-free milk. For the raptor far Western, 0.5–1 μg of bacterially expressed GST-tagged 4E-BP1 or PRAS40 were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane by electroblotting. The membrane was blocked with 5% (w/v) fat-free milk in phosphate-buffered saline, 0.02% (v/v) Tween 20™ and then incubated overnight at 4 °C with the Myc-raptor lysate described above. The membrane was then probed with mouse anti-Myc antibody followed by incubation with hors eradiser peroxidase-conjugated anti-mouse antibody and ECL detection. Equal protein loading between samples was verified both by blotting with anti-GST antibody and staining with Coo massie Brilliant Blue. The 14-3-3 far Western blot (14), also referred to as 14-3-3 in vitro overlay assay, was used here to study the direct binding of 14-3-3 proteins to PRAS40. Forty micrograms of HEK293 cellular extract were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% (w/v) fat-free milk in phosphate-buffered saline, 0.02% (v/v) Tween 20™ and then incubated overnight at 4 °C with the Myc-raptor lysate described above. The membrane was then probed with mouse anti-Myc antibody followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody and ECL detection. Equal protein loading between samples was verified both by blotting with anti-GST antibody and staining with Coo massie Brilliant Blue. The 14-3-3 far Western blot (14), also referred to as 14-3-3 in vitro overlay assay, was used here to study the direct binding of 14-3-3 proteins to PRAS40. Forty micrograms of HEK293 cellular extract were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% (w/v) fat-free milk in phosphate-buffered saline, 0.02% (v/v) Tween 20™ and then incubated overnight at 4 °C with the Myc-raptor lysate described above. The membrane was then probed with mouse anti-Myc antibody followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody and ECL detection. Equal protein loading between samples was verified both by blotting with anti-GST antibody and staining with Coo massie Brilliant Blue.

Table 1: Small interfering RNAs used in this study

| siRNA       | Sequence                      |
|-------------|-------------------------------|
| PRAS40      | 5′-CCUUCGUGUGUACGCGGUGCAGGAUUG-3′ |
| Raptor      | 5′-CAGUAUACUGGUAAGAAGAAAGCAAGC-3′ |
| Scrambled   | 5′-CCUGUGACAGUGAGGUAUACUUCUUU-3′ |

Glucose (4.5 g/liter) Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G and transfected as described earlier (15). Cells were starved of serum for 16 h and, in some cases, depleted of amino acids by incubation in Dulbecco’s phosphate-buffered saline supplemented with 4.5 g/liter d-glucose, sodium pyruvate, and minimal essential medium vitamins for 90 min. Where indicated, cells were treated with 100 nm rapamycin (mTOR inhibitor) for 30 min prior to insulin stimulation (100 nm, 25 min). Cells were harvested in extraction buffer containing 1% (v/v) Triton X-100 as previously described (13), supplemented with 50 μM Mg-132. Lysates were preclarified by centrifugation at 16,000 × g for 10 min at 4 °C. The detergent Triton X-100, employed here, has been previously reported to destabilize protein–protein interactions within the mTOR complex 1 (2). Thus, for immunoprecipitation and kinase assays, we made use of an alternative extraction buffer containing the zwitterionic detergent CHAPS (0.3% (v/v)), as described in Ref. 2, with modifications; 50 μM Mg-132 was added to the extraction buffer, and lysates were preclarified by centrifugation at low speed (800 × g for 10 min). HA, Myc, raptor, rictor, and mTOR immunoprecipitates were prepared by incubating 1 mg of total protein with 40 μl of 50% (v/v) protein G-Sepharose slurry and the appropriate antisera for 3 h at 4 °C.

Vectors and siRNA Oligonucleotides—The vectors for FLAG-tagged Rheb, TSC1, and TSC2 were generous gifts of Dr A. Tee (Cardiff, UK). The Myc-raptor construct was a kind gift from Dr. J. Parra. Anti-PRAS40 was raised in sheep using the peptide immunogen reported in Ref. 9 and was obtained from the Division of Signal Transduction Therapy (Dundee). Peroxidase-conjugated anti-digoxigenin antibody was purchased from Roche Applied Science. Background information on siRNA oligonucleotide duplexes (Invitrogen) were used, in combination, for transient knockdown of PRAS40 or raptor (Table 1). Scrambled siRNA oligonucleotides, with identical

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nucleotide composition but scrambled sequence, were used as negative controls (Table 1).

Expression and Purification of GST Fusion Proteins in E. coli—
Expression and purification of the GST-tagged 4E-BP1 and PRAS40 proteins in E. coli were carried out as described previously (16).

mTOR Kinase Assays—mTOR, raptor, or rictor immunoprecipitates from growing HEK293 cells were prepared as described above. Kinase reactions were performed by incubating immunoprecipitates with 2 μg of purified GST-4E-BP1 or -PRAS40, 0.2 μCi of [γ-32P]ATP, 200 μCi unlabeled ATP, 4 mM MnCl2, and kinase buffer (17) in a final volume of 40 μl. Kinase reactions were carried out at 37 °C for 20 min, after which time they were stopped with excess (80 mM) EDTA.

In Vivo [32P] Radiolabeling and Two-dimensional Peptide Mapping—HEK293 cells were transfected with 10 μg of HA-PRAS40 wild type or mutants. 24 h following transfection, cells were starved of serum for 14 h. The cells were then depleted of phosphate in Krebs-Ringer buffer (107 mM NaCl, 5 mM KCl, 3 mM CaCl2, 1 mM MgSO4, 6.2 mM K2HPO4, 10 mM NaHCO3, 10 mM d-glucose, 0.1% (v/v) bovine serum albumin, 20 mM HEPES (pH 7.6), and 0.0015% (w/v) phenol red) supplemented with the following 10−X amino acid mixture (0.6 mM Trp, 2 mM Met, 2.5 mM His, 4 mM Tyr, 4 mM Cys, 4 mM Phe, 5 mM Arg, 10 mM Lys, 8 mM Thr, 8 mM Val, 8 mM Ile, 8 mM Leu, 40 mM Gln) for 1 h, followed by radiolabeling with [32P]orthophosphate for 3.5 h in the same buffer. During labeling, cells were, in some cases, deprived of amino acids for 3.5 h, followed by insulin stimulation (100 nM, 25 min). Where indicated, cells were also treated with rapamycin (100 nM, 30 min) prior to insulin stimulation. Cells were lysed, HA-PRAS40 was immunoprecipitated and resolved by SDS-PAGE, and band pieces containing radiolabeled HA-PRAS40 were excised from the gel. HA-PRAS40 was reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 for 15 min, alkylated with 50 mM iodoacetamide in 100 mM NH4HCO3 for 30 min, dehydrated in 100% (v/v) acetonitrile, and subjected to in-gel proteolytic digestion with 25 μg/ml chymotrypsin in 20 mM NH4HCO3, 0.1% n-octyl glucoside for 16 h at 25 °C with constant agitation. Proteolytic digests were separated by electrophoresis at 800 V for 1 h15 min in first dimension buffer (2.5% (v/v) formic acid, 7.8% (v/v) acetic acid) followed by chromatography in second dimension buffer (62% (v/v) isobutyric acid, 1.9% (v/v) n-butanol, 4.8% (v/v) pyridine, and 2.9% (v/v) acetic acid). Radiolabeled peptides were detected using the Typhoon™ PhosphorImager.

Quantitation and Statistical Analysis—Western blot data were scanned and quantitated using the Odyssey® infrared imaging system (Li-cor® Biosciences). Other Western blot data obtained by ECL were quantitated using ImageJ software (available on the World Wide Web). Statistical significance was determined using Student’s t test.

RESULTS

The Binding of PRAS40 to 14-3-3 Proteins Requires Signaling through mTORC1—It was previously reported that, in PC12 cells, the binding of endogenous PRAS40 to 14-3-3 proteins in the standard overlay (“far Western”) assay requires both amino acids and insulin (10). The data in Fig. 1A confirm that this is also the case in human embryonic kidney 293 (HEK293) cells.

We use HEK293 cells in preference to the widely employed HEK293T line, since the latter shows constitutive activation of phosphatidylinositol 3-kinase/Akt signaling, as indicated by the high level of phosphorylation of Thr246 in PRAS40 in serum-starved cells (supplemental Fig. S1). Probably as a consequence of elevated phosphatidylinositol 3-kinase/Akt signaling, mTORC1 signaling is also basally active, as judged from the elevated phosphorylation of S6, a substrate for the mTORC1-activated S6 kinases. These effects are probably a consequence of the transformation of HEK293 cells by SV40 that encode large and small T-antigens, previously shown to activate Akt signaling (18). In contrast, in HEK293 cells, Akt and mTORC1 signaling show only low basal activity and are markedly activated by treatment of the cells with insulin (supplemental Fig. S1), as they are physiologically. Extracellular signal-regulated kinase (mitogen-activated protein kinase) signaling is not basally active in HEK293T cells (supplemental Fig. S1).

Earlier studies showed that Thr246 can be phosphorylated by Akt/protein kinase B and that its phosphorylation is required for 14-3-3 binding (9). However, insulin induces substantial phosphorylation of Thr246 in PRAS40 even in the absence of amino acids (Fig. 1A), where 14-3-3 binding is not observed. This suggested that an additional amino acid-dependent input is required for 14-3-3 binding to PRAS40. The requirement for amino acids and insulin suggested that mTORC1 might play a role in regulating the binding of 14-3-3 proteins to PRAS40. Rapamycin modestly inhibits 14-3-3 binding to PRAS40; the partial nature of this inhibition may be similar to certain other effects of mTORC1 (such as the phosphorylation of some sites in 4E-BP1, which is largely insensitive to rapamycin (12, 19)). To ascertain whether PRAS40 binding to 14-3-3 proteins is indeed controlled by mTORC1, we used siRNA to deplete HEK293 cells of raptor, a key component of mTORC1 that is required for its downstream signaling. RNA interference was effective in knocking down the expression of raptor and in inhibiting signaling from mTORC1, as judged by the impaired insulin-induced phosphorylation of Ser65 and Thr70 in 4E-BP1 and of multiple sites in ribosomal protein S6, a substrate for the S6 kinases (Fig. 1B). Raptor knockdown also substantially impaired the insulin-promoted binding of 14-3-3 proteins to PRAS40 (Fig. 1B), indicating that PRAS40 is indeed regulated by mTORC1. It did not, however, affect insulin-induced phosphorylation of Ser473 in Akt (which is catalyzed by a distinct complex, mTORC2 (17)).

PRAS40 Binds Raptor via a TOS Motif—Other targets for mTORC1, such as 4E-BP1 and S6 kinase 1 (4, 5), bind raptor, and this is essential for their control by mTORC1 (5, 6). Since raptor is also required for regulation of 14-3-3 binding to PRAS40, we asked whether PRAS40 could bind raptor, using a raptor overlay assay (14). PRAS40, expressed as a GST fusion in E. coli, was resolved by SDS-PAGE and transferred to a membrane, which was probed with lysate from cells expressing Myc-tagged raptor. Myc-raptor bound GST-PRAS40 but not GST (Fig. 1C). Binding of Myc-raptor to GST-PRAS40 was strikingly

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interacts with 4E-BP1 and the S6 kinases via their short TOS motifs, and these motifs are required for their control by mTORC1 (4, 5). The F114A mutation in the 4E-BP1 TOS motif (FEMD) abolishes raptor binding (5, 6) (also see Fig. 1C). PRAS40 contains a sequence resembling a TOS motif (129FVMDE133 (Fig. 1D)). Fig. 1C shows that mutation to alanine of each of the first, third, or fourth residues in this region abolished the binding of PRAS40 to raptor. As an alternative to this assay, we also expressed HA-tagged PRAS40 in HEK293 cells and subjected the lysates to immunoprecipitation with anti-HA antisera. Wild type HA-PRAS40 co-immunoprecipitated with endogenous raptor, in accordance with other recent data (20, 21), but the F129A mutant did not (Fig. 1E), showing that the interaction of PRAS40 with raptor requires the TOS-like motif.

Since raptor interacts with mTOR, we asked whether PRAS40 is also associated with mTOR. PRAS40 is clearly present in mTOR immunoprecipitates, as are the known mTOR-binding partners raptor and rictor (Fig. 2A). Raptor is a component of mTORC1 but not of mTORC2, which contains rictor. Immunoprecipitation with anti-raptor resulted in the co-precipitation of mTOR and PRAS40 (Fig. 2B), whereas anti-rictor immunoprecipitated mTOR but not PRAS40. Together, these data suggest that PRAS40 stably associates with mTORC1 but not mTORC2.

Treatment of serum- and amino acid-starved HEK293 cells with insulin caused a modest reduction in the amount of PRAS40 that co-immunoprecipitated with raptor (Fig. 2C). Quantitation of data from three independent experiments revealed that insulin decreased the association of PRAS40 with raptor by 75% \((p < 0.05)\). Amino acids alone had no effect on the raptor-PRAS40 co-precipitation, but potentiated the ability of insulin to induce dissociation of PRAS40 from raptor. Treatment with rapamycin essentially abolished co-precipitation of raptor with PRAS40 and mTOR (Fig. 2C).

**FIGURE 1. PRAS40 binds raptor.** A, HEK293 cells were starved of serum (16 h) and, in some cases, subsequently starved of amino acids (90 min). The cells were then treated with rapamycin (100 nm, 30 min), where indicated, prior to stimulation with insulin (100 nm, 25 min). Lysates were subjected to 14-3-3 far Western blot analysis, using a digoxigenin-labeled 14-3-3 probe, or Western blot analysis using the indicated antisera. B, HEK293 cells were transfected with a 50 nM concentration of each of two siRNA oligonucleotide duplexes targeting the raptor mRNA or negative control scrambled siRNAs (as listed in Table 1). 24 h later, cells were starved of serum and then insulin-stimulated as in A. Cells were harvested, and 20 μg of (protein) lysate was analyzed by SDS-PAGE/immunoblot or by far Western blot as in A. C, purified GST-PRAS40 or indicated mutants. GST and wild type or F114A mutant 4E-BP1 (0.8 μg of each protein, expressed in E. coli) were subjected to SDS-PAGE/ electroblotting and then incubated with cell lysate containing Myc-raptor (far western) as described under “Experimental Procedures.” Similar membranes were also probed with anti-GST or stained with Coomassie to assess the levels of the proteins used. D, comparison of the sequence of residues 129–133 of human PRAS40 with known TOS motifs in other targets for mTORC1. E, HEK293 cells were transfected with 2 μg of HA-PRAS40 DNA or with an equal amount of empty vector. 24 h later, cells were serum-starved as in A and lysed. HA immunoprecipitates were prepared and analyzed by immunoblot using anti-HA or anti-raptor, as indicated. F, as E, but cells were stimulated with insulin following serum starvation. Lysates were analyzed by 14-3-3 far Western or by Western blot using the indicated antisera.

stronger in this assay than binding to GST-4E-BP1, used as a positive control. (The relative amounts of the fusion proteins may be compared by the GST blot; Fig. 1C, bottom). Raptor
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To test the functional significance of the PRAS40 TOS motif, we expressed wild type or F129A mutant PRAS40 in HEK293 cells. The F129A mutation almost abolished 14-3-3 binding in the far Western assay (Fig. 1F), consistent with the idea that mTORC1 regulates PRAS40 via the PRAS40 TOS motif and with the finding that raptor is required for the regulation of PRAS40 (Fig. 1B).

PRAS40 Is Phosphorylated by mTORC1—Other proteins that possess TOS motifs are phosphorylated by mTOR in vitro (e.g. see Ref. 22). As shown in Fig. 3A, mTOR immunoprecipitates can phosphorylate GST-PRAS40 (but not GST itself; not shown). Mutation of Phe129 to alanine within the TOS motif in PRAS40 markedly decreased its phosphorylation by mTOR (Fig. 3A and C), suggesting that it may be a substrate specifically for mTORC1. Quantitation of the data revealed that phosphorylation of the F129A mutant was decreased to 65% of that of wild type PRAS40 ($p < 0.05$). To test this, we used antibodies for raptor or rictor to immunoprecipitate mTORC1 or mTORC2 separately. mTORC1 robustly phosphorylated recombinant PRAS40 (Fig. 3B), whereas the labeling seen with mTORC2 was only at the background level (no IP lane). Thus, PRAS40 is a specific substrate for mTORC1. After the initial submission of this manuscript, Oshiro et al. (23) showed that PRAS40 is indeed phosphorylated by mTORC1, both in vitro and in vivo, and that the main site of modification is Ser183. We too observe that mutation of Ser183 to alanine decreases PRAS40 phosphorylation by mTOR in vitro, to the same extent as the F129A mutation.

PRAS40 Is Subject to Phosphorylation at Multiple Sites in Vivo—To study whether mTORC1 regulates PRAS40 phosphorylation in vivo, we expressed PRAS40 in HEK293 cells. The cells were subsequently transferred to phosphate-free medium, radiolabeled with $[^{32}P]$orthophosphate, and in some cases treated with rapamycin. After lysis, the radiolabeled PRAS40 was purified by immunoprecipitation and SDS-PAGE, and then digested with chymotrypsin (which yielded better resolved peptide maps than other proteases tested, including trypsin or Glu-C; not shown). The resulting phosphopeptides were displayed by two-dimensional mapping and autoradiography (Fig. 4). It is clear from the maps that the in vivo phosphorylation of PRAS40 is complex, with at least 11 phosphopeptides reproducibly being observed. Many of them occur in diagonal “ladders” where less negatively charged peptides run further in chromatography (i.e. are more hydrophobic).
This is consistent with these being variants of the same peptide(s) in different states of phosphorylation (e.g. see Ref. 24). Inspection of the sequence of PRAS40 does indeed reveal several potential chymotryptic peptides that contain multiple serine or threonine residues.

To study further the possible role of mTORC1 in regulating PRAS40 phosphorylation in vivo, we labeled cells in medium containing insulin with or without amino acids (Fig. 4, A and B). In Fig. 4C, amino acid-fed, insulin-stimulated cells were treated with rapamycin. Careful inspection of the complex peptide maps from PRAS40 revealed that, although most spots were present in all three cases, amino acid starvation caused a change in the pattern of negatively charged peptides (left-hand side of maps). In amino acid-fed cells that have been treated with insulin, three peptides (termed a–c in Fig. 4A) are observed. Amino acid starvation (which impairs mTORC1 signaling in these cells; e.g. see Fig. 1A) reproducibly led to the disappearance of peptide a and the appearance of a new spot (d), which carried a net positive charge (Fig. 4B). This behavior is consistent with amino acid starvation eliciting the partial dephosphorylation of a multiply phosphorylated peptide (e.g. at just one site). A similar pattern is seen when cells are treated with rapamycin to inhibit mTORC1. The effect was slightly less marked than when cells are starved of amino acids, as is also the case for the phosphorylation of certain sites in 4E-BP1 (T37/46 (12)) that are phosphorylated by mTORC1 (e.g. see Ref. 25). Similarly, the effect of rapamycin on 14-3-3 binding to PRAS40 is also less marked that the effect of amino acid starvation (Fig. 1A). Although several other peptides are observed on these maps, no reproducible changes in their behavior were seen in response to amino acid starvation or rapamycin. The apparent differences between the patterns in Fig. 4, A and B, probably reflect differences in efficiency of protease digestion or resolution in the second dimension.

It seemed likely that the binding of 14-3-3 proteins to PRAS40 involved both an mTORC1-regulated phosphorylation site (corresponding to the input from amino acids that is needed for 14-3-3 binding) as well as Thr246, which accounts for the requirement for insulin (9).

The motif search program ScanSite (available on the World Wide Web) predicts Ser183 as a potential 14-3-3 binding site in PRAS40. To test the role of Ser183 in the binding of PRAS40 to 14-3-3, we compared the binding of wild type PRAS40 and the S183A mutant to 14-3-3. Mutation of Ser183 to alanine abolished PRAS40 binding to 14-3-3 proteins (Fig. 5A). Mutation of other selected serine/threonine residues (Thr246, Ser146, and Ser187) to alanine in PRAS40 did not affect 14-3-3 binding (data not shown). It appears that the mTORC1-mediated phosphorylation of Ser183 allows direct binding of 14-3-3 to this residue.

Surprisingly, the S183A mutation reduced the phosphorylation of Thr246. Ser183 is essential for 14-3-3 binding (Fig. 5A); it is possible that Ser183-dependent 14-3-3 binding protects Thr246 from dephosphorylation. This notion is consistent both with the observations that depletion.
of raptor (Fig. 1B) or mutation of the TOS motif in PRAS40 (Fig. 1F) reduces Thr^{246} phosphorylation and with the ability of 14-3-3 binding to protect other partners against dephosphorylation (26–28).

Since mutation of Ser^{183} to alanine abolished 14-3-3 binding, we tested whether mutation of Ser^{183} to an acidic residue (Asp or Glu) permitted insulin-induced 14-3-3 binding in the absence of amino acids. Fig. 5B indicates that such mutations do not support 14-3-3 binding in the presence or absence of amino acids. We also introduced acidic residues in place of Thr^{246}, which is also known to be critical for 14-3-3 binding (Fig. 5A; see also Ref. 9), to test whether such mutations would bypass the requirement for insulin in the binding of PRAS40 to 14-3-3s. Again, they did not allow 14-3-3 binding (Fig. 5C). It is unlikely that the mutants fail to bind 14-3-3 because they affect the folding of PRAS40, since the protein is denatured prior to far Western analysis. It is more probable that, at least in the case of PRAS40, phosphorylation at serine or threonine rather than negative charge per se is required for stable interaction with 14-3-3 proteins.

**Binding of PRAS40 14-3-3 Is Sensitive to Manipulating the Upstream Control of mTORC1**—The above data suggested that, as well as being a component of mTORC1, PRAS40 is also regulated by mTORC1. To test this further, we co-expressed PRAS40 with Rheb, a small G-protein that, in its GTP-bound form, activates the kinase activity of mTORC1 (29). Overexpression of Rheb activates mTORC1 in amino acid-starved cells (8, 29). Insulin was unable to elicit the phosphorylation of S6 in amino acid-deprived HEK293 cells. Ectopic expression of Rheb permitted insulin to stimulate S6 phosphorylation in amino acid-deprived cells, and this effect was abolished by rapamycin (Fig. 6A). Rheb also allowed insulin to promote 14-3-3 binding to ectopically expressed PRAS40 (which is not otherwise seen in amino acid-starved cells). This too was blocked by rapamycin (Fig. 6A).

The tuberous sclerosis complex, comprising TSC1 and TSC2, acts as a GTPase activator protein for Rheb, converting it to its inactive GDP-bound form. Akt-mediated phosphorylation of TSC2 is believed to impair the GTPase activity of TSC1/2, allowing Rheb to accumulate in its GTP-bound form and activate mTORC1 (29, 30). Conversely, expression of TSC1/2 (negative regulators of Rheb/ mTORC1) inhibited phosphorylation of 4E-BP1 as well as amino acid- and insulin-induced 14-3-3 binding to PRAS40 (Fig. 6B). Together, these data strongly indicate that PRAS40 lies downstream of mTORC1 in addition to being regulated directly by Akt/protein kinase B (9, 20, 21).

Since Thr^{246} lies in a sequence context (RXRXXT) similar to those phosphorylated by other AGC family kinases (31), it was possible that PRAS40 was phosphorylated (e.g. by the S6 kinases or RSKs). Several lines of evidence argue strongly against a role for these enzymes in phosphorylating Thr^{246} in PRAS40. First, insulin induces Thr^{246} phosphorylation in amino acid-deprived cells (Fig. 1A) but cannot activate S6ks (as revealed by the lack of S6 phosphorylation) (Fig. 1A). Second, rapamycin does not decrease Thr^{246} phosphorylation in endogenous PRAS40 (Fig. 1A) but does completely inhibit S6 phosphorylation. Third, p90RSK enzymes may be excluded, since insulin does not act-

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**FIGURE 6.** 14-3-3 binding to PRAS40 is reciprocally regulated by Rheb and TSC1/2. A, HEK293 cells were co-transfected with 3 μg of HA-PRAS40 DNA or empty vector and 2 μg of FLAG-Rheb. 24 h later, cells were starved of serum for 14 h and were also deprived of amino acids for 90 min prior to stimulation with 100 nm insulin for 25 min where indicated. Cells were treated with 100 nm rapamycin during the last 30 min of amino acid starvation (prior to insulin stimulation) where noted. Cells were lysed, and anti-HA immunoprecipitates were prepared and analyzed by far Western blotting using a digoxigenin-labeled 14-3-3 probe. Total lysates were analyzed by immunoblot as indicated. B, HEK293 cells were co-transfected with 2 and 3 μg of FLAG-TSC1 and -TSC2, respectively, or with empty vector. In all cases, cells were also transfected with 0.1 μg of rat Myc-4E-BP1 or 2 μg of HA-PRAS40. Cells were transfected with 0.1 μg of rat Myc-4E-BP1 or 2 μg of HA-PRAS0, as noted. 24 h later, cells were starved of serum and subsequently deprived of amino acids and/or stimulated with insulin, where indicated. Cells were harvested, and anti-HA (for PRAS40) or anti-Myc (for 4E-BP1) immunoprecipitates were prepared. Immunoprecipitates were analyzed by SDS-PAGE/immunoblot as indicated. Samples of lysate were analyzed with anti-FLAG. IP, immunoprecipitation; WB, Western blot.
vate their upstream kinases, extracellular signal-regulated kinases, in HEK293 cells (supplemental Fig. S1; see also Ref. 32). Fourth, in cells carrying a mutation in 3-phosphoinositide-dependent kinase-1 such that Akt/protein kinase B cannot be activated, S6Ks and RSKs still can be switched on (33). Studies using such cells revealed that insulin-like growth factor 1-induced phosphorylation of Thr246 in PRAS40 is lost, showing that S6K and RSK do not contribute significantly to this.

We observed that phosphorylation of endogenous PRAS40 at Thr246 is largely insensitive to rapamycin (Fig. 2) (20, 21, 23, 34). It appears to be a component of their upstream kinases, extracellular signal-regulated kinases, in HEK293 cells (supplemental Fig. S1; see also Ref. 32). Fourth, in cells carrying a mutation in 3-phosphoinositide-dependent kinase-1 such that Akt/protein kinase B cannot be activated, S6Ks and RSKs still can be switched on (33). Studies using such cells revealed that insulin-like growth factor 1-induced phosphorylation of Thr246 in PRAS40 is lost, showing that S6K and RSK do not contribute significantly to this.

Reduced 4E-BP1 Phosphorylation in Cells Overexpressing PRAS40 Is Not Apparently Due to Competition for Raptor Binding—Earlier studies (20, 21) have shown that overexpression of PRAS40 inhibits mTORC1 signaling and have suggested that PRAS40 is a negative upstream regulator of mTORC1. We also find that overexpressing PRAS40 inhibits signaling downstream of mTORC1 in HEK293 cells (as judged by the phosphorylation of 4E-BP1 at multiple sites) (Fig. 7A). Overexpression of PRAS40 inhibited both the amino acid-induced phosphorylation of Thr37/Thr46 and the insulin-stimulated phosphorylation of Thr70 and Ser65 in endogenous 4E-BP1, without impairing the ability of insulin to activate Akt/protein kinase B (Fig. 7A).

The finding that PRAS40 contains a functional TOS motif (this study) (see also Refs. 23 and 34) provides a potential explanation for the latter effect (i.e. that overexpressed PRAS40 competes with other TOS motif-containing proteins for binding to raptor/mTORC1 and thereby inhibits their regulation). We put this idea to the test. We co-expressed PRAS40 with rat 4E-BP1 wild type or F129A mutant (Fig. 7B). Wild type PRAS40 and the F129A mutant (which cannot bind raptor) inhibited mTORC1 signaling to a similar extent (Fig. 7B; note that the numbering of rat 4E-BP1 differs by −1 from the human protein), apparently ruling out this explanation. Thus, mutation of the TOS motif alone is not enough to affect the ability of PRAS40 to inhibit mTORC1.

PRAS40 Is Required for Signaling Downstream of mTORC1, but Not for Upstream Signaling to This Complex—PRAS40 binds stably to mTORC1 and co-immunoprecipitates with it (Fig. 2) (20, 21, 23, 34). It thus appears to be a component of this complex. We therefore asked whether knocking down PRAS40 expression affected signaling downstream of this complex. It was possible that this might enhance signaling, since it would decrease competition between different TOS motif-containing partners for binding to raptor. For example, knocking out 4E-BP1/2 leads to enhanced activation of S6K1 (35). We studied the phosphorylation states of two mTORC1 targets, 4E-BP1 and S6, in lysates from control and PRAS40 knockdown cells. The phosphorylation of some sites in 4E-BP1 (Thr37 and Thr46) is induced by amino acids alone, whereas others (Ser65 and Thr70) require insulin (Fig. 8A) (12). Knocking down PRAS40 strongly decreased the phosphorylation of S6 at all of the sites tested without affecting total S6 levels (Fig. 8, A and B). Depleting PRAS40 impaired insulin-stimulated phosphorylation of Ser65 and Thr70 in 4E-BP1 (Fig. 8, A and B). Interestingly, it also markedly decreased the amino acid-dependent phosphorylation of Thr37/Thr46 (12) in the absence of insulin (Fig. 8A). Similar data were obtained in 10 separate experiments. Scrambled siRNAs or mock transfection did not impair mTORC1 signaling when compared with appropriate controls (Fig. 8, A and B; data not shown). Knocking down PRAS40 decreased the insulin-stimulated phosphorylation of S6 (Ser235/Ser236) and of Ser65 in 4E-BP1 to 33% (p < 0.05, n = 3) and 26% (p < 0.05, n = 3), respectively, of the values for the scrambled control. Thus, PRAS40 is required for both the amino acid- and the insulin-stimulated inputs to 4E-BP1 phosphorylation.

FIGURE 7. Overexpression of PRAS40 impairs mTORC1 signaling. A, HEK293 cells were co-transfected with 2 μg of HA-PRAS40 wild type and 5 μg of enhanced green fluorescent protein plasmid DNA. Transfection efficiency was monitored by estimating enhanced green fluorescent protein expression. 90–100% transfection efficiency was observed. Maximal transfection efficiency was desired to study the effects of exogenous PRAS40 on endogenous 4E-BP1. 24 h following transfection, cells were starved of serum for 16 h and then stimulated with insulin (100 nM, 25 min) and lysed. Whole cell lysates were analyzed by SDS-PAGE/immunoblot using the antisera indicated. B, HEK293 cells were co-transfected with vectors for rat Myc-4E-BP1 (0.1 μg) plus human HA-PRAS40 (wild type or F129A). 24 h later, cells were starved of serum (16 h) and in some cases also deprived of amino acids (90 min) prior to stimulation with insulin (100 nM, 25 min) as indicated. Myc-4E-BP1 was immunoprecipitated (IP) from the lysates and analyzed by SDS-PAGE/immunoblot as noted. Lysates were also analyzed with anti-HA to monitor PRAS40 overexpression.
It was important to establish whether the effects of PRAS40 knockdown were specific to mTORC1 or whether they also affected signaling via Akt to TSC2. Knocking down PRAS40 had no effect upon the insulin-induced phosphorylation of Akt at either Thr308 or Ser473 (Fig. 8A and B), indicating that PRAS40 is not needed for mTORC2 function, consistent with its absence from this complex. Importantly, these data also indicate that the impairment of insulin-activated mTORC1 signaling that is seen in PRAS40 knockdown cells is not due to interference with the upstream phosphatidylinositide 3-kinase/Akt pathway. To verify this, we examined the phosphorylation of Akt sites in glycogen synthase kinase 3β and TSC2. Knocking down PRAS40 had no effect on insulin-induced phosphorylation of glycogen synthase kinase 3α/β (Fig. 8B) or TSC2 (at Ser939, an Akt site (36); Fig. 8A). Thus, (i) PRAS40 plays a positive role in amino acid and/or insulin-stimulated mTORC1 signaling, and (ii) knocking down PRAS40 does not impair upstream signaling by insulin via Akt to TSC2.

We considered it important to test the effect of depleting PRAS40 on mTORC1 signaling in another cell line. Transient depletion of PRAS40 in HeLa cells again decreased phosphorylation of S6 at Ser235/236, without affecting mTORC2 activity, as determined by Akt phosphorylation at Ser473 (Fig. 8C).

Taken together, our data place PRAS40 downstream of mTORC1. Why does knocking down PRAS40 impair mTORC1 signaling? Since PRAS40 associates with mTORC1, it was possible that PRAS40 played a role in the assembly of mTOR-raptor complexes. To test this, we used siRNA to knock down PRAS40 expression and then immunoprecipitated mTOR and looked at the amounts of associated raptor (Fig. 9A). A modest, but reproducible, decrease in raptor binding was observed (Fig. 9A, top). Quantitation of the data revealed that PRAS40 depletion slightly decreased raptor binding to mTOR by about 40%, but this was not statistically significant (p value = 0.13, n = 3). There was no discernible change in the overall levels of mTOR or raptor (Fig. 9A, bottom). It is possible that PRAS40 may play a minor role in the stability of the mTORC1 complex, and this may, in part, explain the impairment of mTORC1 signaling seen when it is knocked down.

However, knocking down PRAS40 did not affect the in vitro kinase activity of mTORC1 measured against 4E-BP1 (Fig. 9B), as observed by others (23). It is important to note that it is unclear whether such in vitro assays fully or accurately reflect the in vivo activity of mTORC1.

DISCUSSION

The present data define PRAS40 as a novel target for regulation by mTORC1. After the initial submission of this manuscript, two other studies on PRAS40 appeared, both showing that PRAS40 contains a functional TOS motif (23, 34). PRAS40 is only the third type of protein to be shown to possess a functional TOS motif (the others being the S6 kinases and 4E-BPs,
although hypoxia-inducible factor 1α has recently also been reported to contain a TOS motif (37).

One interpretation of the present findings and earlier data concerning the insulin- and amino acid-dependent association of PRAS40 with 14-3-3 proteins is that this involves two phosphorylation sites, Thr\(^{246}\) (which is regulated by insulin) and another site that is controlled by mTORC1 and (thus by amino acids). The recent study by Oshiro et al. (23) revealed that Ser\(^{183}\) is a substrate for mTORC1 in vitro and is regulated by mTORC1 in vivo. Our data show that this residue is required for association with 14-3-3 proteins, making it likely that two sites involved in the amino acid- and insulin-induced association of PRAS40 with 14-3-3 are the Akt site, Thr\(^{246}\), and the mTORC1 site, Ser\(^{183}\). This provides a molecular mechanism for the control of 14-3-3 binding to PRAS40 by amino acids and insulin, although the functional significance of this interaction remains to be clarified.

Other recent studies (20, 21) suggested that PRAS40 was an upstream negative regulator of mTORC1 and that the binding to it of 14-3-3 acted to release this inhibitory effect. Since this study, together with the data described in Ref. 23, indicate that PRAS40 is a downstream target for mTORC1, the earlier interpretation may need reassessment.

Our initial in vivo labeling studies reveal that PRAS40 is phosphorylated at many sites, with up to 11 distinct phosphopeptides being seen on two-dimensional maps. Further work will be required to study more fully the phosphorylation of PRAS40. Both our data and those of Oshiro (23) indicate that mTORC1 phosphorylates at least one other site in PRAS40 (in addition to Ser\(^{183}\)).

Our data are generally in good agreement with the other recent reports (20, 21, 23, 34) that PRAS40 interacts with raptor via its TOS motif and dissociates in response to the addition of amino acids or insulin. These and other observations have led to the suggestion that PRAS40 may impair signaling to other mTORC1 targets, such as 4E-BP1 and S6K1 (which also contain TOS motifs) by competing with them for access to a limiting amount of mTOR. Our data are in general consistent with that model for the normal regulation of signaling downstream of mTORC1.

However, we also find that knocking down PRAS40 expression actually impairs signaling downstream of mTORC1. It is interesting to note that 14-3-3 proteins promote rapamycin-sensitive (TORC1) signaling in budding yeast, probably downstream of the TOR proteins (38). It is thus tempting to speculate that the binding of 14-3-3 to PRAS40 may play a related role in mammalian cells, although yeast does not appear to have an ortholog of PRAS40.

It appears puzzling that either knocking down PRAS40 or overexpressing inhibits mTORC1 signaling. These observations may reflect roles for PRAS40 in both long-term (positive) regulation of signaling from mTORC1 and, in the longer term, the integrity and/or function of this complex. This is consistent with our finding that the depletion of PRAS40 leads to somewhat decreased recovery of raptor in mTOR immunoprecipitates, perhaps reflecting a role for PRAS40 in the assembly of mTORC1. This would be consistent with the suggestion that PRAS40 may interact with mTOR as well as with raptor (34). It is possible that the inhibitory effect on mTORC1 signaling of PRAS40 (F129A), in which the TOS motif is destroyed, reflects residual interaction between other regions of PRAS40 and mTOR or raptor, which disrupt downstream signaling. In the case of another substrate for mTORC1, 4E-BP1, it appears that regions in addition to the TOS motif are involved in its association with mTORC1. For example, data from Eguchi and colleagues (39) have suggested that the phosphorylation status of 4E-BP1 regulates its interaction with mTORC1. Similarly, phosphorylated residues, and adjacent features, in PRAS40 may also affect its binding to raptor, as has already been shown to be the case for Ser\(^{183}\) (23).

Several studies have recently pointed to a role for PRAS40 in cell survival (40, 41); understanding the function and complex regulation of PRAS40, a downstream target of mTORC1, clearly requires further investigation.

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