Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation

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Growth factor induced activation of phosphoinositide 3-kinase and protein kinase B (PKB) leads to increased activity of the mammalian target of rapamycin (mTOR). This subsequently leads to increased phosphorylation of eIF4E binding protein-1 (4EBP1) and activation of p70 ribosomal S6 protein kinase (p70S6K), both of which are important steps in the stimulation of protein translation. The stimulation of translation is attenuated in cells deprived of amino acids and this is associated with the attenuation of 4EBP1 phosphorylation and p70S6K activation. It has been suggested that PKB regulates mTOR function by phosphorylation although direct phosphorylation of mTOR by PKB has not been demonstrated previously. In the present work, we have found that PKB directly phosphorylates mTOR and, using phosphospecific antibodies, we have shown this phosphorylation occurs at Ser2481. Insulin also induces phosphorylation on Ser2481 and this effect is blocked by wortmannin but not rapamycin, consistent with the effect being mediated by PKB. Amino-acid starvation rapidly attenuated the reactivity of the Ser2481 phosphospecific antibody with mTOR and this could not be restored by either insulin stimulation of cells or incubation with PKB in vitro. Our findings demonstrate that mTOR is a direct target for PKB and support the conclusion that regulation of phosphorylation of Ser2481 is a point of convergence for the counteracting regulatory effects of growth factors and amino acid levels.

Key words: eIF4E binding protein-1, p70s6 kinase, phosphoinositide 3-kinase.

INTRODUCTION

The macrolide ester, rapamycin, acts to block a limited range of growth-factor-induced responses in cells; the best understood of these are the pathways involved in regulating the rate of protein translation [1,2]. In particular, rapamycin blocks growth-factor-induced phosphorylation of eIF4E binding protein-1 (4EBP1) [3–8] and activation of p70 ribosomal S6 protein kinase (p70S6K), both of which are important steps in the stimulation of protein translation. The stimulation of translation is attenuated in cells deprived of amino acids and this is associated with the attenuation of 4EBP1 phosphorylation and p70S6K activation. It has been suggested that PKB regulates mTOR function by phosphorylation although direct phosphorylation of mTOR by PKB has not been demonstrated previously. In the present work, we have found that PKB directly phosphorylates mTOR and, using phosphospecific antibodies, we have shown this phosphorylation occurs at Ser2481. Insulin also induces phosphorylation on Ser2481 and this effect is blocked by wortmannin but not rapamycin, consistent with the effect being mediated by PKB. Amino-acid starvation rapidly attenuated the reactivity of the Ser2481 phosphospecific antibody with mTOR and this could not be restored by either insulin stimulation of cells or incubation with PKB in vitro. Our findings demonstrate that mTOR is a direct target for PKB and support the conclusion that regulation of phosphorylation of Ser2481 is a point of convergence for the counteracting regulatory effects of growth factors and amino acid levels.

Key words: eIF4E binding protein-1, p70s6 kinase, phosphoinositide 3-kinase.

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Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; 4EBP1, eIF4E binding protein-1; p70S6K, p70 ribosomal S6 protein kinase; mTOR, mammalian target of rapamycin.
pathways using transient transfection with constitutively active PKB [26,27] or stable cell lines expressing a chimaeric mutant oestrogen-receptor-PKB construct that is acutely activated on addition of 4-hydroxytamoxifen [22]. In these studies, activated PKB stimulated p70 S6K activity and 4EBP1 phosphorylation, with each of these effects remaining sensitive to rapamycin, whereas dominant-negative forms of PKB blocked the phosphorylation of 4EBP1 and activation of p70 S6K. Moreover, these studies demonstrated that PKB causes activation of 4EBP1 kinase activity present in mTOR immunoprecipitates [22], indicating that PKB is regulating the activity of mTOR. The obvious mechanism by which PKB might affect mTOR function is by direct phosphorylation. Previous studies by Scott and Lawrence [28] have provided evidence that activation of PKB leads to a phosphorylation event in the region between amino acids 2433–2450 of the mTOR sequence. It was suggested that this might involve direct phosphorylation by PKB as this region leads to a phosphorylation event in the region between amino acids 2433–2450 of the mTOR sequence. It was suggested that this might involve direct phosphorylation by PKB as this region contains two potential PKB phosphorylation sites (Thr2446 and Ser2448). However, they were unable to show that mTOR was directly phosphorylated by PKB in vitro which lead them to speculate about the possible existence of an mTOR kinase downstream of PKB, although no such kinase has yet been identified [28].

In the present study, we have shown that PKB does indeed directly phosphorylate mTOR on Ser2448. Insulin also induces phosphorylation on this site, with this effect being blocked by the PI3-kinase inhibitor wortmannin, but not by the mTOR inhibitor rapamycin, which is consistent with insulin’s effects also acting through PKB. Reactivity of the Ser2448 phosphospecific antibody with mTOR was rapidly reduced by amino-acid starvation of cells and reactivity was not restored by insulin stimulation of amino-acid deprived cells or by incubation of mTOR immunoprecipitated from these cells with PKB in vitro. We conclude that this provides evidence that the phosphorylation of this site correlates with the ability of insulin and amino acids to signal through mTOR, indicating that phosphorylation at this site plays a key role in modulating the signalling pathways involved in regulating protein translation.

MATERIALS AND METHODS

Radiochemicals were from Amersham. All other reagents are from Sigma unless stated otherwise. Synthetic PtdIns(3,4,5)P3 was provided by Dr Piers Gaffney (University College London, London, U.K.). Recombinant glutathione S-transferase fusion proteins, including the entire coding sequences of PKB and phosphoinositide-dependent kinase 1 were prepared as described previously [29,30]. FLAG-tagged mTOR expression constructs were kindly provided by Dr Stuart Scheiber (Harvard University, Boston, MA, U.S.A.). An antibody was raised in sheep using the phosphopeptide RSRTTRD*YSAGQSV. This corresponds to amino acids 2441–2455 of the mTOR sequence, and * indicates the phosphorylated serine. The antibody was affinity purified on Sepharose coupled to the phosphopeptide and then passed though a column coupled to the dephosphopeptide. The antibodies that did not bind to the column were used as the phosphospecific antibodies and are referred to as mTOR-Ser2448 antibody. A previously described rabbit polyclonal antisera raised to a fusion protein which corresponds to the region between amino acids 668 and 939 of the mTOR sequence [20] was also used, and this is referred to as mTOR antibody.

Kinase assays of mTOR autokinase activity were performed in vitro as described previously [20]. Initial labelling experiments of mTOR were performed in vitro on 10-cm dishes containing 3T3-L1 adipocytes or 6-cm dishes containing CHO-IR800 cells. Cells were starved of serum overnight were then transferred to phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) containing 100 μCi/ml 32P. Cells were labelled for 90 min at 37°C and then incubated with insulin for 0–30 min. The cells were washed once with ice-cold PBS and lysed at 4°C in lysis buffer [10 mM Tris/HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na2VO4, 1% (v/v) Triton X-100, 1 mM PMSF containing 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin]. Lysates were clarified by centrifugation at 15000 g for 10 min at 4°C. Total mTOR was immunoprecipitated using mTOR polyclonal antisera [20], and was separated by SDS/PAGE (7.5%, gel). The gel was fixed, dried and the bands visualized using a Fuji FLA 2000 phosphorimager. Endogenous expression of mTOR was found to be low, therefore, in experiments designed to map phosphorylation sites, a FLAG-tagged mTOR expression construct was transiently transfected into HEK-293 cells to increase levels of expression and to gain sufficient incorporation of 32P into mTOR. Mapping of phosphorylation sites was performed as described previously [31].

For assays of PKB activity towards mTOR, HEK-293 cells were incubated overnight in serum-free DMEM and stimulated with insulin as shown in the Figure legends. In amino-acid starvation experiments, cells were incubated in Dulbecco’s PBS with 0.1g/l CaCl2 for 0–60 min. Cells were lysed, on ice, for 10 min in buffer containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 5 mM EDTA, 0.1 M Tris/HCl (pH 7.4), 1 mM Na2VO4, 2 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation at 14000 g for 10 min at 4°C. The supernatant was incubated for 90 min with the mTOR antibody [20]. Immuno-complexes were then incubated with Protein A-agarose for 30 min and the precipitates were washed twice in lysis buffer, once in 0.5 M LiCl/0.1 M Tris/HCl, pH 8.0, and once in PKB assay buffer (0.1 mM Tris/HCl, pH 7.4/20 mM MgCl2).

Immunoprecipitates were subjected to incubation in vitro with PKB, as indicated in the Figure legends, and were resuspended in 10 μl of PKB assay buffer. For PKB assay, 1 μg of recombinant PKB was pre-incubated with 1 μg of phosphoinositide-dependent kinase 1 and 8 μg PtdIns(3,4,5)P3 for 5 min at 25°C to allow complex formation. The assay was started by the addition of 50 μM ATP (final concentration); [γ-32P]ATP was included as indicated in the legends of Figures 2 and 3. Samples were incubated in a final volume of 20 μl at 30°C for 15 min.

All samples were separated by SDS/PAGE (MiniProtein 7.5%, gel) at 180 V for 1 h. Where incorporation of 32P-label into mTOR was being assessed, the gels were fixed, dried and the bands were visualized using a Fuji FLA 2000 phosphorimager. For blotting experiments, the proteins were transferred on to PVDF membranes by wet transfer (45 min at 70 V). To assess phosphorylation at Ser2448, membranes were blotted using the phospho-specific mTOR-Ser2448 antibody in Tris-buffered saline (50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 7.6) containing 0.1% (v/v) Tween-20 and 5% (w/v) BSA. To assess total mTOR levels, membranes were blotted with the mTOR antibody as described previously [20]. Blots were incubated with horseradish-peroxidase-labelled secondary antibody, visualized using enhanced chemiluminescence and the images were captured using a cooled CCD camera system (Fuji LAS1000).

RESULTS AND DISCUSSION

The aim of this study was to determine whether mTOR function was regulated by direct phosphorylation of mTOR. We therefore first investigated whether levels of phosphate incorporated into
mTOR were increased following insulin stimulation and found insulin provoked a rapid increase in the level of phosphorylation of mTOR in both CHO-IR800 cells and 3T3-L1 adipocytes labelled with $^{32}$P$\left[\text{P}\right]$ (Figure 1). In an attempt to identify the phosphorylation sites involved, the phosphorylated mTOR was purified and the tryptic peptide fragments were analysed by HPLC. Incorporation of $^{32}$P-label into mTOR was very low and only one significantly phosphorylated peptide was detected in mTOR in the unstimulated state (results not shown). This peptide corresponded to amino acids 1819–1867 of mTOR (results not shown). This sequence contains a number of potential phosphorylation sites, including three serine/threonine proline-directed sites, and it was not possible to identify the exact site on which phosphorylation occurred. Following insulin stimulation, a minor phosphopeptide peak appeared but insufficient material was obtained to permit sequencing.

One possible explanation for the increased phosphorylation of mTOR would be an insulin-induced increase in mTOR autokinase activity. To investigate this, mTOR was immunoprecipitated from cells that had been stimulated with insulin and mTOR autokinase activity was assessed. Insulin treatment did not increase mTOR autokinase activity in CHO-IR800, HEK-293 or in A14 cells (NIH-3T3 cells overexpressing insulin receptors) (results not shown). This indicates that an insulin-dependent upstream kinase was acting on mTOR. PKB is activated acutely by insulin and several recent reports suggests PKB lies upstream of mTOR [26,27,32]. Therefore, we tested whether PKB would directly phosphorylate mTOR. Activated PKB stimulated a 2–3 fold increase in the level of $^{32}$P-label in vitro, indicating a direct phosphorylation of mTOR by PKB (Figure 2A). The results of previous studies have suggested that PKB might induce phosphorylation in the region between amino acids 2433 to 2450 of the mTOR sequence and this might be crucial to the activation of mTOR [28]. The region surrounding Ser$^{2448}$ is of particular interest as it is contained within a very good consensus PKB phosphorylation site [33]. As we were unable to map the insulin-stimulated phosphorylation sites in mTOR, we chose to use phosphospecific antibodies as an alternative approach for analysing phosphorylation at the Ser$^{2448}$ site as these have been successfully employed to study phosphorylation of other PKB targets, such as the Bcl2 binding protein BAD. The specificity of the mTOR Ser$^{2448}$ antibody was confirmed by Western blotting. Immunoprecipitation of endogenous mTOR from HEK-293 cells was performed with the mTOR antibody and FLAG epitope-tagged mTOR was expressed in HEK-293 cells and immunoprecipitated using antibodies to the FLAG tag. Western blots of both of these immunoprecipitates with the mTOR-Ser$^{2448}$ antibody revealed a single band which co-migrated exactly with the band recognized by the mTOR antibody, whereas PKB incubation had no effect on the total phosphorylation of mTOR-induced by PKB in vitro (Figure 2A). We next used the phosphospecific antibody to determine whether the phosphorylation of mTOR induced by insulin stimulation was due to phosphorylation at Ser$^{2448}$. HEK-293 cells were stimulated with insulin, the mTOR was immunoprecipitated and these were

![Figure 1](image1.png) Insulin-induced increase in phosphorylation of mTOR

CHO-IR800 cells (A) or 3T3-L1 adipocytes (B) were labelled with $^{32}$P$\left[\text{P}\right]$ as described in the Materials and methods section. Cells were stimulated with 1 μM insulin for the indicated times. In each cell type similar results were obtained in at least two independent experiments performed in duplicate.

![Figure 2](image2.png) PKB- and insulin-induced phosphorylation of mTOR at Ser$^{2448}$

Immunoprecipitates from unstimulated HEK-293 cells were performed using the mTOR antibody (A and B). Activated PKB was incubated with the immunoprecipitates and kinase assays were performed in vitro using either $^{32}$P-labelled (A) or unlabelled (B) γ-ATP. Immunoprecipitates were separated by SDS/PAGE and gels were either fixed and dried (A) or transferred to PVDF and Western blotted with mTOR-Ser$^{2448}$ antibody (B). The results were similar in a total of three independent experiments performed in duplicate. To determine whether insulin stimulated phosphorylation at Ser$^{2448}$, serum-starved HEK-293 cells were incubated with either rapamycin (100 nM) or wortmannin (100 nM) for 10 min before stimulation with insulin (100 nM) or vehicle for 5 min. Cells were lysed and immunoprecipitated with mTOR antibody. Immunoprecipitates were separated by SDS/PAGE, transferred to a PVDF membrane and Western blotted with mTOR-Ser$^{2448}$ antibody (C). Similar results were obtained in three independent experiments.
Western blotted with the mTOR-Ser2448 phosphospecific antibody. The reactivity of mTOR with the mTOR-Ser2448 phosphospecific antibody was increased 1.7 ± 0.03-fold (mean ± S.E.M., n = 4) in insulin-stimulated cells (Figure 2C) and the extent of this increase closely matched the increase in total phosphorylation observed in the cell-labelling studies (Figure 1). The insulin-stimulated phosphorylation of mTOR at Ser2448 was not blocked by rapamycin but was blocked by the PI 3-kinase inhibitor, wortmannin, at doses which were sufficient to block insulin stimulation of PKB activity but not high enough to block mTOR autokinase [19,20] (Figure 2C). This again indicates that the increased Ser2448 phosphorylation was not due to the autokinase activity of mTOR but was caused by an upstream kinase, the activation of which requires PI 3-kinase. Taken together, these results provide strong evidence that PKB can indeed directly phosphorylate mTOR and that this is likely to explain the insulin-induced phosphorylation of mTOR. This provides the first evidence for a direct effect of PKB on mTOR.

It has been reported previously that as little as 30 min amino-acid starvation blocks insulin stimulation of p70S6k and lowers the level of phosphorylation of 4EBP1. Both the insulin and amino-acid effects were shown to act via mTOR [12–14]. This raises the possibility that insulin signalling and amino-acid effects converge at the level of mTOR. Consistent with this we found that, following amino-acid starvation, there was a rapid reduction in the reactivity of the Ser2448 phosphospecific antibody with mTOR in Western blotting, reducing it to almost undetectable levels after 30 min without affecting total levels of mTOR expression (Figure 3A). One interpretation of this result is that phosphorylation of mTOR Ser2448 is being reduced, although it is also possible that the amino-acid deprivation is inducing some other modification of mTOR in the region of Ser2448 (e.g. another phosphorylation) which reduces the ability of the phosphospecific antibody to react with this region. Either way, it indicates that amino-acid deprivation has a profound effect on the region of mTOR surrounding Ser2448.

If the amino-acid deprivation is causing dephosphorylation of mTOR at Ser2448, it is unlikely to be the result of inhibition of PKB as previous studies indicate that the removal of amino acids does not affect basal or insulin-stimulated PKB activity [12,13]. Therefore if the amino-acid starvation is blocking phosphorylation of mTOR at Ser2448 it must be doing so by making the mTOR refractory to phosphorylation at this site. In support of this, we found that reactivity of the phosphospecific antibody with mTOR could not be induced by incubation of mTOR immunoprecipitated from amino-acid deprived cells with activated PKB in a kinase assay in vitro (Figure 3B), nor could it be induced by insulin stimulation of the amino-acid deprived cells (Figure 3C).

Two mechanisms could be envisaged by which amino-acid deprivation might block phosphorylation at Ser2448. First, it is possible that the cells possess a serine phosphatase that targets Ser2448 and is sensitive to changes in amino-acid levels. Indeed, there has been a great deal of speculation about a possible role for protein phosphatase 2A (PP2A) in mediating mTOR functions, although there is no evidence that amino-acid deprivation activates this phosphatase [34]. There are two other arguments against protein phosphatase 2A being the primary force responsible for regulating the phosphorylation on Ser2448. First, the attenuation of p70S6k activity and 4EBP1 phosphorylation induced by amino-acid withdrawal is not blocked by phosphatase inhibitors [12]. Secondly, we found that amino-acid withdrawal caused the immunoprecipitated mTOR to become refractory to rephosphorylation by PKB in vitro. Thus any amino-acid-stimulated phosphatase would need to be very tightly associated with the mTOR to survive the immunoprecipitation in the RIPA buffer system used in these studies.

A more likely possibility is that mTOR is a target for a protein kinase whose activity is regulated by amino-acid levels in the cell. There is precedent for phosphorylation of mTOR causing a phenotype similar to that induced by amino-acid starvation, as increasing cAMP levels in 3T3-L1 adipocytes blocks insulin stimulation of 4EBP1 phosphorylation [28]. Increasing cAMP also blocks the insulin-induced reduction in reactivity of mTOR with an antibody raised to the sequence region surrounding Ser2448. This has been taken as evidence that raised cAMP could be blocking PKB phosphorylation at this site [28]. Like amino-acid deprivation, increasing cAMP had no effect on PKB activity in adipocytes, suggesting a direct effect of cAMP-dependent protein kinase (PKA) on mTOR [28,35]. We were unable to identify any candidate kinases which would be activated by amino-acid deprivation and result in mTOR phosphorylation. It seems unlikely that these effects act via PKA as available information suggests amino-acid starvation does not alter cellular cAMP levels [36]. Another possibility is that an amino-acid depletion might inactivate a kinase responsible for a priming phosphorylation on mTOR needed before PKB can act, and that this priming phosphorylation might be amino-acid dependent. In yeast, the protein kinase GCN2 is inactivated by the accumulation of deacylated tRNA induced by amino-acid starvation.

Figure 3 Amino-acid starvation reduces phosphorylation of mTOR at Ser2448 and makes the phosphorylation of this site refractory to PKB and insulin

HEK-293 cells were incubated in either DMEM or were amino-acid starved in PBS. In all cases the cells were lysed and immunoprecipitation was then performed using the mTOR antibody and products were separated by SDS/PAGE. The results show Western blots with the mTOR-Ser2448 antibody (mTOR-ser2448 blot) and the same blot stripped and reprobed with the mTOR antibody (mTOR blot). (A) Effects of amino-acid starvation. (B) Effects of PKB (Protein kinase B) phosphorylation. Cells were either incubated with DMEM (Amino acids +) or amino-acid starved in PBS for 60 min (Amino acids −) before immunoprecipitation. In the lane marked Protein kinase B +, the immunoprecipitates were incubated with activated PKB and γ-ATP in a kinase assay in vitro before SDS/PAGE. (C) Effect of insulin stimulation in the presence of amino-acid starvation. Cells were either incubated with DMEM (Amino acids +) or amino-acid starved for 60 min (Amino acids −). In the lane marked Insulin +, cells were stimulated with 100 nM insulin before lysis. For each experiment similar results were obtained in a total of three independent experiments.
and it has been speculated that inactivation of a mammalian homologue of GCN2 may be involved in amino-acid starvation-induced events [13]. Although a mammalian homologue of GCN2 has not been identified, there are kinases (e.g. PKR and HI) which have a high degree of homology in the catalytic domain, and members of this family phosphorylate serines in SXXR sequences. Although such mechanisms do not operate in yeast cells (Tor1p and Tor2p lack the appropriate phosphorylation sites), it may be more than coincidence that a SXXR site overlaps the Ser\(^{1448}\) PKB site in mTOR. Indeed, our results could be interpreted as providing support for such a mechanism, as the lack of reactivity of the Ser\(^{1448}\)-phosphospecific antibody with mTOR following amino-acid deprivation could be due to the presence of phosphorylation at sites adjacent to the Ser\(^{1448}\) residue. From the present studies we have not been able to determine whether the decreased reactivity of the Ser\(^{1448}\) antibody with mTOR following amino-acid starvation reflects decreased phosphorylation at this site or phosphorylation of adjacent sites or indeed a combination of both. Further studies will be required to distinguish between these possibilities.

In summary, insulin and PKB stimulate an increase in phosphorylation on Ser\(^{1448}\) of mTOR, which closely parallels the previously described increase in activity of mTOR by insulin and PKB [28]. Amino-acid starvation blocks mTOR function and blocks the ability of insulin to stimulate mTOR activity [12,13]. Our evidence indicates amino-acid starvation either blocks the insulin- and PKB-induced phosphorylation or induces a phosphorylation of residues adjacent to Ser\(^{1448}\) thus preventing access by PKB. Either of these mechanisms is likely to be important in downregulating mTOR activity. These parallels provide strong evidence that the PKB-induced phosphorylation of Ser\(^{1448}\) in mTOR plays a key role in regulating mTOR function and represents the point at which growth-factor signalling and the amino-acid sensing machinery converge to elicit their opposing effects on protein translation.

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REFERENCES


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