Both rapamycin-sensitive and -insensitive pathways are involved in the phosphorylation of the initiation factor-4E-binding protein (4E-BP1) in response to insulin in rat epididymal fat-cells

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There is mounting evidence that in fat and other insulin-sensitive cells activation of protein synthesis may involve the dissociation of a protein (4E-BP1) from eukaryotic initiation factor (eIF)-4E thus allowing formation of the eIF-4F complex. This study compares the effects of insulin and epidermal growth factor (EGF) on the phosphorylation of 4E-BP1 in fat-cells (followed by gel-shift assays and incorporation of ^{32}P) and on its association with eIF-4E. Several lines of evidence suggest that mitogen-activated protein kinase (MAP kinase) is not involved in these effects of insulin. Insulin causes much more extensive phosphorylation and dissociation of 4E-BP1 from eIF-4E than EGF, although EGF activates MAP kinase to a much greater extent than insulin. Moreover, MAP kinase does not phosphorylate 4E-BP1 when it is complexed with eIF-4E. In contrast, insulin activates the 40S ribosomal protein S6 kinase ($p70^{S6K}$) 18-fold compared with a 2-fold activation by EGF, and the time course of this activation is similar to the phosphorylation and dissociation of 4E-BP1. Rapamycin, a specific inhibitor of the activation of this latter kinase, inhibits dissociation of 4E-BP1 from eIF-4E in cells incubated with insulin but reveals a phosphorylated form of 4E-BP1 which remains bound to eIF-4E. It is concluded that in rat epididymal fat-cells, the effects of insulin on 4E-BP1 involves multiple phosphorylation events. One phosphorylation event is rapamycin-insensitive, occurs only on bound 4E-BP1 and does not initiate dissociation. The second event does result in dissociation and is blocked by rapamycin, suggesting that the $p70^{S6K}$ signalling pathway is involved: $p70^{S6K}$ itself is probably not involved directly as this kinase does not phosphorylate 4E-BP1 *in vitro*.

INTRODUCTION

Exposure of fat-cells to various hormones and growth factors increases the phosphorylation of a number of proteins within a few minutes (for a review see ref. [1]). In particular, insulin increases substantially the phosphorylation on both serine and threonine residues of a protein of apparent molecular mass 22 kDa (as judged by SDS/PAGE) [2–6]. This protein has recently been cloned from rat tissues and found to have a molecular mass of 12.4 kDa [7]. Furthermore, two human cDNA clones with a high degree of homology to the rat fat-cell protein have been identified as being involved in the control of mRNA translation through a functional cloning screen for proteins interacting with eukaryotic initiation factor (eIF)-4E. These are termed eIF-4E-binding proteins 1 and 2 (4E-BP1 and 2); 4E-BP1 is the equivalent of the original 22 kDa rat fat-cell phosphoprotein. eIF-4E binds to the 5′-cap (7-methylguanosine) of eukaryotic mRNAs and this is thought to be the initial step in the binding of mRNA to the ribosome during peptide-chain initiation (reviewed in ref. [8]). Both 4E-BP1 and 4E-BP2 have been shown to inhibit cap-dependent mRNA translation [9].

Evidence has also been presented showing that increased phosphorylation of 4E-BP1 causes it to dissociate from eIF-4E [9]. For example, insulin treatment of fat-cells, which leads to substantially increased phosphorylation of 4E-BP1, reduces the amount of 4E-BP1 co-extracted with eIF-4E on the affinity matrix 7-methyl-GTP (m^7GTP)–Sepharose. Since this dissociation should relieve the inhibition of translation, it potentially provides a mechanism by which agents that increase the phosphorylation of 4E-BP1 could stimulate translation. eIF-4E forms a high-molecular-mass complex (termed eIF-4F) which includes two other translation factors eIF-4A (an RNA helicase) and eIF-4G (formerly known as p220 or eIF-4γ, reviewed in ref. [8]); eIF-4A is thought to play a key role in 'unwinding' secondary structure which, when in the 5′-untranslated regions (5′-UTRs) of mRNAs, can inhibit their translation [10]. Thus the dissociation of 4E-BP1, by relieving inhibition of the function of eIF-4E, may allow up-regulation of the translation of mRNAs with 5′-UTRs that are rich in secondary structure. Overexpression of eIF-4E enhances the translation of the mRNAs encoding cyclin D1 [11] and ornithine decarboxylase [12]. The release of eIF-4E from inhibition by 4E-BP1, for example (as the result of its phosphorylation in response to insulin, for example), might be expected to have the same effect. Indeed, insulin does enhance the translation of ornithine decarboxylase in fibroblasts expressing the insulin receptor [13].

In vitro, 4E-BP1 is a substrate for both mitogen-activated protein kinase (MAP kinase) [14,15] and casein kinase 2 [14,16]. In rat fat-cells, the serine residue (Ser-64) that is phosphorylated by MAP kinase *in vitro* also appears to become phosphorylated

Abbreviations used: eIF, eukaryotic initiation factor; EGF, epidermal growth factor: MAP kinase, mitogen-activated protein kinase; $p70^{S6K}$, 40S ribosomal protein S6 kinase; 4E-BP1, eIF-4E-binding protein 1; 5′-UTR, 5′-untranslated region; ECL, enhanced chemiluminescence; DTT, dithiothreitol.

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when fat-cells are exposed to insulin [14]. However, the increased phosphorylation of 4E-BP1 in fat-cells exposed to insulin occurs on both serine and threonine residues within at least two peptides which are separable by two-dimensional thin-layer analysis [3,5]. Hence, it is likely that at least one additional kinase is involved in the phosphorylation of this protein. Multiple forms of 4E-BP1 are also separable by two-dimensional PAGE [5,17] which also suggests that the protein is phosphorylated on multiple sites in intact cells.

In this study, we have compared the effects of insulin and EGF on the phosphorylation of 4E-BP1 in fat-cells and on its association with eIF-4E. Furthermore, we have examined the effects of rapamycin, an inhibitor of the activation of the 40S ribosomal protein S6 kinase ($p70^{S6K}$), and wortmannin, an inhibitor of phosphatidylinositide 3-kinase (PI 3′-kinase). We conclude that at least two protein kinases acting on distinct sites are involved in the phosphorylation of 4E-BP1 in intact cells but neither of these kinases appear to be MAP kinase. Our studies using freshly prepared rat fat-cells confirm and extend recent studies using cultured cell lines (3T3-L1 and aortic smooth muscle cells) [17,18] which also indicate that dissociation of 4E-BP1 from eIF-4E is brought about by a rapamycin-sensitive mechanism and were published while this paper was in preparation.

EXPERIMENTAL

Materials

Male Wistar rats were fed *ad libitum* up to the time of killing on a stock laboratory diet (CRM; Bioshore, Lavender Hill, Cambs., U.K.). Hyperfilm, enhanced chemiluminescence (ECL) Western-blotting kits, [γ-^{32}P]ATP and [^{32}P]P_i were obtained from Amersham International. All chemicals and biochemicals were obtained from Sigma Chemical Co. or BDH, except for pepstatin, antipain and leupeptin (Cambridge Research Biochemicals, Cambridge, U.K.), collagenase (Worthington Diagnostic Systems, Freehold, NJ, U.S.A.), rapamycin (Affiniti Research Products Ltd., Nottingham, U.K.), epidermal growth factor (EGF) (Collaborative Biomedical Products), m^{7}GTP–Sepharose (Pharmacia Biotechnology Inc.) and microcystin and dithiothreitol (DTT) (Calbiochem, Nottingham, U.K.). Immobilon P membrane and Centricon 10 microconcentrators were from Amicon (Gloucester, U.K.). Immunopure Gentle Ag/Ab Elution Buffer was from Pierce, Rockford, IL, U.S.A., and PD10 desalting columns and Mono Q chromatography columns were from Pharmacia. $p70^{S6K}$ was prepared by immunoprecipitation from extracts of insulin-treated CHO.T cells.

General techniques

SDS/PAGE [19] was performed using acrylamide/bisacrylamide gels (15%:0.4%, w/v) and a Bio-Rad Protean II mini-gel system. Densitometry was carried out using a Joyce–Loebl Chromoscan 3 linked to a Hewlett–Packard 9000/3000 computer [20].

Preparation of cell extracts

Fat-cells were prepared from rat epididymal fat pads with modifications as described [21,22]. Cells (routinely 170–200 mg dry cell weight) were incubated in gassed Krebs–Henseleit buffer (1.5 ml) containing 10 mM Hepes, 2 mg/ml glucose and 1% BSA with further additions as indicated in the Figure legends. In studies involving [^{32}P]P_i, cells were incubated in medium containing initially 0.4 mM P_i and about 1000 c.p.m./pmol. Fat-cells were extracted in 50 mM β-glycerophosphate, pH 7.4, containing 1.5 mM EGTA, 0.5 mM Na_3VO_4, 1 mM DTT, 1 μM microcystin and the proteinase inhibitors pepstatin, antipain and leupeptin (1 μg/ml each), 2 mM benzamidine and 0.1 mM PMSF.

Western blotting and immunoprecipitation

Antiserum against eIF-4E was raised as described [23] and affinity-purified before use as previously described [24]. Anti-peptide antibodies to 4E-BP1 were raised in rabbits and used as described by Diggle *et al.* [6].

For immunoprecipitation, samples were incubated with anti-4E-BP1 serum (1:100 dilution) plus 5 mg of Protein A–Sepharose for 2 h with tumbling at 4 °C. Beads were pelleted briefly by centrifugation and washed three times (1 ml each wash) in 50 mM Hepes, pH 7.4, containing 20 mM EDTA, 50 mM NaF, 0.1% (v/v) Triton X-100, 1 μg/ml each pepstatin, antipain and leupeptin and 2 mM benzamidine. A final wash was performed in 1 ml of the above buffer at one-tenth the concentration. 4E-BP1 was eluted by boiling the beads in 0.1 ml of one-tenth concentration Laemmli sample buffer, followed by an equivalent volume of water.

Isolation and analysis of eIF-4E and 4E-BP1

eIF-4E and bound 4E-BP1 were purified from fat-cell extracts by tumbling extracts (1 ml) prepared with m^{7}GTP–Sepharose (25 μl of suspension) for 90 min at 4 °C. The beads were pelleted rapidly, washed as described above and eluted with 25 μl of 100 μM m^{7}GTP.

Preparative-scale isolation of reticulocyte eIF-4E–4E-BP1 was performed by applying 1 ml of rabbit reticulocyte lysate [diluted 1:1 with 20 mM Hepes/KOH, pH 7.6, containing 100 mM KCl, 2 mM EDTA, 10% (v/v) glycerol, 0.5 mM DTT, 2 mM benzamidine and pepstatin, antipain and leupeptin (1 μg/ml each)] to a column (bed volume 1 ml) of m^{7}GTP–Sepharose. Unbound protein was removed from the column by washing with the above buffer. eIF-4E and associated 4E-BP1 were eluted with buffer (see above) containing 100 μM m^{7}GTP. Peak fractions were concentrated (Centricon 10 membrane; Amicon), dialysed against the same buffer to remove m^{7}GTP and stored at −70 °C.

Protein kinase assays

The activities of MAP kinase and $p70^{S6K}$ in fat-cell extracts were measured in anti-p42/p44 or anti-$p70^{S6K}$ immunoprecipitates using the appropriate synthetic peptide substrate as described previously [25]. Immunoprecipitates were washed three times in extraction buffer before assay.

Preparation of recombinant 4E-BP1

Full-length 4E-BP1 cDNA was prepared by PCR from a rat fat-cell cDNA library (a gift from Dr. G. Holman, University of Bath, Bath, U.K.). 4E-BP1 cloned into pET11a [AMS Biotechnology (UK) Ltd.] was expressed in *Escherichia coli* BL21(DE3)pLysS strain in the presence of the chaperonins GroES and GroEL in pREP4 [26]. *E. coli* (5 litres) expressing 4E-BP1 were pelleted by centrifugation (7000 ***g*** for 7 min) and sonicated (15 ml aliquots; 3 × 10 s bursts) in 10 mM KH_2PO_4, pH 7.4, containing 2 mM EDTA, 1 mM DTT, 1 μg/ml each proteinase inhibitors pepstatin, antipain and leupeptin and 0.5 mM PMSF. After centrifugation at 30000 ***g*** for 20 min, the

supernatant was subjected to chromatography on a Mono Q HR 10/10 column equilibrated in the above buffer but without the PMSF. Proteins were eluted with a linear gradient of NaCl to 0.5 M in the same buffer. 4E-BP1, identified by western blotting and eluted between 100 and 200 mM NaCl, was then applied to a 4E-BP1 anti-peptide antibody column (prepared as described previously [24]). 4E-BP1 was eluted with Immunopure Gentle Ag/Ab Elution Buffer, desalted on a PD10 column and concentrated using a Centricon 10 microconcentrator.

In vitro phosphorylation studies

Recombinant 4E-BP1 or a complex of eIF-4E and 4E-BP1 isolated from rabbit reticulocyte lysates was phosphorylated in 10 mM β-glycerophosphate, pH 7.4, with additions as for buffer B above and in the presence of 100 μM [γ-^{32}P]ATP (specific radioactivity 1000–2000 c.p.m./pmol) and 5 mM $MgCl_2$. Samples were incubated for 15 min at 30 °C and reactions were stopped by the addition of Laemmli sample buffer. Phosphoproteins were separated by SDS/PAGE and visualized by radioautography using preflashed Amersham Hyperfilm in cassettes with intensifying screens.

RESULTS AND DISCUSSION

Effects of insulin, EGF and isoprenaline on 4E-BP1 phosphorylation and its association with eIF-4E

Earlier studies have shown that, in ^{32}P-labelled fat-cells, insulin rapidly causes the increased phosphorylation of 4E-BP1 which results in its decreased mobility on SDS/PAGE [4,6]. A shift in mobility with phosphorylation has also been shown in 3T3-L1 adipocytes in response to insulin [15,17,27].

Figure 1(A) shows the effects of insulin on the pattern of immunoreactive species of 4E-BP1 separated by SDS/PAGE in total cell extracts (a), in samples of 4E-BP1 associated with eIF-4E [isolated by affinity chromatography on m^{7}GTP–Sepharose (c)] as well in the material not retained by this affinity matrix (b). In agreement with earlier studies [6], two major species of 4E-BP1 were apparent in total extracts of untreated cells with the faster migrating species usually corresponding to at least 50 % of the total 4E-BP1. Insulin caused the disappearance of much of this species as well as the appearance of a third, even more slowly migrating species. Most of the 4E-BP1 retained by the affinity matrix in control cells was the fastest moving species and very little, if any, 4E-BP1 remained bound to eIF-4E in cells incubated with insulin. Conversely, as expected, the unbound material (not associated with eIF-4E) from insulin-treated cells contained the two slower migrating species of 4E-BP1. These observations are consistent with the idea that increased phosphorylation of 4E-BP1 causes its dissociation from eIF-4E [9,15]. Similar observations have been made with 3T3 L1 adipocytes [17] but the effects of insulin were less marked in this system as all three species separated by SDS/PAGE were present in about equal amounts in cells incubated in the absence of insulin and the fastest migrating species was still evident in insulin-treated cells.

In 20 separate fat-cell preparations, the effect of insulin treatment for 15 min resulted in an almost total dissociation of 4E-BP1 from eIF-4E (to 8.6 ± 2.4 % of control; mean ± S.E.M.). The strength of the eIF-4E signal was consistently decreased in samples from insulin-treated cells relative to controls (to 69 ± 13 % of control; see also figures 1A, 1C and 2A). It is possible that the increased association of eIF-4E with initiation complexes containing mRNA, eIF-4G and/or eIF-4A may reduce its ability (or availability) to bind to the m^{7}GTP–Sepharose affinity matrix, perhaps because a proportion of eIF-4E becomes more tightly associated within ribosomal complexes.

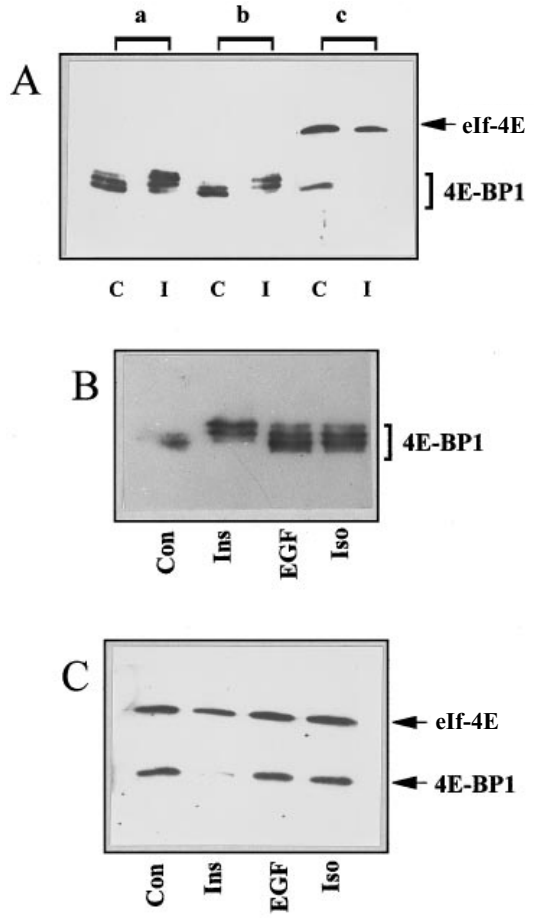

Figure 1 Effects of hormones on the association of 4E-BP1 with eIF-4E in rat fat-cells

Fat-cells (170–200 mg dry weight) were incubated in the absence or presence of 83 nM insulin, 100 nM EGF or 3 μM isoprenaline for 15 min, and extracts were prepared and subjected to either immunoprecipitation with anti-4E-BP1 antibody or m^{7}GTP–Sepharose affinity chromatography. The products were subject to SDS/PAGE and Western blotting with a mixture of anti-4E-BP1 and anti-eIF-4E antibodies using ECL. Amounts of material in each lane correspond to the same volumes of cell extracts. Results are typical of more than six separate experiments. (**A**) Cells were treated with (I) or without (C) insulin as described above. Total 4E-BP1 was prepared by immunoprecipitation of cell extracts (a): eIF-4E (and associated 4E-BP1) was prepared by incubation of extracts with m^{7}GTP–Sepharose (c). 4E-BP1 not bound to the m^{7}GTP–Sepharose affinity matrix from the above extracts was isolated by immunoprecipitation (b). The exposure for lanes in c was six times longer than for the other lanes. (**B**) 4E-BP1 not bound to m^{7}GTP–Sepharose (see above) was isolated by immunoprecipitation of control (Con), insulin- (Ins), EGF- and isoprenaline- (Iso) treated cell extracts. (**C**) eIF-4E (and associated 4E-BP1) was prepared by incubation of extracts with m^{7}GTP–Sepharose. Lanes are as in (**B**).

The data in Figure 1(A) also indicate that in rat fat-cells the amount of 4E-BP1 associated with eIF-4E even in control cells is only a small proportion of the total 4E-BP1 present. First, a much shorter ECL exposure time was needed to detect 4E-BP1 in the unbound fraction (b) than in the bound fraction (c). Secondly, there is little difference in the intensity of the fastest migrating species in control total cell extracts and control unbound 4E-BP1 although identical amounts of cell extract were loaded. This indicates that only a small proportion is bound to eIF-4E and removed by treatment with the m^{7}GTP–Sepharose matrix.

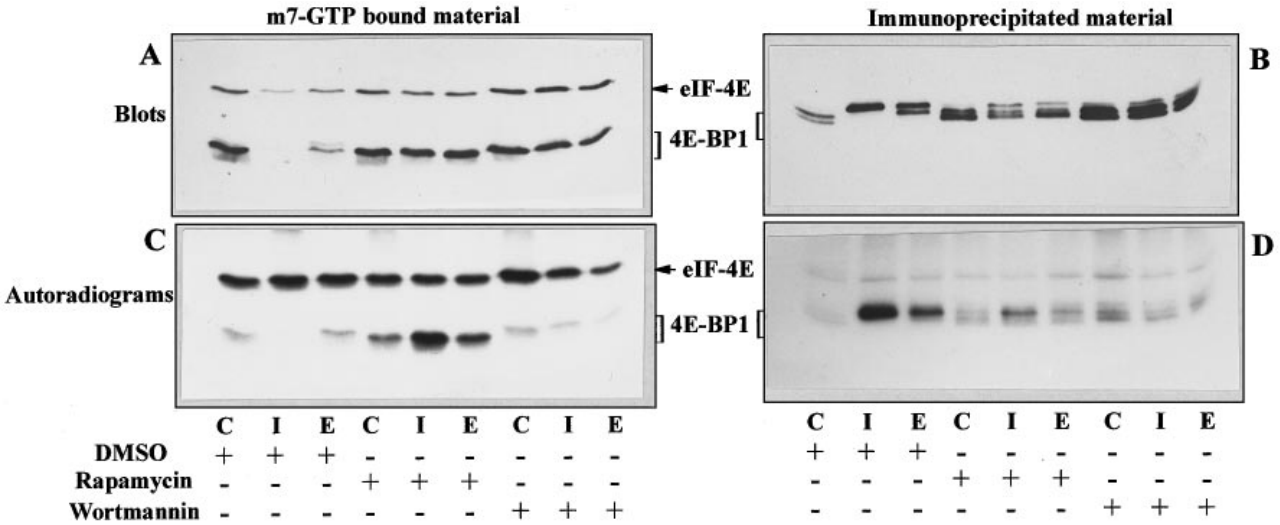

Figure 2 Effects of rapamycin and wortmannin on the phosphorylation of 4E-BP1 and its association with eIF-4E

Fat-cells were incubated and extracts subjected to successive m^7GTP–Sepharose affinity chromatography (**A,C**) and immunoprecipitation (**B,D**) as in Figure 1 except that the cells were preincubated with medium containing [^{32}P]P_i for 60 min (see the Experimental section). Fat-cells were then incubated in the same medium for 30 min with rapamycin (20 nM) or wortmannin (100 nM) before addition to insulin (I; 83 nM) or EGF (E; 100 nM) for a further 10 min. All incubations were carried out in the presence of 0.01% DMSO. After SDS/PAGE and transfer to poly(vinylidene difluoride) membranes the membranes were radioautographed for 72 h (**C**) or 24 h (**D**) and then blotted with a mixture of anti-4E-BP1 and anti-eIF-4E antibodies and developed by ECL for 40 s (**A**) or 15 s (**B**). Results are typical of four experiments.

Figures 1(B) and 1(C) show that EGF and isoprenaline cause changes in the ratio of the species of 4E-BP1 which are separated by SDS/PAGE but that these effects are less pronounced than those seen with insulin and are associated with less dissociation of 4E-BP1 from eIF-4E (see also Figures 2A and 2B). The decrease in the mobility of 4E-BP1 seen in response to EGF is slightly greater in Figure 2(B), indicating that a certain degree of variability is associated with the effects of the growth factor. Figure 2(D) shows that this greater shift in response to EGF in this experiment is associated with a corresponding increase in the dissociation of 4E-BP1 from eIF-4E.

In further experiments, the effects of insulin and EGF on the phosphorylation of the various species of 4E-BP1 separated by SDS/PAGE were determined directly from the steady-state incorporation of ^{32}P from radiolabelled P_i in the incubation medium (Figure 2). These showed that there was little or no incorporation of ^{32}P into the fastest migrating species which binds to eIF-4E in the absence of hormones (Figure 2C). In contrast, there was extensive incorporation of ^{32}P into the slower migrating forms which do not bind to eIF-4E and which are more prevalent in cells exposed to insulin, and to a lesser extent in cells exposed to EGF (Figure 2D). These studies also show that eIF-4E is phosphorylated in rat fat-cells (Figure 2C). If a correction is made for the smaller amount of eIF-4E bound to m^7GTP–Sepharose in the hormone-treated cells, it is evident that insulin causes at least a doubling of the phosphorylation of this initiation factor and that EGF may also cause a modest increase. Increases in the phosphorylation of eIF-4E with insulin have also been shown in 3T3-L1 [28], NIH-3T3 and HIR 3.5 fibroblasts [13].

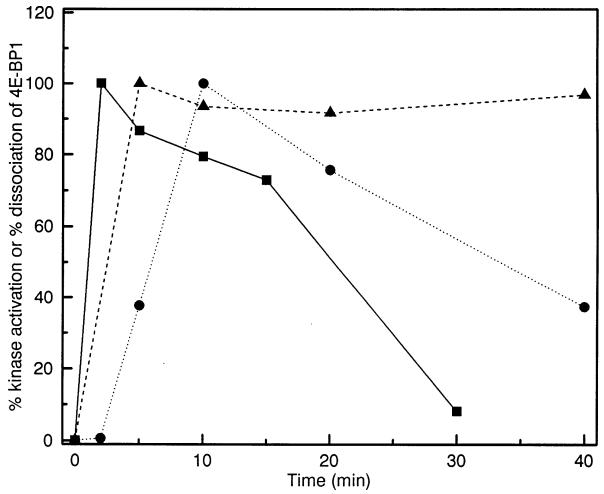

Figure 3 Time course of activation of MAP kinase and p70^{S6K} and dissociation of 4E-BP1 in response to insulin

Freshly isolated fat-cells were incubated for the times indicated. Activities of MAP kinase and p70^{S6K} are expressed as a percentage of the maximum activation in the presence of insulin and are means ± S.E.M. for observations on two to four separate cell preparations. ■, MAP kinase; ●, p70^{S6K}. The basal and maximal activities of MAP kinase were 0.55 and 1.35 pmol of ^{32}P incorporated into substrate peptide/min per g dry weight of fat-cells respectively for observations on two separate cell preparations. The values for p70^{S6K} were 4.53 and 87 pmol of ^{32}P incorporated into substrate peptide/min per g dry weight of fat-cells respectively for observations on four separate cell preparations. Also shown is the extent of dissociation of 4E-BP1 (▲) determined by densitometric scanning.

Role of MAP kinase and p70^{S6K} in the phosphorylation of 4E-BP1 in rat fat-cells

We and others have previously shown that insulin and EGF activate MAP kinase in fat-cells [25,29]. In experiments carried out under the same conditions as those used in the present study, both insulin and EGF activated MAP kinase in isolated fat-cells, with maximal stimulation of activity seen after 2–5 min, but EGF gave a considerably higher degree of activation of this

Table 1 Effects of rapamycin and wortmannin on the activation of p70^{S6K} in fat-cells by insulin, EGF and isoprenaline

Fat-cells ($n = 4$–15 separate fat-cell preparations) were incubated for 30 min with 20 nM rapamycin or 100 nM wortmannin before the addition of 83 nM insulin, 100 nM EGF or 3 μM isoprenaline for 10 min. All incubations were carried out in the presence of 0.01% (v/v) DMSO. Results are expressed as percentage activity in cells incubated in the presence of 0.01% (v/v) DMSO alone [control (mean ± S.E.M.) = 5.1 ± 0.63 pmol of ^{32}P incorporated into substrate peptide/min per g dry weight of fat-cells for observations on nine separate cell preparations]. Significance as assessed by Student's t test is indicated as follows; $^{**}P < 0.001$, $^{*}P < 0.01$.

	Kinase activity (% of control)		
Added hormone	No inhibitor	Wortmannin	Rapamycin
None	100	102 ± 9	116 ± 13
Insulin	1810 ± 308**	87 ± 6	114 ± 9
EGF	269 ± 42*	93 ± 7	118 ± 12
Isoprenaline	290 ± 42**	104 ± 24	98 ± 8

kinase (11.3 ± 2.2-fold) then insulin (4.3 ± 0.8-fold). In contrast, isoprenaline had no effect on MAP kinase (101 ± 18; $n = 6$; mean ± S.E.M.; relative to control = 100) as previously reported [29]. Given that EGF has a smaller effect than insulin on the phosphorylation of 4E-BP1 or its association with eIF-4E, these data cast doubt on the role of MAP kinase in the phosphorylation of 4E-BP1. Moreover, maximal dissociation of 4E-BP1 from eIF-4E occurs between 5 and 10 min and is sustained for at least 60 min whereas the activation of MAP kinase by insulin is transient and barely detectable after 30 min (Figure 3).

In fat-cells, insulin rapidly activated p70^{S6K} with a maximal stimulation of approx. 18-fold occurring after about 10 min, followed by only a slow decline for the next 30–40 min, whereas EGF and isoprenaline both produced very modest activation (Figure 3, Table 1). Stimulation of p70^{S6K} activity in isolated fat-cells treated with insulin has been reported previously [25,30]. Our results differ from those of Lin and Lawrence [30] who reported only a 2-fold increase in p70^{S6K} with both insulin and EGF. The reason for this difference is unclear but it should be noted that these researchers used a different assay procedure which may be less specific than that used in the present study.

We have previously reported that rapamycin does not significantly affect the activation of MAP kinase in fat-cells [25]. In contrast, rapamycin totally abolished stimulation of p70^{S6K} activity by insulin, EGF and isoprenaline, while having no significant effect on basal levels of kinase activity (Table 1). Furthermore preincubation with wortmannin (100 nM), an inhibitor of PI 3′-kinase, also abolished the stimulation of p70^{S6K} by insulin, EGF and isoprenaline (Table 1).

Both wortmannin and rapamycin block the effects of insulin and EGF on the dissociation of 4E-BP1 from eIF-4E (Figure 2A). In 11–23 experiments the amount of 4E-BP1 bound was decreased by insulin to 8.6 ± 2.4% of control, whereas the corresponding values in the presence of insulin plus rapamycin (20 nM) or wortmannin (100 μM) were 94 ± 11% or 134 ± 25% respectively. Rapamycin exerted a half-maximal effect on the proportion of 4E-BP1 in the fastest migrating species at about 0.5–1 nM (not shown). The half-maximal effect on the activation of p70^{S6K} by insulin was similar at about 0.1–0.5 nM.

Wortmannin also reverses the effects of the hormones on the phosphorylation of 4E-BP1 as indicated by the incorporation of ^{32}P (Figures 2C and 2D) and indicated by the pattern of species separated by SDS/PAGE (Figures 2A and 2B). The effects of rapamycin on the phosphorylation of 4E-BP1 are more complex. In the presence of insulin, and to a lesser extent EGF, rapamycin reveals a highly phosphorylated species of 4E-BP1 which is bound to eIF-4E (Figure 2C). This species is the fastest migrating form of 4E-BP1 on SDS/PAGE. In four experiments, in the presence of insulin, rapamycin increased the incorporation of ^{32}P into 4E-BP1 bound to eIF-4E by 1002 ± 209% whereas the incorporation of ^{32}P into the unbound fraction of 4E-BP1 was markedly decreased (Figure 2D). In the absence of any other effectors rapamycin increased the phosphorylation of 4E-BP1 approx. 2-fold (Figure 2C) but this increase is much smaller than that seen in the presence of rapamycin plus insulin.

To investigate further which kinase(s) might be responsible for phosphorylating 4E-BP1, *in vitro* phosphorylation studies were carried out. These employed either free recombinant 4E-BP1 or 4E-BP1 complexed to eIF-4E purified from rabbit reticulocyte lysates (the complexes were isolated using m^{7}GTP–Sepharose). As previously reported [14], recombinant 4E-BP1 was an excellent substrate for MAP kinase (Figure 4). In contrast, 4E-BP1 complexed to eIF-4E was not significantly phosphorylated by MAP kinase under the same conditions, perhaps because the residue (or residues) phosphorylated by MAP kinase is occluded in the 4E-BP1–eIF-4E complex (see also ref. [17]). These results lend further support to the idea that MAP kinase is not the kinase involved in the phosphorylation of 4E-BP1 *in vivo* as had been previously suggested [14, 15]. Free 4E-BP1 is also a substrate for casein kinase 2, although the degree of phosphorylation by this kinase is far less than that catalysed by MAP kinase. In contrast with MAP kinase, however, association of 4E-BP1 with eIF-4E does not prevent the ability of casein kinase 2 to phosphorylate 4E-BP1 using either rabbit (Figure 4) or rat complex (results not shown). Indeed, the 4E-BP1 appears to be a better substrate for casein kinase 2 when complexed to eIF-4E. It has previously been reported that free recombinant 4E-BP1 is not a substrate for the p70^{S6K} [14]. We also find that 4E-BP1, either free or complexed with eIF-4E, is not phosphorylated by p70^{S6K} although under the same conditions this kinase caused extensive phosphorylation of the ribosomal protein S6 present in 40S subunits (Figure 4).

General conclusions

It is clear from the results of this study that both rapamycin-sensitive and -insensitive pathways are involved in the phosphorylation of 4E-BP1 in response to insulin in rat epididymal fat-cells. Other recent studies using cultured cell lines [17,18] have observed that rapamycin blocks the dissociation of 4E-BP1 from eIF-4E. Since this was associated with a reversal of the changes in migration of 4E-BP1 on SDS/PAGE, it was concluded that rapamycin acts by blocking the phosphorylation of 4E-BP1. However, the use of ^{32}P incorporation to follow phosphorylation directly in the present study has revealed that at least two distinct phosphorylation events occur in the presence of insulin. One phosphorylation event is rapamycin-insensitive and does not result in dissociation of 4E-BP1. Moreover, this phosphorylation does not cause any change in the mobility of the fastest migrating form of 4E-BP1 on SDS/PAGE. This event appears to be restricted to 4E-BP1 bound to eIF-4E since in the presence of rapamycin this phosphorylated form of 4E-BP1 is only found in the fraction of 4E-BP1 retained by m^{7}GTP–Sepharose and is not found in the unbound fraction (Figure 2). The second phosphorylation event is rapamycin-sensitive and does result in dissociation of 4E-BP1 from eIF-4E. This event is associated with marked changes in the migration of 4E-BP1 such that, in the presence of insulin, essentially all the 4E-BP1 is converted into the slower migrating species. EGF activated both events, but only to a much smaller extent than insulin. Wort-

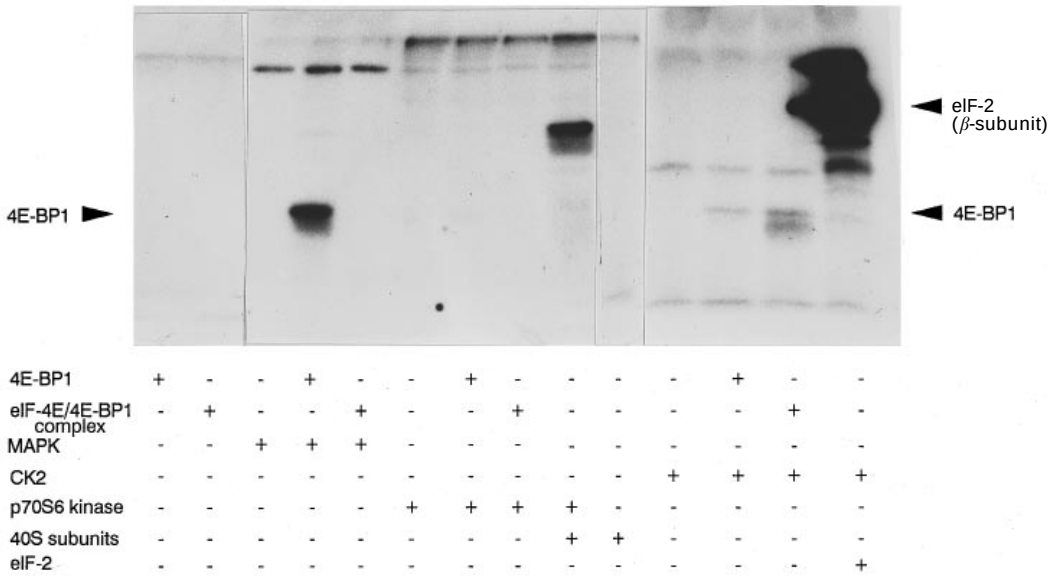

Figure 4 ***In vitro*** **phosphorylation of 4E-BP1 by MAP kinase, casein kinase 2 and p70^{S6K}**

Phosphorylation was carried out using [γ-^{32}P]ATP and 5 units/ml MAP kinase (MAPK; 1 unit phosphorylates 1 μmol of myelin basic protein/min per mg), 0.1 unit/ml casein kinase 2 (CK2; 1 unit phosphorylates 1 nmol of casein/min per mg) or p70^{S6K} immunoprecipitated from 0.2–0.3 mg of insulin-treated CHO.T cells (extracted in buffer as for fat-cells). eIF-2 (β-subunit) serves as a positive control for casein kinase 2. Incubations were for 15 min at 30 °C and reactions were stopped by the addition of Laemmli sample buffer. Samples were separated by SDS/PAGE and revealed by radioautography for 16 h. Recombinant 4E-BP1 and 4E-BP1 complexed to eIF-4E, approx. 1 μg; eIF-2, 5 μg; 40S ribosomal subunits, 9 μg. Results are typical of three experiments.

mannin is able to block both phosphorylation events essentially completely.

It seems likely that two different protein kinases are involved in the phosphorylation of 4E-BP1 in cells exposed to insulin. Insulin may act by stimulating both kinases but other mechanisms are theoretically possible; for example, insulin may inhibit some specific protein phosphatase.

The identities of the kinases involved in the two phosphorylation events are unknown. However, a number of general conclusions can be drawn. MAP kinase (ERK1/ERK2) is probably not involved because of the relative effects of EGF, the time course of MAP kinase activation and the inability of MAP kinase to phosphorylate 4E-BP1 when it is complexed with eIF-4E (Figure 4). We attempted to obtain further evidence using the MAP kinase kinase (MEK) inhibitor PD098159 but it caused little or no inhibition of MAP kinase activation in rat fat-cells (results not shown). This is in contrast with 3T3-L1 adipocytes where it has been found that the inhibitor does block the effects of insulin on MAP kinase activity but not the effects of insulin on 4E-BP1 [17]. Unbound 4E-BP1 is clearly a good substrate for MAP kinase *in vitro* but it is not a substrate when bound to eIF-4E (Figure 4) [17]. However, it is difficult to see how this could be a complete explanation of why 4E-BP1 is not phosphorylated by MAP kinase within rat-fat-cells. A large proportion of the 4E-BP1 is not bound to eIF-4E even in the absence of hormones, yet little of the unbound 4E-BP1 becomes phosphorylated in the presence of EGF plus rapamycin (Figure 2D), although there is extensive activation of MAP kinase under these conditions [25]. Obvious explanations for this might be compartmentation within the cell or that the 'free' 4E-BP1 is in fact bound to another protein.

The kinase involved in the rapamycin-sensitive phosphorylation event that causes dissociation is probably not p70^{S6K} itself as *in vitro* this kinase does not phosphorylate 4E-BP1 (Figure 4). Rather it seems more likely that it is some other kinase in the p70^{S6K} signalling pathway. However, we have not eliminated the possibility that p70^{S6K} only acts on 4E-BP1 after its phosphorylation by the rapamycin-insensitive kinase. It is possible that casein kinase 2 may be involved in the rapamycin-insensitive phosphorylation of 4E-BP1 since this kinase is able to phosphorylate the binding protein, albeit to a lesser extent than MAP kinase, and is activated by insulin in rat fat-calls [16]. Interestingly, although casein kinase 2 can phosphorylate both free and bound 4E-BP1, *in vitro* at least the eIF-4E–4E-BP1 complex appears to be a better substrate than unbound 4E–BP1. However, it has to be concluded that it is as yet unclear whether the kinase involved in intact cells in the rapamycin-insensitive event is casein kinase 2.

Other studies have implicated the p70^{S6K} pathway in the regulation of the translation of certain mRNAs which contain polypyrimidine tracts at their 5′ ends [31] and in the control of elongation via the phosphorylation state of elongation factor eEF-2 [32]. Taken together with the findings in the present study, it seems that this signalling pathway may have multiple inputs into the regulation of gene expression at the level of mRNA translation. It has previously been suggested that the effect of rapamycin on the translational up-regulation of the polypyrimidine-tract-containing mRNAs may involve phosphorylation of ribosomal protein S6 [31], since this is blocked by rapamycin. As the present data show that this drug also affects the association of eIF-4E with 4E-BP1 and thus presumably its activity, the control of the translation of polypyrimidine-tract-containing mRNAs may involve eIF-4E instead of or in addition to S6 [33].

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