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Insulin-stimulated phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway

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Abstract The cap-binding initiation factor 4E (eIF4E) is regulated by phosphorylation and by the inhibitory binding protein 4E-BP1. Here we show that insulin-induced phosphorylation of eIF4E is not significantly affected by rapamycin, but is sensitive to wortmannin, which inhibits phosphatidylinositol 3′-kinase and blocks the activation of MAP kinase. Since PD098059, an inhibitor of MAP kinase activation, also blocks insulin-induced phosphorylation of eIF4E, the MAP kinase pathway seems to mediate this effect. Phosphorylated eIF4E can still bind to 4E-BP1. These data illustrate that (i) distinct signalling pathways mediate the phosphorylation of eIF4E and 4E-BP1 and (ii) phosphorylation of eIF4E, unlike that of 4E-BP1, does not lead directly to the release of 4E-BP1.

Key words: MAP kinase; Insulin; Translation; eIF; Rapamycin

1. Introduction

Initiation factor 4E (eIF4E) plays a key role in mRNA translation in eukaryotic cells by binding the 5′-cap structure (7-methylguanosine) which is present at the 5′-end of all cellular cytoplasmic mRNAs [1–3]. eIF4E undergoes phosphorylation at a single major site now identified as Ser-209 close to the C-terminus of this 216 amino acid protein [4,5]. Phosphorylation of eIF4E is increased under a variety of conditions where rates of translation are enhanced. These include treatment of cells with insulin or growth factors or other mitogens (reviewed in [6–8]). Despite the well-characterised correlation between eIF4E phosphorylation and increased rates of translation, it is still not clear how phosphorylation influences the biological activity of eIF4E, although it has been reported that phosphorylated eIF4E has a higher affinity for the mRNA cap than does its nonphosphorylated counterpart [9]. It is also the case that phosphorylated eIF4E seems to be associated preferentially with other translation factors, i.e. the other components of the eIF4F complex [2,3,10]. Earlier studies addressing the role of eIF4E phosphorylation employed mutants of eIF4E in which the residue then considered to be the site of phosphorylation (Ser-53) was altered to Ala to preclude phosphorylation [11–13]. These results are now clearly invalid since this is not the main site of phosphorylation.

Two additional proteins which interact with eIF4E have recently been discovered. These are termed 4E-BP1 and 4E-BP2 (eIF4E binding proteins 1 and 2). Each inhibits cap-dependent mRNA translation implying that they block the function of eIF4E in peptide-chain initiation [14]. 4E-BP1 (also known as PHAS-I) is a phosphoprotein whose state of phosphorylation increases in response to insulin [14–18] or insulin-like growth factor-1 (IGF-1 [19]). This causes its dissociation from eIF4E and should result in the alleviation of the inhibition of eIF4E. Recent work from the group of Sonenberg indicates that 4E-BP1 prevents the assembly of the eIF4F complex and hence efficient translation initiation [20,21]. The insulin- or IGF-1-induced phosphorylation of 4E-BP1 has been shown, in several cell-types, to be inhibited by the immunosuppressant rapamycin, a specific inhibitor of the signalling pathway which leads to activation of the p70 ribosomal protein S6 kinase (p70S6k) [17–19,22]. Consistent with its effect on 4E-BP1, rapamycin blocks translation in mammalian cells and in *Saccharomyces cerevisiae* [22,23].

We have examined the signal transduction pathway which leads to the phosphorylation of eIF4E. Our data indicate that eIF4E phosphorylation is not blocked by rapamycin, while the phosphorylation of 4E-BP1 is blocked by this drug, as in other cell types. Thus, the phosphorylation of the two proteins in response to insulin is mediated via distinct signalling pathways. Furthermore, eIF4E phosphorylation is blocked by interventions which prevent activation of the MAP kinase pathway, indicating that eIF4E is a target for signalling via this pathway.

2. Materials and methods

2.1. Chemicals and biochemicals

m^7GTP-Sepharose was from Pharmacia Biotech Inc. [γ-^{32}P]ATP was purchased from Amersham plc. Chinese hamster ovary (CHO.T) cells were kindly provided by Dr. L. Ellis (Houston, TX). Materials for tissue culture were obtained from Gibco Life Technologies, Inc. Microcystin, wortmannin, rapamycin and CHAPS were from Calbiochem. Unless otherwise stated, all other reagents were from Sigma. Antiscrum to 4E-BP1 was generously provided by Dr. T.A. Diggle and Professor R.M. Denton (University of Bristol). PD098059 was a kind gift from Parke-Davis (through Dr. A.R. Saltiel).

2.2. Cell culture and treatment of cells

CHO.T cells were grown and maintained in culture as described previously [24]. For experiments, plated cells were grown to 80% confluence in medium containing 10% [v/v] serum and then starved of serum for 24 h prior to treatment and extraction. Treatments with agonists and/or inhibitors of signal transduction were as described in each figure legend. Cells were then washed and extracts prepared as described in [24].

2.3. Electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described earlier [25]. Blots were developed using the Enhanced Chemiluminescence kit (Amersham plc). Isoelectric focusing of eIF4E was performed as described [5,26] using Ampholines (Pharmacia) in the pH range 3.5–10.

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2.4. Isolation of initiation factors

eIF4E was isolated as described previously for analysis by isoelectric focusing [5]. In the case of SDS-PAGE, proteins were removed from the m^7GTP-Sepharose matrix by boiling in concentrated SDS sample buffer. Immunoprecipitation of 4E-BP1 was as described by Diggle et al. [27].

2.5. Assessment of the activation of protein kinases

MAP kinase was assayed using the peptide substrate 'Thr-669' exactly as described previously [28] except that crude extracts rather than immunoprecipitates were used. The activation state of p70 S6 kinase was assessed by means of its migration on SDS-PAGE, where increased phosphorylation of the enzyme, which leads to its activation, causes it to migrate more slowly. The method has been described previously [29].

3. Results

3.1. Interaction of eIF4E with 4E-BP1 in CHO.T cells

In order to examine the degree of association between 4E-BP1 and eIF4E, cell extracts were processed to isolate eIF4E and associated 4E-BP1 by affinity chromatography on m GTP-Sepharose. The resulting samples were then subjected to SDS-PAGE and immunoblotting. Insulin caused a marked decrease in the amount of 4E-BP1 recovered after this step (Fig. 1A). Rapamycin blocked the dissociation of 4E-BP1 induced by insulin (data not shown), as has been reported for other cell types [14,17–19,22,30]. On SDS-PAGE, 4E-BP1 migrates as a doublet (Fig. 1B). A third, faster migrating species was also visible in some experiments. When 4E-BP1 was immunoprecipitated from control and insulin-treated cells it could be seen that there was an apparent shift in its migration upon insulin treatment (as reported earlier [17–19,22], and apparently resulting from its phosphorylation). This shift was entirely blocked by rapamycin, which also blocked the band shift for, and therefore the activation of, p70 S6 kinase (Fig. 1C).

3.2. Rapamycin does not prevent phosphorylation of eIF-4E

In order to test whether rapamycin also blocks the phosphorylation of eIF4E which occurs in these cells in response to insulin [26], the phosphorylation state of eIF4E was assessed by the isoelectric focusing/immunoblotting procedure that we have described elsewhere [26] (Fig. 1D). Insulin results in a rapid (within 7.5 min) and marked increase in the state of phosphorylation of eIF4E and this increase was not affected by rapamycin.

Thus, the phosphorylation of eIF4E in response to insulin appears to involve a distinct signalling pathway from that leading to the phosphorylation of 4E-BP1. The pathway which regulates eIF4E phosphorylation is therefore also distinct from that leading to activation of p70 S6k. These data also imply that the increase in the phosphorylation state of eIF4E induced by insulin is not merely a consequence of the dissociation of eIF4E from 4E-BP1, which is a possible mechanism. Rather, it is due to other events, e.g. the activation of an eIF4E kinase such as that recently described by the group of Damuni [31].

3.3. Phosphorylation of eIF-4E by insulin or serum is blocked by wortmannin

Other pathways which could lead to enhanced phosphorylation of eIF4E include the MAP kinase pathway which is also activated by insulin in CHO.T cells [24,28]. As we have reported previously, the activation of MAP kinase is substantially blocked by wortmannin [28], which is a potent and selective (although not absolutely specific) inhibitor of phosphatidylinositol-3′-kinase (PI 3′-kinase) [32].

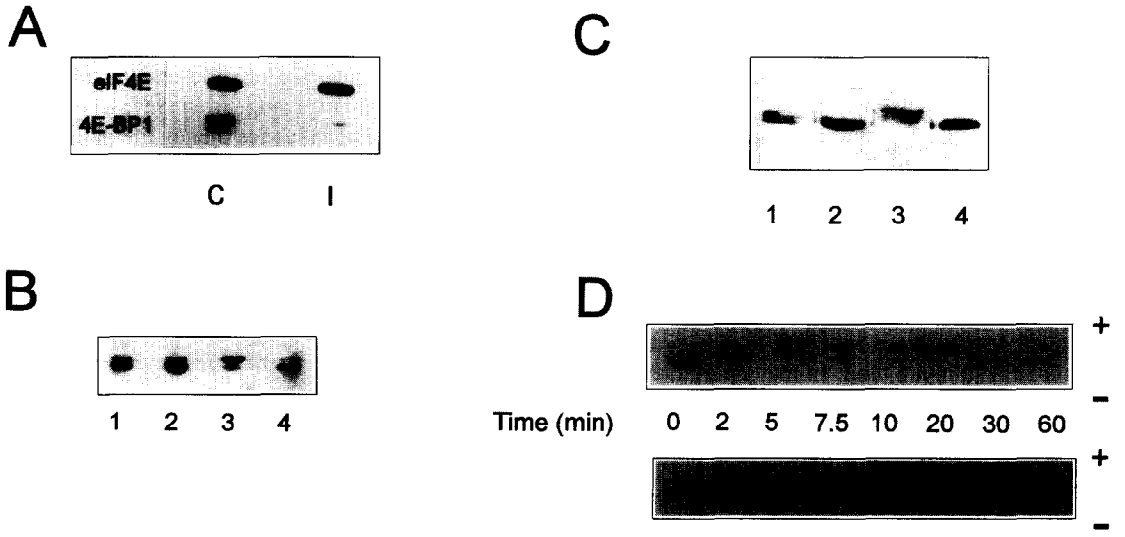

Fig. 1. Effects of insulin and rapamycin on eIF4E and 4E-BP1. In each panel, serum-starved CHO.T cells were used to prepare extracts for analysis. Each panel shows data typical of those obtained from at least three independent experiments. (A) Cells were incubated for 10min in the absence (C) or presence (I) of 2.5 nM insulin. eIF4E was isolated from the cell extracts by affinity chromatography on m^7GTP-Sepharose. Samples were analysed by SDS-PAGE followed by Western blotting using an affinity-purified anti-eIF4E antibody and an anti-4E-BP1 antiserum. (B,C) Cells were incubated for 30 min in the presence of DMSO carrier (lanes 1,3) or 20 nM rapamycin (lanes 2,4) before a further 5 min incubation with no addition (lanes 1,2) or 10 nM insulin (lanes 3,4). Cell extracts were prepared and used both to immunoprecipitate 4E-BP1 (B) and to analyse the band shift for p70S6 kinase (C). Both panels show Western blots from SDS-PA gels. (D) CHO.T cells were incubated for 30 min in the presence of DMSO (upper blot) or 20 nM rapamycin (lower blot) before being challenged with 10 nM insulin for varying times, as indicated. eIF4E was isolated as before and analysed by isoelectric focusing (IEF) followed by Western blotting. The direction of electrophoresis is indicated.

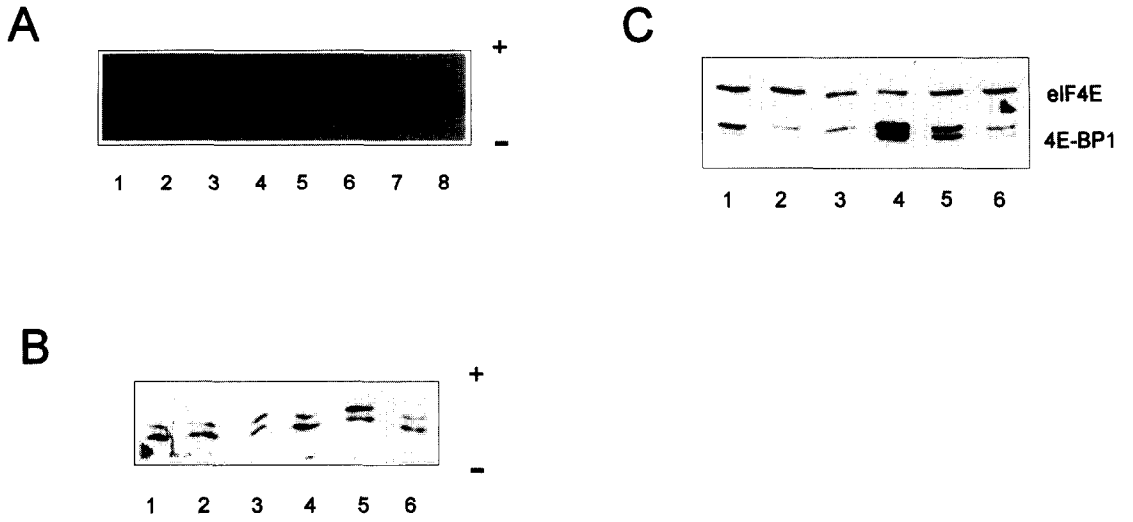

Fig. 2. Increased phoshorylation of eIF4E is mediated by the MAP kinase pathway. (A) Serum-starved CHO.T cells were incubated for 15 min in the presence of DMSO carrier (lanes 1–4) or 100 nM wortmannin (lanes 5–8), before challenging for a further 10 min with no addition (lanes 1,5), 10 nM insulin (lanes 2,6), 1 μM PMA (lanes 3,7) or 10% (v/v) foetal calf serum (lanes 4,8). eIF4E was isolated from cell extracts (see Fig. 1) and analysed by IEF followed by Western blotting with an affinity-purified anti-eIF4E antibody. The direction of electrophoresis is indicated. (B) Cells were incubated for 1 h in the presence of DMSO carrier (lanes 1,3,5) or 50 μM PD098059 (lanes 2,4,6) before challenging for a further 10 min with no addition (lanes 1,2), or with insulin at 2 nM (lanes 3,4) or 5 nM (lanes 5,6). Cell extracts were used to perform IEF for eIF4E as described above. (C) Cells were incubated for 1 h in the presence of DMSO carrier (lanes 1–3) or 50 μM PD098059 (lanes 4–6) before challenging for a further 10 min with no addition (lanes 1,4), or with insulin at 2 nM (lanes 2,5) or 5 nM (lanes 3,6). Cell extracts were used to analyse the association of 4E-BP1 with eIF4E, as described in the legend to Fig. 1.

Wortmannin largely blocked the increase in phosphorylation of eIF4E induced by insulin, phorbol ester or serum (Fig. 2A). This finding suggests that the phosphorylation of eIF4E requires a signalling pathway involving PI 3′-kinase. PI 3′-kinase is thought to lie upstream of p70 S6 kinase, but the involvement of this pathway in eIF4E phosphorylation was ruled out by the data described above.

3.4. Effect of inhibitors of the MAP kinase cascade

The above data obtained with wortmannin were consistent with the idea that the increase in the phosphorylation of eIF4E is mediated via the MAP kinase signalling pathway. However, since other signalling pathways are also downstream of PI 3′-kinase (e.g. protein kinase B, PKB [33,34]), it was important to test directly the role of the MAP kinase pathway. To do this we made use of a recently described inhibitor (PD098059) of the activation of the MAP kinase kinase (MEK) which blocks the activation of MAP kinase in a variety of cell types [19,35–37]. This compound also blocks activation of MAP kinase in CHO.T cells provided low concentrations of insulin are used. In five separate experiments, PD098059 decreased the activity of MAP kinase seen in response to 2 or 5 nM insulin to 7.6 ± 3.4 or 29.2 ± 8.7%, respectively, of the values seen with hormone alone. Other workers have also reported that PD098059 fails to block completely the activation of MAP kinase in response to high doses of agonist [35,36].

PD098059 also inhibited the insulin-induced increase in eIF4E phosphorylation seen at such doses of insulin (Fig. 2B). It should be noted that at higher insulin concentrations, where PD098059 only partly blocks activation of MAP kinase, the effect on eIF4E phosphorylation was also only partial, lending further weight to the idea that eIF4E lies downstream of MAP kinase.

Although PD098059 tended to increase the amount of 4E-BP1 associated with eIF4E in control cells, it had no effect whatsoever on the ability of insulin to induce the release of 4E-BP1 from eIF4E (Fig. 2C). Thus, the effect of this compound on the insulin-induced phosphorylation of eIF4E cannot be ascribed to impaired dissociation of 4E-BP1 (and hence possible occlusion of the phosphorylation site in eIF4E). The increased association of 4E-BP1 with eIF4E in control cells treated with PD098059 was associated with a rise in the relative proportion of the protein in the fastest-migrating, and presumably least phosphorylated, form. The observation that PD098059 did affect the basal level of association of 4E-BP1 with eIF4E was surprising given an earlier report that this compound did not influence this or the phosphorylation state of 4E-BP1 in 3T3-L1 adipocytes [18].

The simplest explanation for these observations would be that eIF4E itself is a substrate for either MAP kinase or another kinase in this pathway. We therefore tested the abilities of MAP kinase and two kinases known to be activated by

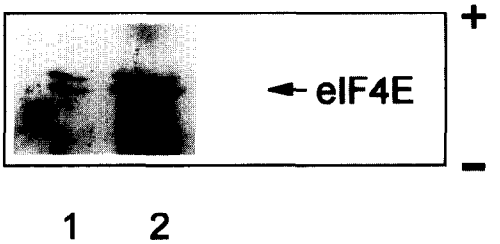

Fig. 3. Analysis of the phosphorylation state of eIF4E bound to 4E-BP1. CHO.T cell extracts were used to isolate either eIF4E by affinity chromatography (lane 1) or 4E-BP1 by immunoprecipitation (lane 2). In both cases, proteins were eluted from the washed Sepharose pellets by mixing with urea and concentrated IEF sample buffer (see Section 2). Samples were analysed by IEF and Western blotting using an affinity purified anti-eIF4E antibody. The direction of electrophoresis is indicated.

MAP kinase to phosphorylate purified eIF4E. The latter two enzymes are MAP kinase-activated protein kinase-1 (an isoform of p90 ribosomal protein S6 kinase [38]) and MAP kinase-activated protein kinase-2 (which may lie on a separate signalling pathway in vivo [39]). None of these enzymes was able to phosphorylate eIF4E purified from rabbit reticulocytes, although each was active as assessed using standard substrates (data not shown).

3.3. Phosphorylated eIF-4E still interacts with 4E-BP1

To test whether phosphorylated eIF4E can still bind to 4E-BP1, 4E-BP1 immunoprecipitates were subjected to isoelectric focusing analysis and immunoblotting using an anti-eIF4E antibody (Fig. 3). These data indicate that phosphorylated eIF4E can bind to 4E-BP1 and thus that the phosphorylation of eIF-4E does not play a major role in the regulation of this interaction.

4. Discussion

The data described here show that the insulin-induced phosphorylation of eIF4E is mediated by a signalling pathway distinct from that which leads to the dissociation of 4EBP1 from eIF4E. The latter event involves the phosphorylation of 4E-BP1 [14,30] and is blocked by rapamycin [17–19,22]. Morley and Pain [8] have also recently reported that rapamycin failed to block the increased phosphorylation of eIF4E (termed eIF4α in their paper) which is induced following the activation of T-lymphocytes.

Our findings strongly point to a key role for the MAP kinase pathway in mediating the insulin-induced increase in eIF4E phosphorylation. The specific inhibitor of the activation of MAP kinase kinase (PD098059), which blocked the stimulation of MAP kinase by the concentrations of insulin used here, also completely eliminated the increase in eIF4E phosphorylation. Since none of the known kinases which are activated downstream of MAP kinase kinase (i.e. MAP kinase and MAP kinase-activated kinases-1 and -2) was able to phosphorylate eIF4E, it is likely that a further protein kinase, whose activation is linked to the MAP kinase pathway, mediates this effect. As the activation of MAP kinase is linked via Raf and MAP kinase kinase to Ras, our data are entirely consistent with earlier findings pointing to a role for Ras in mediating the phosphorylation of eIF4E [40,41]. In particular, a dominant negative mutant of Ras was shown to block the nerve growth factor-induced phosphorylation of eIF4E is PC 12 cells [40]. Makkinje et al. [31] have shown that an insulin-stimulated protamine kinase can phosphorylate eIF4E at the appropriate site (Ser-209 [4,5]) in vitro. Its mode of activation by insulin is so far unknown: one might speculate that this kinase is activated by insulin in a Ras- and MAP kinase-dependent manner.

Our observation that PD098059 affects the phosphorylation of 4E-BP1, and its association with eIF4E, in our hands suggests that there is some input from the MAP kinase pathway to the regulation of this protein at least in CHO.T cells. In addition, the large increase in the amount of 4E-BP1 associated with eIF4E in PD098059-treated cells (Fig. 2C) also indicates that even in serum-starved CHO.T cells, only a small proportion of the eIF-4E is bound to 4E-BP1. This is an advantage in separating the insulin-stimulated phosphorylation of eIF-4E from the dissociation of the factor from 4E-BP1; it is even more unlikely that this latter event plays a part in the increase in phosphorylated eIF4E.

It is thus clear that at least two distinct signalling pathways are involved in the control of the initiation of mRNA translation, the MAP kinase pathway and that which includes p70 S6 kinase [17–19,22].

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