The binding of PRAS40 to 14-3-3 proteins is not required for activation of mTORC1 signalling by phorbol esters/ERK

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INTRODUCTION

mTOR (mammalian target of rapamycin) forms two main types of complex, termed mTORC1/2 (mTOR complexes 1 and 2) [1,2]. Signalling through mTORC1 is stimulated by amino acids and agents such as insulin and is generally inhibited by rapamycin (reviewed in [3]).

mTORC1 contains several protein components that are not present in mTORC2. One of these is raptor (regulatory associated protein of mTOR), the orthologue of the yeast TORC1 component KOG1 [4]. Raptor fulfills a 'scaffolding' function by binding proteins that are substrates for phosphorylation by mTOR and recruiting them to mTORC1 [5–7]. Such mTORC1 substrates include the 4E-BPs (eukaryotic initiation factor 4E-binding proteins) and ribosomal protein S6Ks (S6 kinases) [3,8]. Their binding to raptor, and their phosphorylation at specific sites by mTOR, is dependent upon their TOS (TOR signalling) motifs [5–7,9,10].

A further specific mTORC1 component was recently discovered. This protein is termed PRAS40 (proline-rich Akt substrate of 40 kDa) [11,12]. PRAS40 binds 14-3-3 proteins (which interact with phosphoproteins) and this interaction depends upon the phosphorylation of PRAS40 at Thr246, as well as upon an amino-acid-dependent input [13,14]. In response to insulin, the phosphorylation of Thr246 is catalysed by PKB (protein kinase B) [15]. PKB signalling also leads to the activation of mTORC1 [16,17]. Initial results indicated that PRAS40 acts as a negative regulator of mTORC1 and it has been proposed that PKB-mediated phosphorylation of PRAS40 overcomes this negative regulation, perhaps due to dissociation of PRAS40 from mTORC1 due to binding of PRAS40 to 14-3-3 proteins [11].

It was subsequently shown that PRAS40 itself possesses a TOS motif and that this is required for its interaction with raptor [14,18,19]. Furthermore, mTORC1 can phosphorylate PRAS40 (at Ser183 [19]), and this site and Thr246 are required for binding to 14-3-3 proteins [14,15].

As mentioned above, insulin activates mTORC1 via PKB. PKB phosphorylates a GAP (GTPase-activator protein) complex containing TSC (tuberous sclerosis complex) 1 and TSC2 (reviewed in [17,20–22]). TSC2 stimulates the hydrolysis of GTP bound to the small G-protein Rheb. GTP-bound Rheb, through an as yet undefined mechanism, activates mTORC1 signalling [23]. PKB phosphorylates TSC2 at two major sites (Ser198 and Thr246), and this is thought to inactivate its GAP activity toward Rheb and to increase mTORC1 signalling [20,21,24]. Ectopic expression of either TSC2 or PRAS40 inhibits signalling downstream of mTORC1. The relationship between these two mechanisms of mTORC1 inhibition is so far unclear. These proposed mechanisms are, of course, not mutually exclusive.

mTORC1 signalling can also be activated by MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/ERK signalling, e.g. in response to α1-adrenergic agonists in cardiomyocytes [25] and to phorbol esters in HEK (human embryonic kidney)-293 cells [26,27]. Earlier results suggest that this regulation also involves phosphorylation and inactivation of TSC2, although the sites of phosphorylation differ...
from those acted upon by PKB [28,29], and it is unclear whether TSC2 is regulated by ERK itself [30] or by its downstream effectors, the RSKs (ribosomal S6 kinases) [27–29].

Given the existence of two models for the activation of mTORC1 signalling by insulin, we have studied whether PRAS40 is controlled by the MEK/ERK pathway. The results of the present study reveal that although mTORC1 signalling is regulated by the MEK/ERK pathway, PRAS40 is not; this pathway induces neither PRAS40 phosphorylation at Thr389 nor its binding to 14-3-3 proteins. The findings of the present study should prompt a re-examination of the proposals that PRAS40 either regulates upstream signalling to mTORC1 or downstream signalling from that complex.

EXPERIMENTAL

Biochemicals and other reagents

General laboratory chemicals were purchased from Sigma–Aldrich and Fisher Scientific. Rapamycin, LY294002, PD098059 and BIM (bisindolylmaleimide I) were from Calbiochem. Recombinant human insulin, PMA and 4α-phorbol 12,13-didecanoate were purchased from Sigma–Aldrich. Digoxigenin, trypsin and endoprotease Glu-C were from Roche Applied Science. Tissue culture reagents were from Invitrogen. BI-D1870 was purchased from the Division of Signal Transduction Therapy, College of Life Sciences, University of Dundee, Dundee, Scotland, U.K.

Sources of antisera

The sources of antisera used in the present study have been described previously [14]. In addition, in the present study we have also used antisera against tubulin (Santa Cruz Biotechnology).

Raptor and 14-3-3 far-Western blotting procedures

These were performed exactly as described previously [14].

Cell culture, treatments and transfections

HEK-293 cells were propagated and transfected as described previously [14]. At 24 h following transfection, cells were starved of serum for 16 h, and in some instances also depleted of amino acids for a further 90 min in Dulbecco’s PBS supplemented with 4.5 g/l d-glucose, sodium pyruvate and MEM vitamins. The cells were then pre-treated with signalling inhibitors, where indicated: 100 nM rapamycin (30 min), 50 μM PD098059 (45 min), 30 μM LY294002 (45 min) or 10 μM BI-D1870 (1 h), followed by stimulation with 100 nM insulin (25 min) or PMA (1 μM unless otherwise indicated) for 25 or 45 min, as stated in the Figure legends.

Cell harvesting and protein immunoprecipitation

Cells were harvested with our standard extraction buffer containing 1% (v/v) Triton X-100 as previously described [31], supplemented with 50 μM MG132 and lysates were pre-cleared by centrifugation at 16000 g for 10 min at 4°C. HA (haemagglutinin) immunoprecipitates were prepared by incubating 1 mg of total protein with anti-HA antisera and 35 μl of 50% (v/v) protein G-Sepharose slurry for 3 h at 4°C, mixing end-over-end. The immunoprecipitates were washed twice with lysis buffer [31] and resuspended in 50 μl of 5× sample buffer containing 5% (v/v) 2-mercaptoethanol. Triton X-100 detergent disrupts protein–protein interactions within mTORC1. To study the association of PRAS40 with raptor, we have employed an extraction buffer containing an alternative detergent (0.3% CHAPS), as described previously [14].

Vectors

The pcDNA3.1 Myc/His-tagged rat 4E-BP1 vector has been described previously [32] as was the pCMV5 HA-tagged human PRAS40 vector [14]. Site-directed mutagenesis of PRAS40 and 4E-BP1 was performed using the Stratagene QuikChange® system according to manufacturer’s protocol.

SDS/PAGE, Western and far-Western blotting

SDS/PAGE and immunoblotting were performed as described previously [33]. The methods for the 14-3-3 ‘far-Western overlay’ and the preparation of 14-3-3 probe have also been described previously [14].

32P in vivo radiolabelling and two-dimensional phosphopeptide mapping

The procedures for 32P in vivo radiolabelling and two-dimensional phosphopeptide mapping have been described in detail previously [14]. However, in the present study, we made use of alternative proteases, trypsin and endoprotease Glu-C. Radiolabelled PRAS40 was subjected to in-gel proteolytic digestion with 10 μg/ml trypsin in 20 mM NH4HCO3/0.1% (w/v) n-octyl glucoside for 16 h at 30°C with vigorous agitation. In some instances, radiolabelled PRAS40 was doubly digested with 10 μg/ml trypsin and 10 μg/ml endoprotease Glu-C. All other steps were carried out as described previously [14].

RESULTS AND DISCUSSION

PMA activates mTORC1 signalling but does not induce binding of PRAS40 to 14-3-3

Recent work has shown that PRAS40 associates with mTORC1, and its overexpression decreases the phosphorylation of other mTORC1 substrates, including 4E-BPs and S6K [11,12,14,18,19]. This observation prompted the model that PRAS40 negatively regulates mTORC1 activity by acting upstream of this complex [11,12]. In response to insulin, PKB phosphorylates PRAS40 at Thr246 and consequently induces the binding of PRAS40 to 14-3-3 proteins [13,15]. Since insulin also activates mTORC1 signalling, it has been suggested that phosphorylation of PRAS40 at Thr246 and its binding to 14-3-3 may overcome this inhibition, thus allowing insulin to activate mTORC1 [11,12]. It was therefore of interest to examine whether other agents that turn on mTORC1 signalling also regulate PRAS40 binding to 14-3-3.

Treatment of HEK-293 cells with the phorbol ester PMA resulted in the increased phosphorylation of ribosomal protein S6 at four sites that are substrates for S6Ks and the increased phosphorylation of 4E-BP1 at Ser244 (Figure 1A), showing that it was mediated by mTORC1 and confirming that PMA does indeed activate mTORC1 signalling in HEK-293 cells. In contrast, phosphorylation of S6 at Ser235/236 was only partially blocked by rapamycin (Figure 1A). This is in accordance with studies using cells in which both S6K genes have been knocked out, which demonstrated that Ser240/244 phosphorylation, but not that of Ser235/236, was absolutely dependent on the S6Ks [34] which lie downstream of mTORC1. PMA activates ERK but not PKB in HEK-293 cells (Figure 1A; [26] see also Figures 1C and 1D). Rapamycin did not affect
ERK activation, as judged by its phosphorylation at Thr\(^{202}\)/Tyr\(^{204}\) (Figure 1A).

When tested across a 100-fold concentration range of PMA, robust ERK activation was observed at 1 \(\mu\)M (Figure 1B). Although in the experiment shown, substantial activation of ERK was also seen at 0.1 \(\mu\)M, this was not a consistent observation. We therefore chose to use the highest concentration of PMA (1 \(\mu\)M) in subsequent experiments. To test whether PMA-induced ERK phosphorylation was specific to active phorbol esters, we also studied the ability of an inactive analogue (4\(\alpha\)-phorbol 12,13-didecanoate) to switch on ERK signalling. Treatment of HEK-293 cells with 1 \(\mu\)M 4\(\alpha\)-phorbol 12,13-didecanoate failed to activate ERK, as shown in Figure 1(B). PMA activates several isoforms of PKC (protein kinase C) (\(\alpha\)-\(\epsilon\), \(\eta\) and \(\theta\)) which in turn activate the ERK pathway, probably through the activation of the protein kinase Raf1, which lies upstream of ERK. Several mechanisms have been proposed to explain this, including the direct phosphorylation of Raf kinase-inhibitory protein 1, which relieves its inhibitory effect on Raf activity (for reviews see [35,36]). The broad-spectrum PKC inhibitor BIM completely blocked PMA-induced ERK activation (Figure 1B), even at the highest PMA concentration used in the present study, consistent with the fact that PKC signalling leads to ERK activation. The PMA-induced phosphorylation of 4E-BP1 at Ser\(^{65}\) was also completely eliminated by BIM (Figure 1B).

We next used a well-established overlay assay [13] to examine the ability of PRAS40 to bind 14-3-3 (Figure 1C). As reported previously [13,15], PRAS40 from serum-starved cells cannot interact with 14-3-3, but does so following insulin treatment of the cells. In contrast, PMA treatment did not allow PRAS40 to bind 14-3-3 proteins (Figure 1C). As we were concerned that the time point chosen in the experiment shown (25 min) might not be optimal for regulation of PRAS40, we treated cells with PMA for two different times prior to cell lysis (Figure 1D). No
phosphorylation of PRAS40 at Thr\textsuperscript{246} or 14-3-3 binding was observed at 25 or 45 min in multiple experiments (Figure 1D) although ERK activity was increased at both time points. Thus PMA can activate mTORC1 signalling without inducing PRAS40 phosphorylation at Thr\textsuperscript{246} or its binding to 14-3-3.

One possible interpretation of this result is that the activation of mTORC1 by PMA occurs by a mechanism that is quite distinct from that used by insulin and which is proposed to involve the alleviation of the inhibitory effect of PRAS40 on mTORC1. We therefore tested whether overexpressing PRAS40 impaired the activation of mTORC1 signalling by PMA, as it does for insulin [11,12,18,19]. As shown in Figure 2(A), overexpression of PRAS40 did inhibit the activation of mTORC1 by PMA, as manifested by decreased phosphorylation of 4E-BP1 at Thr\textsuperscript{36}, Thr\textsuperscript{246} and Ser\textsuperscript{64}. Overexpressing PRAS40 also blocked the PMA-induced activation of S6K (Figure 2B) as indicated, first, by the suppression of its phosphorylation at Thr\textsuperscript{389} (Figure 2B, upper panel) and, secondly, by the loss of the more slowly migrating hyperphosphorylated species (Figure 2B, lower panel).

**Thr\textsuperscript{246} is the only major insulin-regulated phosphorylation site in PRAS40**

Phosphorylation of Thr\textsuperscript{246} in PRAS40 has been shown to be required for insulin-induced binding of 14-3-3 to PRAS40 [11,14,15]. The inability of PMA to induce 14-3-3 binding might be explained by the absence of Thr\textsuperscript{246} phosphorylation of PRAS40 following PMA treatment (Figures 1C and 1D). However, it remained possible that insulin might also induce the phosphorylation of additional sites that are not affected by PMA.

To study this, we expressed wild-type HA–PRAS40 or a T246A mutant in HEK-293 cells. Cells were subsequently starved of serum, transferred to phosphate-free medium, metabolically labelled with \[^{32}P\]Pi, and in some cases also treated with insulin. Cells were then lysed and HA–PRAS40 was isolated by immunoprecipitation followed by SDS/PAGE. Following autoradiography, the radiolabelled PRAS40 band was excised and digested with trypsin. The resulting phosphopeptides were resolved by two-dimensional mapping. Tryptic digestion of PRAS40 isolated from cells that had not been treated with insulin generated two major phosphopeptides plus a cluster of labelled peptides close to the origin (Figure 3A). Treatment with insulin resulted in the appearance of a single well-defined peptide (Figure 3B): this species was absent from the map for the T246A mutant from insulin-stimulated cells (Figure 3C), indicating that Thr\textsuperscript{246} is the major site controlled by insulin.

Thr\textsuperscript{24} is also in a context similar to the consensus for phosphorylation by AGC family kinases, i.e. RXRXX\textsuperscript{Y/T} [37] and might therefore be a target for insulin-activated kinases such as PKB or S6Ks. We therefore also compared peptide maps derived from wild-type or T24A mutant PRAS40 (Figures 3D and 3E) isolated from cells that had been treated with insulin. In this case, the immunopurified PRAS40 was digested with trypsin and Glu-C, as this gave better-resolved peptide maps with respect to the peptide containing Thr\textsuperscript{24}. Comparison of the maps for wild-type PRAS40 and the T24A mutant revealed that one phosphopeptide was absent from the latter (Figures 3D and 3E), indicating that Thr\textsuperscript{24} is indeed phosphorylated in living cells. However, this species was still present in peptide maps of wild-type PRAS40 from cells that had, or had not, been treated with insulin (results not shown). Thus, phosphorylation of Thr\textsuperscript{24} is not regulated by amino acids or by insulin.

Mutation of Thr\textsuperscript{24} to an alanine residue had no effect on the phosphorylation of PRAS40 at Thr\textsuperscript{246} or on its ability to bind 14-3-3 (Figure 3F). Thus Thr\textsuperscript{24} plays no role in 14-3-3 binding and cannot be involved in its induction by insulin/amino acids.

**Mutation of Thr\textsuperscript{246} to a serine residue allows PMA to induce 14-3-3 binding to PRAS40**

The above studies are consistent with the idea that PKB can phosphorylate Thr\textsuperscript{246} in PRAS40. Previous work indicated that RSKs [including an enzyme that is also called MAPKAP-K1 (MAPK-activated protein kinase 1)] preferentially phosphorylates

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Figure 2 Ectopic expression of PRAS40 impairs the ability of PMA to activate mTORC1

(A) HEK-293 cells were co-transfected with 0.1 μg Myc-tagged rat 4E-BP1 and 2 μg of either HA-tagged PRAS40 or the corresponding empty vector. Cells were starved of serum for 16 h and then, in some instances, deprived of amino acids for 90 min, followed by stimulation with insulin (100 nM, 25 min) or PMA (1 μM, 25 min). Cells were lysed, and samples were analysed either directly (SDS/PAGE and Western blot analysis using the indicated antibodies, upper panels) or subjected to immunoprecipitation (IP) using anti-Myc followed by Western blot analysis for total S6K–4E-BP1 or the indicated phosphorylation sites. (B) HEK-293 cells were co-transfected with vectors for HA-tagged S6K and HA-tagged PRAS40 or the corresponding empty vector (in each case, using 0.7 μg of DNA). Cells were starved and stimulated as detailed in (A). Cells were harvested and lysates analysed for phosphorylation of S6K by SDS/PAGE and Western blot analysis using either a phosphospecific antibody for Thr\textsuperscript{389} in S6K (pT389 S6k; upper panel) or anti-HA to detect co-translationally-expressed S6K. This also detects HA–PRAS40, where present. Note that S6K runs as multiple differentially phosphorylated bands (the most phosphorylated one migrating most slowly).

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Figure 3 Thr\textsuperscript{246} is the only major insulin-regulated phosphorylation site in PRAS40

Phosphorylation of Thr\textsuperscript{246} in PRAS40 has been shown to be required for insulin-induced binding of 14-3-3 to PRAS40 [11,14,15]. The inability of PMA to induce 14-3-3 binding might be explained by the absence of Thr\textsuperscript{246} phosphorylation of PRAS40 following PMA treatment (Figures 1C and 1D). However, it remained possible that insulin might also induce the phosphorylation of additional sites that are not affected by PMA.

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ERK, PRAS40 and mTORC1 signalling

Figure 3  *In vivo* analysis of the phosphorylation of wild-type PRAS40 and mutants

(A-E) HEK-293 cells were transfected with 10 μg of wild-type (WT) HA–PRAS40 or the indicated mutants. Serum-starved cells were metabolically labelled with [32P]Pi, and treated with insulin (100 nM, 25 min; INS) where indicated. After lysis, HA–PRAS40 was isolated by immunoprecipitation, subjected to protease digestion with trypsin (A–C) or trypsin and Glu-C (D and E), and the resulting phosphopeptides were resolved by two-dimensional mapping and detected by autoradiography. The direction of chromatography and polarity of electrophoresis are shown on the top left-hand panel. ‘X’ marks the origin (i.e. where the sample was applied). Circles denote the positions of peptides that are discussed in the text. (F) HEK-293 cells were transfected with 2 μg of vectors for wild-type PRAS40 or the T24A mutant plasmid. Cells were starved of serum (16 h) and then treated with insulin (100 nM, 25 min) as indicated. Overexpressed PRAS40 was immunoprecipitated (IP) with anti-HA antisera and samples were analysed for Thr246 phosphorylation or 14-3-3 binding by Western or far-Western blotting respectively.

serine rather than threonine residues [37]. We therefore mutated Thr246 to a serine residue. In contrast with the wild-type protein, PMA was able to induce 14-3-3 binding to this mutant, the extent of binding being similar to that seen after insulin treatment (Figure 4A). It was notable that the level of 14-3-3 binding seen for this mutant after either insulin or PMA treatment was much higher than for wild-type PRAS40 (Figure 4A; compare the signals for wild-type and T246S in short and long exposures of the overlay; expression levels were similar as judged from the HA blot in the lower part of this panel).

MEK1/ERK signalling promotes phosphorylation of PRAS40(T246S)

The finding that PMA induces the binding of 14-3-3 proteins to the PRAS40(T246S) mutant could be explained if the residue in position 246 now underwent PMA-induced phosphorylation. We therefore tested whether the PRAS40(pThr246) phosphospecific antibody could cross-react with PRAS40(pSer246). As shown in Figure 4(B), a signal was indeed observed with the PRAS40(T246S) mutant from insulin-treated cells, showing that the antibody does cross-react with the mutant. A signal was also seen for this mutant after treatment of cells with PMA, showing that PMA brings about the phosphorylation of the mutant PRAS40, as well as its binding to 14-3-3. This demonstrates that the reason PMA does not allow 14-3-3 binding to wild-type PRAS40 is that PMA does not induce the phosphorylation of Thr246. Altering this residue to a serine is sufficient to allow both phosphorylation and 14-3-3 binding. The PMA-induced phosphorylation of Ser246 was almost entirely inhibited by treatment of cells with the MEK1 inhibitor PD098059, indicating that it is mediated via the MEK/ERK pathway (Figure 4B). Similarly, inhibition of MEK/ERK signalling decreased the PMA-induced binding of the PRAS40(T246S) mutant to 14-3-3 (Figure 4B).

The RSK inhibitor BI-D1870 inhibits PMA-induced phosphorylation of the PRAS40(T246S) mutant

The observation that the MEK1 inhibitor PD098059 inhibits both the PMA-induced phosphorylation of the PRAS40 mutant at
Ser$^{246}$ and 14-3-3 binding (Figure 4B) indicated that this requires signalling via MEK and therefore probably ERKs. However, the sequence around Ser$^{246}$ does not match the consensus for phosphorylation by MAPKs (which is Ser–Pro or Thr–Pro). As noted, it does, however, closely fit the consensus for RSKs, AGC kinases with a similar substrate specificity to PKB [37].

To test directly the role of RSKs in phosphorylating Ser$^{246}$ in the mutant PRAS40, we made use of the recently described RSK inhibitor BI-D1870 [38]. BI-D1870 inhibited the PMA-induced phosphorylation of GSK3 (glycogen synthase kinase 3) (Figure 4B; see also [38]), a substrate for RSKs [39]) but did not impair ERK activation (if anything, an increase was seen, Figure 4B). BI-D1870 completely blocked PMA-induced phosphorylation of Ser$^{246}$ in PRAS40, illustrating that this requires RSK activity as well as PMA-induced 14-3-3 binding (Figure 4B).

Given that Ser$^{246}$ lies in a suitable context for phosphorylation by RSKs [37], the simplest explanation is that RSKs act directly on Ser$^{246}$ (but cannot phosphorylate wild-type PRAS40 as it has a threonine residue at this position). This is in agreement with the observation that RSK1 preferentially phosphorylates serine rather than threonine residues [14].

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Figure 4  The PRAS40(T246S) mutant binds 14-3-3 in response to PMA

(A-D) HEK-293 cells were transfected with 2 μg of wild-type HA-tagged PRAS40 or the indicated mutants (T246A, T246S). Cells were starved of serum (16 h) and, in some instances, subsequently deprived of amino acids. Where indicated, cells were pre-treated with rapamycin (100 nM, 30 min), PD098059 (50 μM, 45 min) or BI-D1870 (10 μM, 1 h) and then treated with PMA (1 μM, 25 min) or insulin (100 nM, 25 min). Samples of lysate were analysed directly by SDS/PAGE and Western blot analysis or subjected to HA-immunoprecipitation followed by Western or far-Western blotting for 14-3-3 binding. (E) The KSLP and SALP motifs in 4E-BP1 do not function as RAIP-type motifs. HEK-293 cells were transfected with 0.1 μg of wild-type Myc-tagged rat 4E-BP1 plasmid DNA or the indicated mutants (having KSLP or SALP in place of the RAIP motif). Cells were starved of serum (16 h) and then, where indicated, also starved of amino acids (90 min) followed by stimulation with insulin (100 nM, 25 min). Samples of cell lysate were analysed by Western blot using the indicated antisera.

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mTORC1 signalling promotes PMA-induced binding of 14-3-3 to PRAS40(T246S)

Previous results [14] have indicated that binding of 14-3-3 to wild-type PRAS40 requires two phosphorylation sites, Thr246 (induced by insulin and catalysed by PKB [15]) and Ser183 (induced by amino acids and catalysed by mTORC1) [14,19]. Rapamycin decreases the level of 14-3-3 binding seen in response to insulin (see also Figure 4C). Rapamycin also impaired the ability of PMA to promote 14-3-3 binding to the T246S mutant of PRAS40 (Figure 4C). In addition, the PMA-induced 14-3-3 binding requires the presence of amino acids in the medium (Figure 4D), as is also the case for insulin-induced 14-3-3 binding [13,14]. The amino acid status of the cells does not influence the activation of ERK by PMA or the state of phosphorylation of Ser183 (Figure 4D). This is consistent with the idea [14] that 14-3-3 binding requires the phosphorylation of PRAS40 both at Ser183 by mTORC1 [19] and at residue 246 by PKB (or also by RSK for the T246S mutant).

In the case of 4E-BP1, the amino-acid-dependent input to its control involves a 4-amino-acid motif (R13AIP; [32]). PRAS40 contains two somewhat similar sequences, S173ALP and K182SLP. To test whether either of these acts as RAIP-like motifs we introduced them into 4E-BP1 in place of the RAIP feature. As shown in Figure 4(E), neither of these motifs could promote the amino-acid- or insulin-stimulated phosphorylation of multiple sites that is a feature of wild-type 4E-BP1. This finding strongly suggests that the SALP and KSLP sequences do not function as RAIP-like motifs in PRAS40.

The ability of PRAS40 to inhibit mTORC1 signalling is not related to 14-3-3 binding

It has been suggested that the 14-3-3 binding may overcome the inhibitory effect of PRAS40 on mTORC1 signalling, e.g. by bringing about its dissociation from the mTORC1 protein complex [11,18]. The observation that PMA induces 14-3-3 binding to the PRAS40(T246S) mutant but not to the wild-type protein gave us an opportunity to evaluate this idea: if the above mechanism is correct, then the PRAS40(T246S) mutant should impair PMA-induced mTORC1 signalling much more weakly than wild-type PRAS40.

To test this, we co-expressed rat 4E-BP1 with either wild-type or mutant PRAS40 over a similar range of levels (Figure 5A) and examined the effect on PMA-induced phosphorylation of 4E-BP1 (at Thr389 and Ser424, which are all controlled by mTORC1 [40]). Expression of either wild-type or mutant PRAS40 inhibited 4E-BP1 phosphorylation at these sites, and did so to an almost identical extent (Figure 5A). As expected, no binding of 14-3-3 to wild-type PRAS40 was observed, whereas the T246S mutant bound well (Figure 5A), as also seen in Figures 4(A) and 4(B). To test the effect of wild-type PRAS40 or the T246S mutant on S6K, cells were co-transfected with HA–S6K and either the wild-type or T246S version under study. As shown in Figure 5(B), expression of increasing amounts of PRAS40 inhibited the PMA-induced phosphorylation of S6K, as illustrated both by the impairment of its phosphorylation at Thr389 and the loss of slower-moving more highly phosphorylated species. The extent of the effect was indistinguishable between wild-type PRAS40 and the PRAS40(T246S) mutant.

Thus the extent to which PRAS40 impairs mTORC1 signalling is independent of its ability to bind 14-3-3 proteins. 14-3-3 binding may, however, play other roles in the regulation of PRAS40 function.

PMA does not induce the dissociation of PRAS40 from mTOR/raptor

In the presence of amino acids, insulin causes the dissociation of PRAS40 from raptor (Figure 5C; [11,14]) and it has been suggested that this release is required to switch on the signalling downstream of mTORC1 [11,18] (by making raptor available to interact with other proteins that contain TOS motifs). In contrast, PMA did not bring about the release of PRAS40 from raptor (Figure 5C), even though PMA and ERK signalling do activate mTORC1 signalling (Figure 1A) [25–27]. Therefore PMA activates mTORC1 signalling through a mechanism that does not involve the dissociation of PRAS40 from raptor.

Concluding comments

The results presented in the current study demonstrate that although PMA activates mTORC1 signalling, it does not induce either the phosphorylation of PRAS40 at Thr246 or its binding to 14-3-3 proteins. It has previously been suggested that 14-3-3 binding relieves an inhibitory effect of PRAS40 upstream of mTORC1 [11], thereby allowing mTORC1 signalling to be activated by insulin.

Results from our group [14] and others [18,19] have shown that PRAS40 actually lies downstream of mTORC1, which probably provides the amino-acid-dependent input to [13] its control. The observation that insulin promotes the release of PRAS40 from raptor prompted the suggestion that the release of PRAS40 from mTORC1 is required to allow other substrates (4E-BPs, S6Ks, etc.) to bind to raptor and undergo mTORC1-mediated phosphorylation [18].

In the present study we show that although PMA activates mTORC1 signalling, it does not induce the phosphorylation of Thr246 in PRAS40, the binding of PRAS40 to 14-3-3 proteins or the release of PRAS40 from mTORC1. This indicates that 14-3-3 binding and, in particular, the release of PRAS40 from mTORC1 are not required for the stimulation of mTORC1 signalling.

These considerations lead to at least two possible conclusions. First, it is possible that PMA (and downstream ERK signalling) activates mTORC1 signalling by a mechanism that is quite distinct from that employed by insulin (i.e. by one that does not involve the alleviation of the proposed inhibition of mTORC1 by PRAS40). This would be consistent with our present findings that overexpression of mutants of PRAS40 which show grossly altered regulation inhibits mTORC1 signalling to a similar extent to wild-type PRAS40. Examples include PRAS40(F129A) [14] which fails to undergo regulation by insulin and the PRAS40(T246S) mutant which is regulated by PMA whereas wild-type PRAS40 is not (the present study). The release of PRAS40 in response to insulin may reflect the increased phosphorylation of PRAS40: an example of this would be the fact that phosphomimetic mutants of 4E-BP1 (also a target for mTORC1) exhibit decreased binding to raptor [41]. Previously it has been shown that PMA, like insulin, induces the phosphorylation of TSC2 [27,28], and it has consequently been argued that similar mechanisms, involving inhibition of the Rheb-GAP activity of TSC2, serves to activate mTORC1 in response to either insulin or PMA.

These considerations prompt a second possibility, i.e. that PRAS40 is not involved in the control of mTORC1 but rather in its downstream effects. Other studies have suggested that PRAS40 plays an anti-apoptotic role. For example, knocking down PRAS40 increased the sensitivity of tumour cells to pro-apoptotic stimuli [42], and overexpression of PRAS40 protects motor neurons after spinal cord injury [43]. Interestingly, the closest relative of PRAS40 in Drosophila, Lobe, appears to be required for cell survival in the early development of the eye:
loss of Lobe function leads to cell death, involving both caspase-dependent and -independent mechanisms \[44\]. It is thus possible that PRAS40, a PKB substrate, is involved in the well-known anti-apoptotic effects of PI3K (phosphoinositide 3-kinase)/PKB signalling \[45\]. It may, for example, play a role in coupling nutrient availability (sensed by the mTORC1 pathway) to cell survival. Additional work is obviously needed to elucidate fully the roles of PRAS40.

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