Dissociation of the eukaryotic initiation factor-4E/4E-BP1 complex involves phosphorylation of 4E-BP1 by an mTOR-associated kinase

Kate J. Heesom*, Richard M. Denton

Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK

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Abstract mTOR immunoprecipitates contain two 4E-BP1 protein kinase activities. One appears to be due to mTOR itself and results in the phosphorylation of 4E-BP1 on residues T_{36} and T_{45}, as shown previously by others. The other is a kinase which can be separated from mTOR and which phosphorylates 4E-BP1 within a peptide(s) containing residues S_{64} and T_{69}. This phosphorylation, which occurs predominantly on S_{64}, results in the dissociation of 4E-BP1 from eIF-4E.

Key words: mTOR; 4E-BP1; Phosphorylation; mRNA translation initiation; Insulin

1. Introduction

Insulin plays an important role in the overall regulation of protein synthesis [1]. In fat cells, insulin increases substantially the phosphorylation of eIF-4E binding protein-1 (4E-BP1) [2–6]. In unstimulated cells, the binding of 4E-BP1 to the mRNA cap-binding protein, eIF-4E, inhibits cap-dependent translation by blocking the association of eIF-4E with eIF-4G, and hence the formation of the eIF-4F complex which is necessary for translational initiation [7–10]. Phosphorylation of 4E-BP1 in response to insulin results in its dissociation from eIF-4E, allowing the formation of competent eIF-4F complexes [7,9,11–14].

The phosphorylation of 4E-BP1 in insulin-stimulated fat cells is complex and appears to occur on at least five sites via both rapamycin-sensitive and -insensitive signalling pathways [12,15,16]. However, the kinases involved in the phosphorylation of this important translational regulator have not been definitively identified.

A number of studies have suggested that the mammalian target of rapamycin (mTOR) plays a key role in the regulation of protein synthesis (see [17] for review). mTOR is a large protein (predicted $M_r = 289$ kDa) containing several structural motifs including a binding site for the rapamycin-FKBP12 complex (FKBP12, FK506-binding protein of 12 kDa) and a C-terminal catalytic domain that is homologous to that found in phosphatidylinositol 3-OH kinases (PI 3-kinases), although mTOR has not been shown to possess intrinsic lipid kinase activity [18].

Several recent papers have suggested that 4E-BP1 is phosphorylated in vitro by mTOR [19–23]. In this report we confirm that mTOR immunoprecipitates are able to phosphorylate a recombinant eIF-4E/4E-BP1 complex. However, we find that this phosphorylation is achieved by two separate protein kinases, one of which appears to be mTOR itself and the other an mTOR-associated kinase. We have investigated the effects of these two kinase activities on the dissociation of 4E-BP1 from the eIF-4E/4E-BP1 complex.

2. Materials and methods

2.1. Materials

Male Wistar rats (160–210 g) were fed ad libitum on a stock laboratory diet (CRM; Bioshore, Manea, Cambs., UK) until the time of killing. Reagents were as described previously [12] with the following additions; trypsin was from Boehringer Mannheim; TLC plates (cellulose fibre) were from Kodak (Rochester, NY, USA).

2.2. Preparation of rat epididymal fat cell extracts

Isolated rat epididymal fat cells were prepared and incubated as described previously [12]. Fat cells were extracted in 2.5 ml/g dry cell weight of ice-cold buffer A (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 1 μM microcystin-LR and pepstatin, antipain and leupeptin, each at 1 μg/ml) by vortex mixing [24].

2.3. Immunoprecipitation of mTOR

Peptides having sequences identical to positions 2433–2450 (DTNAKGNKRSRTRTDSYS) and 1272–1290 (ARRVSKDDWLEWLRRLSLE) in mTOR [25] were coupled to keyhole limpet haemocyanin and these conjugates were used to immunise rabbits, as described previously [26]. The resulting crude sera were designated Ab 1 and Ab 2, respectively. Fat cell extracts were incubated for 2 h at 4°C with 5 mg protein-A Sepharose and anti-mTOR serum at a dilution of 1:200. The immunoprecipitated material was washed twice with 50 mM HEPES, pH 7.4, containing 500 mM NaCl, twice with 50 mM HEPES, pH 7.4, containing 150 mM NaCl and once with assay buffer B (10 mM HEPES, pH 7.4, containing 50 mM β-glycerophosphate, 150 mM NaCl, 10 mM $MnCl_2$).

2.4. Measurements of 4E-BP1 kinase activity in mTOR immunoprecipitates

The immunoprecipitated mTOR pellet was re-suspended in assay buffer B containing microcystin-LR (1 μM), DTT (1 mM) and the PKA inhibitor, IP20 (200 μM) and incubated for 30 min at 30°C in a total volume of 50 μl with recombinant eIF-4E/4E-BP1 complex or free 4E-BP1 (equivalent to 0.5 mg/ml 4E-BP1; prepared and purified as described [16]), 5 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP (typically 1000 cpm/pmol). Reactions were stopped by the addition of Laemmli sample buffer and phosphoproteins were separated by SDS-PAGE and detected by autoradiography with pre-flashed Amersham Hyperfilm in cassettes with intensifying screens.

2.5. Proteolytic digestion, two-dimensional TLC mapping and phospho-amino acid analysis of 4E-BP1

Radiolabelled 4E-BP1 generated as described above was digested overnight at 37°C in 100 mM NEM, pH 8.0, containing 10 μg trypsin and then for a further 4 h with an additional 10 μg trypsin. The resulting peptides were mapped by TLC with a pH 3.5 buffer [pyridine/acetic acid/water (1:10:189, v/v)] for the electrophoresis dimension and chromatography with pyridine/acetic acid/butanol/water (10:3:15:12, v/v) buffer in the second dimension [27]. The positions

*Corresponding author. Fax: (44) (117) 9288274.
E-mail: k.heesom@bris.ac.uk

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of radiolabelled peptides were identified by autoradiography. The phospho-amino acid content of the radiolabelled 4E-BP1 was determined as described previously [16].

2.6. Analysis of mTOR protein levels using Western blotting

Samples to be analysed for mTOR levels were run on SDS-PAGE, transferred to PVDF membrane, blocked with 10% (w/v) milk powder and probed overnight with a 1:1000 dilution of an antibody raised to a region of the mTOR protein between amino acids 668 and 939 [28]. After rinsing, the blot was probed with a 1:10,000 dilution of an anti-rabbit IgG-horse radish peroxidase-linked antibody and the mTOR band visualised using Amersham ECL Western Blotting detection reagents.

2.7. Analysis of the dissociation of 4E-BP1 from eIF-4E

Following the phosphorylation of the eIF-4E/4E-BP1 complex by various kinase fractions as described above, the assay mixture was tumbled for 90 min at 4°C with 30 μl of m^7GTP-Sepharose-4B slurry. The supernatant was removed and the pellet washed with 1 ml buffer B. Proteins present in the supernatant and pellet were separated by SDS-PAGE and visualised by autoradiography.

3. Results

3.1. Immunoprecipitation of 4E-BP1 kinase activity using antibodies raised to mTOR

The ability of mTOR immunoprecipitates to phosphorylate a complex of eIF-4E and the 4E binding protein, BP1, was assessed using two antibodies raised to different regions of the mTOR protein. Extracts of rat epididymal fat cells were immunoprecipitated with either Ab 1 or Ab 2 and the resulting immunoprecipitated material was assayed for its ability to phosphorylate recombinant 4E-BP1 in a complex with eIF-4E (Fig. 1a). Of the two antibodies used, only Ab 1 was able to immunoprecipitate kinase activity towards the eIF-4E/4E-BP1 complex. This kinase activity appeared to decrease when the fat cells were treated with insulin, but remained at control levels when the cells were treated with EGF. No significant kinase activity was identified in Ab 2 immunoprecipitates from either control or insulin-treated extracts (Fig. 1a).

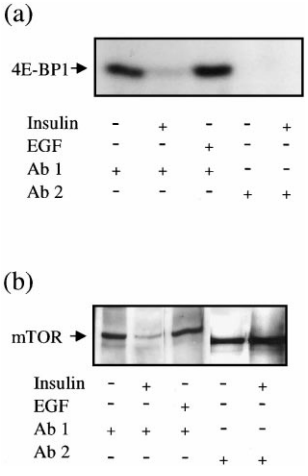

Fig. 1. Immunoprecipitation of 4E-BP1 kinase activity using mTOR Ab 1 and 2. Rat epididymal fat cells were incubated for 10 min at 37°C without additions or with the addition of 83 nM insulin or 100 nM EGF prior to extraction in buffer A. The fat cell extracts were then tumbled for 2 h at 4°C with 5 mg protein-A Sepharose and a 1:200 dilution of mTOR Ab 1 or 2. The immunoprecipitated material was washed extensively and divided into two. Half of the immunoprecipitate was assayed for its ability to phosphorylate recombinant eIF-4E/4E-BP1 complex (a) and half was blotted for the presence of mTOR (b), as described in Section 2. The results are typical of at least three separate experiments.

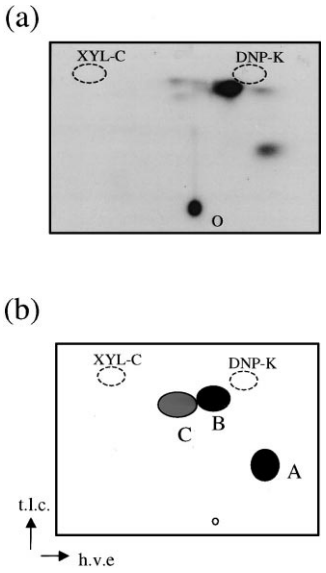

Fig. 2. Two-dimensional thin layer analysis of radiolabelled 4E-BP1. Extracts from control fat cells were immunoprecipitated with mTOR Ab 1 and the immunoprecipitate used to phosphorylate eIF4E/4E-BP1 complex. The radiolabelled 4E-BP1 was digested with trypsin and the resulting peptides separated in two dimensions using TLC. The position of the radiolabelled peptides was determined using autoradiography (a). The migration of the two markers, xylene cyanol (XYL-C) and DNP-lysine (DNP-K), as well as the migration of the three tryptic peptides from 4E-BP1 which become phosphorylated in response to insulin in rat adipocytes is shown in the key (b). The results are typical of four separate experiments.

When the amount of mTOR present in these extracts was assessed by western blotting, it was found that substantially less mTOR was immunoprecipitated by Ab 1 from insulin-treated fat cells compared to control extracts, whereas Ab 2 immunoprecipitated equivalent levels of mTOR from control and insulin-treated cells (Fig. 1b). The lower levels of mTOR protein in Ab 1 immunoprecipitates from insulin-treated cells presumably accounts for the decreased phosphorylation of 4E-BP1 seen under these conditions.

To some extent, these data confirm the work of Scott et al. [20], which suggested that an antibody equivalent to Ab 1 immunoprecipitated less mTOR (and hence less 4E-BP1 kinase activity) from insulin-treated 3T3-L1 adipocytes due to phosphorylation of mTOR within the Ab 1 epitope in response to insulin. This phosphorylation appeared to substantially reduce the binding of Ab 1 to mTOR. However, this group also reported the immunoprecipitation of an insulin-stimulated 4E-BP1 kinase activity with an antibody raised to the same region of mTOR as Ab 2. In the present study we found that Ab 2 did not immunoprecipitate significant 4E-BP1 kinase activity, even from insulin treated fat cells, although mTOR levels in these immunoprecipitates were equivalent to those in Ab 1 immunoprecipitates from control cells (Fig. 1b). The reason for this discrepancy is unclear.

To identify the sites within 4E-BP1 which were phosphorylated by immunoprecipitates generated using Ab 1, the labelled 4E-BP1 was digested with the protease trypsin, and the resulting peptides subjected to two-dimensional thin layer analysis (Fig. 2a). The mTOR immunoprecipitates phosphorylated 4E-BP1 within two of the three tryptic peptides which

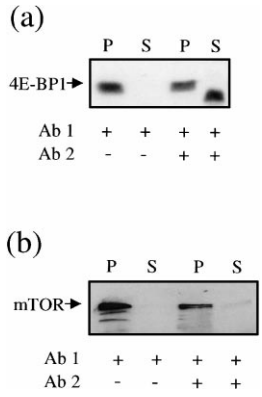

Fig. 3. Effect of mTOR Ab 2 on Ab 1 immunoprecipitates. Extracts from control fat cells were immunoprecipitated using Ab 1 and the immunoprecipitates washed as described in Section 2. After washing, the protein A pellet was incubated for 30 min at 4°C in 40 μl assay buffer B with or without a 1:20 dilution of Ab 2. The supernatant was then removed from the pellet and the pellet was washed as described above and re-suspended in 40 μl buffer B. Both pellet (P) and supernatant (S) were then assayed for their ability to phosphorylate eIF-4E/4E-BP1 complex (a) and for their mTOR content (b).

show increased phosphorylation in insulin-stimulated fat cells in vivo (Fig. 2b). These peptides have been shown previously to contain the residues S_{64} and T_{69} ('A-peptide') and T_{36} and T_{45} ('B-peptide') [16].

3.2. Isolation of an mTOR-associated 4E-BP1 kinase

The ability of Ab 1 immunoprecipitates to phosphorylate 4E-BP1 was abolished by the presence of detergent (1% NP40) during the fat cell extraction or washing of the immunoprecipitated material (data not shown), in agreement with the work of Nishiuma et al. [29]. This suggested the possibility that the phosphorylation of 4E-BP1 by mTOR immunoprecipitates was due to the presence of an associated kinase which was released from the immunoprecipitate in the presence of detergent.

In addition, the inability of Ab 2 to immunoprecipitate 4E-BP1 kinase activity while clearly immunoprecipitating mTOR, raised the possibility that an mTOR-associated kinase was responsible, at least in part, for the phosphorylation of 4E-BP1 seen with Ab 1 immunoprecipitates. Furthermore, it seemed possible that this kinase could be dissociated from mTOR by Ab 2. To investigate this hypothesis, fat cell extracts were immunoprecipitated using Ab 1, and the immunoprecipitated material was then incubated with Ab 2. Following this incubation, 4E-BP1 kinase activity was present in both the protein A pellet (which contained mTOR) and in the resulting supernatant (which did not contain significant levels of mTOR) (Fig. 3a,b).

The 4E-BP1 kinase activity present in the supernatant could be further purified using Mono-Q anion exchange chromatography or size exclusion chromatography on Superose 6. The kinase appeared to have a native molecular weight of approximately 50 kDa and eluted from the Mono-Q column at 350mM NaCl (data not shown).

The sites within 4E-BP1 phosphorylated by mTOR (present in the protein A pellet) and the mTOR-associated kinase (present in the supernatant) were investigated as described for Fig. 2 (Fig. 4a). Two-dimensional thin layer analysis showed that mTOR phosphorylated 4E-BP1 within tryptic peptide B (shown to contain mainly phosphothreonine) whereas the mTOR-associated kinase phosphorylated 4E-BP1 within tryptic peptide A (shown to contain predominantly phosphoserine, although a small amount of phosphothreonine was detected after prolonged autoradiography). Furthermore, mTOR kinase activity was manganese-dependent and phosphorylated both free 4E-BP1 and 4E-BP1 in a complex with eIF-4E, whereas the mTOR-associated kinase was more active in the presence of magnesium and only phosphorylated 4E-BP1 when complexed to eIF-4E (Fig. 4b).

3.3. Effects of mTOR and the mTOR-associated kinase on the dissociation of 4E-BP1 from eIF-4E

To investigate the effect of the kinases described in the present study on the dissociation of 4E-BP1 from eIF-4E, mTOR immunoprecipitated with Ab 1 was incubated with Ab 2 and the resulting mTOR pellet and mTOR-associated kinase were used to phosphorylate the eIF-4E/4E-BP1 complex in the presence of [γ-^{32}P]ATP, as described above. Following incubation with the kinases, the eIF-4E/4E-BP1 complex was precipitated using m^{7}GTP-Sepharose (which binds eIF-4E and any associated 4E-BP1). Proteins present in the

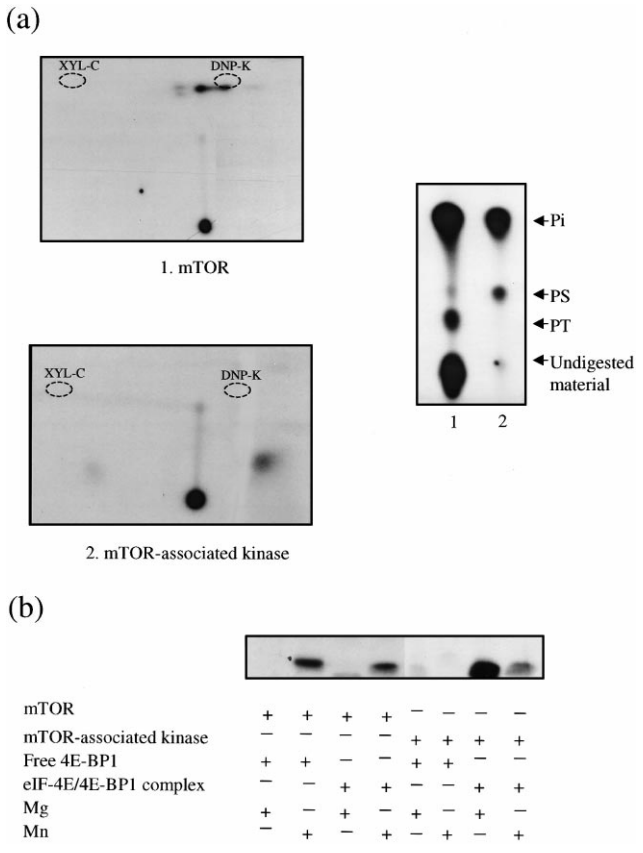

Fig. 4. Phosphorylation of 4E-BP1 by mTOR and the mTOR-associated kinase. mTOR and the mTOR-associated kinase were prepared as described for Fig. 3 and used to phosphorylate eIF-4E/4E-BP1 complex. The radiolabelled 4E-BP1 was digested with trypsin and the resulting peptides separated by two-dimensional TLC as for Fig. 2 (a). The phospho-amino acid content of 4E-BP1 which had been phosphorylated by (1) mTOR or (2) the mTOR-associated kinase is also shown (a). In addition, mTOR and the mTOR-associated kinase were assayed for their ability to phosphorylate free 4E-BP1 or the eIF-4E/4E-BP1 complex in the presence of $MgCl_2$ (5 mM) or $MnCl_2$ (5 mM), as described in Section 2 (b). The results are typical of four separate experiments.

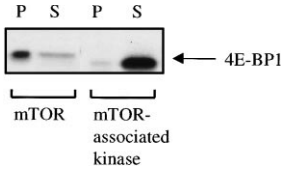

Fig. 5. Effect of mTOR and the mTOR-associated kinase on the dissociation of 4E-BP1 from eIF-4E. mTOR and the mTOR-associated kinase were used to phosphorylate the eIF-4E/4E-BP1 complex as described for Fig. 3. Following incubation for 30 min at 30°C, the kinase assay mixture was tumbled for 90 min at 4°C with m^7GTP-Sepharose slurry, as described in Section 2. The supernatant (S) was then removed and the m^7GTP-Sepharose pellet (P) was washed with 1 ml buffer B and then resuspended in 30 μl buffer B. Proteins present in the pellet and supernatant were separated by SDS-PAGE and the location of labelled 4E-BP1 determined by radioautography. The results are typical of six separate experiments.

resulting m^7GTP-Sepharose pellet or supernatant were then separated by SDS-PAGE and the presence of labelled 4E-BP1 identified by autoradiography (Fig. 5).

It was found that the phosphorylation of 4E-BP1 by mTOR did not appear to result in its dissociation from eIF-4E, since the majority of labelled 4E-BP1 remained in the m^7GTP-Sepharose pellet. However, phosphorylation of 4E-BP1 by the mTOR-associated kinase caused almost complete dissociation from eIF-4E since almost all of the labelled 4E-BP1 was present in the supernatant following m^7GTP-Sepharose treatment. In each case, a proportion (up to 35%) of eIF-4E was found in the supernatant following m^7GTP-Sepharose treatment (data not shown), but since almost all ($>90\%$) of the labelled 4E-BP1 was present in the supernatant following phosphorylation by the mTOR-associated kinase, this is clearly due to dissociation of 4E-BP1 from eIF-4E rather than dissociation of the eIF-4E/4E-BP1 complex from the m^7GTP-Sepharose pellet.

4. Discussion

Experiments in our laboratory and others have shown that in primary rat adipocytes, 4E-BP1 is phosphorylated in response to insulin on five sites which lie within three separate tryptic peptides [15,16]. Four of these sites, S_{64} and T_{69} (within tryptic peptide A) and T_{36} and T_{45} (within tryptic peptide B), fit a Ser/Thr-Pro motif and are phosphorylated via a rapamycin-sensitive signalling pathway. The final site, S_{111} (within tryptic peptide C) does not appear to be a proline-directed site, and is phosphorylated via a rapamycin-insensitive signalling pathway [16].

In the present study we identified two separate kinase activities which were both immunoprecipitated by an antibody raised to a C-terminal region of mTOR (Ab 1). The Ab 1 immunoprecipitate contained kinase activities which phosphorylated the eIF-4E/4E-BP1 complex within tryptic peptides A and B (Fig. 2). At the present time the effect of insulin on these kinase activities remains unresolved, since this hormone appears to substantially decrease the binding of mTOR (and presumably mTOR-associated proteins) to Ab 1 (Fig. 1).

A second antibody raised to a more central region of mTOR (Ab 2) appeared unable to immunoprecipitate significant kinase activity towards the eIF-4E/4E-BP1 complex. This raised the possibility that the phosphorylation of 4E-BP1 by Ab1 immunoprecipitates might be due, at least in part, to the presence of an mTOR-associated kinase, rather than mTOR itself. This hypothesis was also supported by the finding of our group and others [29], that the presence of detergent within the immunoprecipitate abolished the 4E-BP1 kinase activity.

With this in mind, an attempt to isolate the mTOR-associated kinase was undertaken. Crucially, it was found that the addition of Ab 2 to Ab 1 immunoprecipitates resulted in the release of a 4E-BP1 kinase activity into the supernatant, while mTOR itself remained in the protein A pellet. Thus two separable kinase activities were identified. The first remained in the protein A pellet, was manganese-dependent and was more active towards free 4E-BP1 rather than 4E-BP1 in a complex with eIF-4E (3.8 ± 1.8-fold, for three separate experiments). This kinase phosphorylated 4E-BP1 within tryptic peptide B (previously shown to contain residues T_{36} and T_{45} [16]). It is highly likely that this kinase activity represents mTOR itself, and the ability of this kinase to phosphorylate residues T_{36} and T_{45} is in agreement with a number of recent studies [21–23]. However, we cannot exclude the possibility that the phosphorylation of 4E-BP1 by the mTOR-containing pellet is due to another kinase which remains tightly associated with mTOR, even during the procedures employed in the present study.

The second kinase was released into the supernatant upon treatment with Ab 2 and was therefore free of mTOR. This kinase was specific for 4E-BP1 in a complex with eIF-4E, was more active in the presence of magnesium (although it was able to use manganese alone) and phosphorylated 4E-BP1 within tryptic peptide A (previously shown to contain residues S_{64} and T_{69} [16]). Phosphorylation by this mTOR-associated kinase appeared to occur predominantly on serine residues, suggesting that the major site phosphorylated was S_{64} (Fig. 4a). Consistent with this conclusion, we found that the kinase was able to phosphorylate a synthetic peptide containing residues S_{64} and T_{69} (data not shown).

When the effects of these kinases on the eIF-4E/4E-BP1 complex were investigated, it was found that phosphorylation of 4E-BP1 in a complex with eIF-4E by the mTOR-associated kinase caused dissociation of 4E-BP1 from eIF-4E, whereas phosphorylation of the complex by the mTOR-containing pellet was without effect.

This conclusion supports that of Gingras et al. who reported recently that the phosphorylation of 4E-BP1 in the eIF-4E/4E-BP1 complex by mTOR at residues T_{36} and T_{45} did not result in the dissociation of 4E-BP1 from eIF-4E [22]. Two other groups have also claimed that mTOR immunoprecipitates phosphorylate 4E-BP1 at residues T_{36} and T_{45} [21,23], but state that phosphorylation at these residues prevented the association of phosphorylated 4E-BP1 with eIF-4E. However, these in vitro studies did not focus directly on the effect of phosphorylation of 4E-BP1 on its dissociation from the eIF-4E/4E-BP1 complex. It should be noted that while phosphorylation of T_{36} and T_{45} may be enough to prevent the association of 4E-BP1 with eIF-4E, it may not be sufficient to cause dissociation of 4E-BP1 from the eIF-4E/4E-BP1 complex. Burnett et al. have reported that mutants of T_{36} and T_{45} expressed in HEK 293 cells bind constitutively to eIF-4E and hence argue that phosphorylation of these residues in vivo causes the dissociation of 4E-BP1 from eIF-4E [21]. However, Gingras et al. claim that phosphorylation of these residues, although not responsible for dissociation itself, is a

prerequisite for phosphorylation of the other sites in 4E-BP1 [22] and so mutating T_{36} and T_{45} would prevent the phosphorylation of the sites needed to cause dissociation.

Our results show that phosphorylation of 4E-BP1 by mTOR does not result in dissociation of the eIF-4E/4E-BP1 complex. However, phosphorylation by mTOR at residues T_{36} and T_{45} in our study does not appear to be a prerequisite for phosphorylation of the other sites in 4E-BP1, since the mTOR-associated kinase is able to phosphorylate 4E-BP1 in the absence of mTOR. Furthermore, phosphorylation of 4E-BP1 by this mTOR-associated kinase is sufficient to cause dissociation from eIF-4E. This phosphorylation appears to occur predominantly at residue S_{64}. Early studies also suggested that phosphorylation of 4E-BP1 on residue S_{64} resulted in dissociation of the eIF-4E/4E-BP1 complex [30]. More recently, Fadden et al. have shown that phosphorylation of a form of 4E-BP1 mutated at S_{64} by MAP kinase prevented its association with eIF-4E, implying that phosphorylation of S_{64} was not involved in dissociation of the eIF-4E/4E-BP1 complex [15]. Again, this work did not directly examine dissociation. Thus it is possible that phosphorylation of residues other than S_{64} may prevent the association of 4E-BP1 with eIF-4E without actually causing the dissociation of 4E-BP1 from the eIF-4E/4E-BP1 complex.

In conclusion, we have now identified three kinases which phosphorylate 4E-BP1 within the three tryptic peptides which show an increase in phosphorylation in response to insulin. Our previous studies identified an insulin-stimulated kinase from rat adipocytes which phosphorylated 4E-BP1 at S_{111} via a rapamycin-insensitive signalling pathway [16]. The present study has confirmed that mTOR (or a tightly associated kinase) phosphorylates 4E-BP1 at T_{36} and/or T_{45} and has identified an mTOR-associated kinase which phosphorylates 4E-BP1 within a peptide containing residues S_{64} and T_{69}. The effect of insulin on the activities of mTOR and the mTOR-associated kinase is currently under investigation. Of these three kinases, only the mTOR-associated kinase (which appears to predominantly phosphorylate 4E-BP1 at residue S_{64}) causes the dissociation of 4E-BP1 from the eIF-4E/4E-BP1 complex. All three kinases appear to be able to act separately to phosphorylate 4E-BP1 in vitro, and further studies are needed to elucidate the order and importance of the individual phosphorylation events and the significance of a potential mTOR/4E-BP1 kinase complex in vivo.

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