Insulin-stimulated kinase from rat fat cells that phosphorylates initiation factor 4E-binding protein 1 on the rapamycin-insensitive site (serine-111)

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The effects of insulin and rapamycin on the phosphorylation of the translation regulator, initiation factor 4E-binding protein 1 (4E-BP1) have been studied in rat fat cells by following changes in the incorporation of ³²P from [³²P]P_i under steady-state conditions. Both unbound 4E-BP1 and 4E-BP1 bound to eukaryotic initiation factor 4E (eIF4E) were isolated from the cells and then digested with trypsin and other proteases; the radiolabelled phosphopeptides were then separated by two-dimensional thin-layer analysis and HPLC. The results provide confirmation of the conclusion of Fadden, Haystead and Lawrence [J. Biol. Chem. (1997) 272, 10240–10247] that insulin increases the phosphorylation of four sites that fit a Ser/Thr-Pro motif (Thr-36, Thr-45, Ser-64 and Thr-69) and that taken together these phosphorylations result in the dissociation of 4E-BP1 from eIF4E. The effects of insulin on the phosphorylation of these sites, and hence dissociation from eIF4E, are blocked by rapamycin. However, the present study also provides evidence that insulin increases the phosphorylation of 4E-BP1 bound to eIF4E on a further site (Ser-111) and that this is by a rapamycin-

insensitive mechanism. Extraction of rat epididymal fat cells followed by chromatography on Mono-S and Superose 12 columns resulted in the separation of both an insulin-stimulated eIF4E kinase and an apparently novel kinase that is highly specific for Ser-111 of 4E-BP1. The 4E-BP1 kinase was activated more than 10-fold by incubation of the cells with insulin and was markedly more active towards 4E-BP1 bound to eIF4E than towards unbound 4E-BP1. The effects of insulin were blocked by wortmannin, but not by rapamycin. A 14-mer peptide based on the sequence surrounding Ser-111 of 4E-BP1 was also a substrate for the kinase, but peptide substrates for other known protein kinases were not. The kinase is quite distinct from casein kinase 2, which also phosphorylates Ser-111 of 4E-BP1. The possible importance of these kinases in the phosphorylation of 4E-BP1 in fat cells is discussed. It is suggested that the phosphorylation of Ser-111 might be a priming event that facilitates the subsequent phosphorylation of Thr-36, Thr-45, Ser-64 and Thr-69 by a rapamycin-sensitive process that initiates the dissociation of 4E-BP1 from eIF4E and hence the formation of the eIF4F complex.

INTRODUCTION

In 1980, Belsham et al. [1] reported the marked insulin-stimulated phosphorylation of a protein in rat fat cells that had an apparent molecular mass of 22 kDa as judged by SDS/PAGE. The protein remained in solution after boiling and after treatment with up to 2% (w/v) trichloroacetic acid; the phosphorylation observed with insulin was rapid, reversible, occurred on both serine and threonine residues and resulted in changes in the electrophoretic migration of the protein [2–4]. The role of this protein only became evident after its cDNA had been cloned and sequenced [5]. Sonenberg and colleagues then realized that this sequence was virtually identical with a cDNA they had cloned by screening a human placental expression library with labelled eukaryotic initiation factor 4E (eIF4E) as a probe [6]. The protein is called eIF4E-binding protein 1 (4E-BP1, or PHAS-I) and has a molecular mass of only approx. 12.5 kDa.

Subsequent studies showed that the binding of 4E-BP1 to eIF4E inhibited cap-dependent translation by blocking the association of eIF4E with eIF4G and hence the formation of the eIF4F complex [6–9]. Phosphorylation of 4E-BP1, which occurs on treatment of rat fat cells and other cells with insulin, results in the dissociation of 4E-BP1 from eIF4E, allowing the formation of competent eIF4F complexes [6,8,10–13].

The phosphorylation of 4E-BP1 in insulin-treated fat cells is complex. Early studies showed multiple forms by single- and two-dimensional electrophoresis [4,14] and by the fact that at least

two major tryptic phosphopeptides containing phosphoserine and phosphothreonine could be separated by two-dimensional thin-layer analysis [14]. More recent studies [15] have indicated that at least five sites might be phosphorylated in insulin-treated fat cells. These are Thr-36, Thr-45, Ser-64, Thr-69 and Ser-82, which all fit a Ser/Thr-Pro motif and are situated on either side of the likely eIF4E-binding site, which lies between Arg-50 and Met-59 [7]. Fadden et al. [15] suggested that the phosphorylation of Thr-45 and Thr-69 might be the most important in actually causing release, rather than phosphorylation of Ser-64, as had been concluded in an earlier study [16].

The kinases involved in the phosphorylation of 4E-BP1 in insulin-treated cells have not been definitively identified. Two insulin-activated kinases have been shown to phosphorylate purified 4E-BP1 *in vitro*; these are mitogen-activated protein (MAP) kinase [extracellular-signal-regulated protein kinase (ERK-1 and ERK-2)] and casein kinase 2 [12,16,17]. 4E-BP1 is an excellent substrate for MAP kinase, which is capable of phosphorylating the proline-directed sites that are phosphorylated in insulin-treated cells [15]. However, there is now considerable evidence that MAP kinase might not be important in the effects of insulin on the phosphorylation of 4E-BP1 in intact cells [11–13,18–20].

In one-dimensional SDS/PAGE, three bands corresponding to 4E-BP1 can be resolved and are designated α , β and γ in order of decreasing electrophoretic mobility. The increase in phosphorylation of 4E-BP1 caused by insulin is associated with a shift

Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP1, eIF4E-binding protein 1 (also known as PHAS-I); ERK, extracellular-signal-regulated protein kinase; MAP, mitogen-activated protein; DTT, dithiothreitol.

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from the α to the β and γ bands. Rapamycin inhibits these band shifts as well as the insulin-induced dissociation of 4E-BP1 from eIF4E [11–13,19,20]. These observations suggest that a protein kinase component of the FKBP-rapamycin-associated protein (FRAP) pathway is likely to be involved in the phosphorylation of 4E-BP1 that causes the dissociation of 4E-BP1 from eIF4E and allows the formation of the eIF4F complex. However, p70 ribosomal S6 protein kinase itself does not phosphorylate either the free or the eIF4E-bound form of 4E-BP1 *in vitro* and thus might not be involved in intact cells [11]. It has been reported recently that FRAP immunoprecipitates contain kinase activity that is able to phosphorylate unbound 4E-BP1 [21–23].

By following the incorporation of [32 P]P_i into 4E-BP1 in rat fat cells, it became evident that there is also a rapamycin-insensitive pathway involved in the phosphorylation of 4E-BP1 in response to insulin [11]. The 4E-BP1 bound to eIF4E in rat fat cells incubated with insulin plus rapamycin was found to be phosphorylated, but this phosphorylation did not result in any change in the mobility of 4E-BP1 on SDS/PAGE, which still migrated as the α form. Importantly, this event seemed to be largely restricted to 4E-BP1 bound to eIF4E, and thus the possibility arises that this phosphorylation might be the first priming event that might facilitate the phosphorylation of the proline-directed sites and hence dissociation [11].

In this paper we explore the phosphorylation of 4E-BP1 in rat fat cells incubated with insulin (in the presence or absence of rapamycin) in greater detail, separating labelled phosphopeptides formed on digestion with trypsin and other proteases by two-dimensional thin-layer analysis and HPLC. These studies yield confirmatory evidence that the rapamycin-sensitive sites phosphorylated by insulin are Thr-36, Thr-45, Ser-64 and Thr-69; in addition we identified a further site (Ser-111), which is phosphorylated by the rapamycin-insensitive mechanism. This site is shown to be phosphorylated by casein kinase 2 and also by an apparently novel kinase from rat epididymal fat cells.

This latter kinase is greatly stimulated in response to insulin by a mechanism that is sensitive to rapamycin and phosphorylates 4E-BP1 bound to eIF4E in preference to unbound 4E-BP1. The activation of this kinase might represent the first stage of the phosphorylation process that eventually results in the dissociation of 4E-BP1 from eIF4E and hence the formation of the eIF4E complex.

EXPERIMENTAL

Materials

Male Wistar rats (150–200 g) were fed *ad libitum* on a stock laboratory diet (CRM; Bioshore, Manea, Cambs., U.K.) until the time of killing. Reagents were as described previously [11] with the following additions: HPLC columns and reagents, which were from Perkin Elmer (Warrington, Cheshire, U.K.); modified trypsin, chymotrypsin, endopeptidase Glu-C and endopeptidase Asp-N (all sequencing grade) were from Boehringer Mannheim; rat liver casein kinase 2 was from Promega (Southampton, Hants., U.K.); TLC plates (cellulose fibre) were from Kodak (Rochester, NY, U.S.A.). Peptides were synthesized by Dr. G. Bloomberg (Department of Biochemistry, University of Bristol, Bristol, Avon, U.K.).

Preparation of extracts of rat fat cells for studies *in vivo* and the purification of 4E-BP1

Isolated rat epididymal fat cells were prepared and incubated as described [11]. Fat cells were extracted in 2.5 ml/g dry cell weight

of ice-cold buffer A [50 mM β -glycerophosphate/1.5 mM EGTA/1 mM benzamidine/0.5 mM Na₃VO₄ (pH 7.4) containing pepstatin, antipain and leupeptin, (1 μ g/ml each), 0.1 mM PMSF, 1 mM dithiothreitol (DTT) and 1 μ M Microcystin-LR] by vortex-mixing [24].

eIF4E was purified with bound 4E-BP1 from fat-cell extracts (1 ml) by tumbling with 25 μ l (packed volume) of m⁷GTP-Sepharose-4B for 1 h at 4 °C. After the supernatant had been removed and the Sepharose pellet had been washed three times in 1 ml of 50 mM Hepes/20 mM EDTA/10 mM NaF/10 mM Na₄P₂O₇ (pH 7.4) containing 0.1 % (v/v) Triton X-100, 1 μ g/ml pepstatin, 1 μ g/ml antipain, 1 μ g/ml leupeptin and 2 mM benzamidine, proteins were eluted by boiling the Sepharose in 3 $\times$ 100 μ l of 10 mM Tris/HCl/1 mM EDTA containing 0.2 % (v/v) 2-mercaptoethanol and Coomassie Brilliant Blue-R (1 mg/ml). From the supernatant removed above, unbound 4E-BP1 was immunoprecipitated as described previously [11,25].

Purification of recombinant eIF4E, 4E-BP1 and eIF4E/4E-BP1 complex

Recombinant 4E-BP1 was expressed in *Escherichia coli* (5 litres of L-broth) and extracted by sonication as described previously [11] in 100 ml of ice-cold extraction buffer B (50 mM Hepes, pH 7.6, containing 100 mM KCl, 2 mM EDTA, 2 mM DTT, 1 μ g/ml pepstatin, 1 μ g/ml leupeptin, 1 μ g/ml antipain and 10 mM PMSF). Recombinant eIF4E was also expressed in *E. coli* (1 litre) as described previously [26] and extracted by sonication in buffer B. To produce eIF4E/4E-BP1 complex, the sonicated extracts of *E. coli* expressing 4E-BP1 and eIF4E were centrifuged (39000 g for 30 min) and the supernatant from the 4E-BP1 expression was mixed with the inclusion pellet from the eIF4E expression. This mixture was made up to 6 M urea/100 mM DTT and dialysed as described for the production of recombinant eIF4E [26]. The eIF4E/4E-BP1 complex was purified by FPLC chromatography with a 1 ml (bed volume) m⁷GTP-Sepharose-4B column equilibrated in extraction buffer B. Proteins were eluted with 5 ml of this buffer containing 100 μ M of m⁷GTP. The eluate was concentrated to approx. 2 ml with a Centricon-10 microconcentrator (Amicon, Gloucester, Glos., U.K.). The concentration and purity of the product was assessed by HPLC on a C₈ column (details below). In this system 4E-BP1 and eIF4E were eluted at 33 and 40 min retention time [52 % and 66 % (v/v) acetonitrile] respectively. Typically, 5 mg of 4E-BP1 at a stoichiometry of 0.9:1 with eIF4E was produced in this way. Unbound 4E-BP1 was produced from eIF4E/4E-BP1 complex by boiling the complex for 15 min and removing the precipitated eIF4E by centrifugation. 4E-BP1 produced in this way was able to rebind to recombinant eIF4E.

Preparation of fat-cell extracts for studies *in vitro* and separation of protein kinase activities with a Pharmacia SMART system

Fat cells were prepared from rat epididymal fat pads as described previously [11] and extracted in 2.5 ml/g dry cell weight of ice-cold Triton-containing buffer C [50 mM Hepes, pH 7.6, containing 0.2 mM EDTA, 2.2 mM EGTA, 100 mM KCl, 1 mM DTT, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 1 μ M microcystin, 1 μ g/ml pepstatin, 1 μ g/ml antipain and 1 μ g/ml leupeptin]. After centrifugation (14000 g for 10 min) and filtration through a 0.22 μ m membrane, the fat-cell extracts (typically 2.5 ml) were applied to a Mono-S PC 1.6/5 column connected to the Pharmacia SMART system. The column had previously been equilibrated in buffer D [50 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 5 % (v/v) glycerol, 0.03 % (v/v) Brij-35,

0.1 % (v/v) 2-mercaptoethanol, 1 $\mu\text{g/ml}$ pepstatin, 1 $\mu\text{g/ml}$ anti-pain and 1 $\mu\text{g/ml}$ leupeptin]. All buffers were degassed and filtered through a 0.22 μm membrane; chromatography was performed at 4 °C. The column was washed with buffer D until the absorbance at 280 nm had returned to baseline values; development was at a flow rate of 50 $\mu\text{l/min}$ with a discontinuous gradient of buffer D containing 1 M NaCl (see Figure 4). Fractions of 100 μl were collected. In some experiments, active fractions (usually 0.6 ml in total) obtained from the Mono-S column were concentrated to 50 μl with a Microcon-10 micro-concentrator (Amicon) and applied to a Superose 12 PC 3.2/30 column equilibrated in buffer D containing 200 mM NaCl. The column was developed at a flow rate of 40 $\mu\text{l/min}$ and 80 μl fractions were collected.

Protein kinase assays

The ability of purified casein kinase 2 or thio-phosphorylated MAP kinase to phosphorylate either the recombinant eIF4E/4E-BP1 complex or unbound 4E-BP1 (1 μg) was assessed by incubating the protein with stated amounts of either kinase at 30 °C for 15 min in a total volume of 25 μl of buffer A containing 10 mM MgCl_2 and 0.1 mM [γ - ^{32}P]ATP (typically 1000 c.p.m./pmol). Similarly, the ability of fractions from the Mono-S or Superose 12 columns to phosphorylate recombinant eIF4E/4E-BP1 complex was assessed by incubating the fraction (20 μl) at 30 °C for 30 min in a total volume of 50 μl containing purified recombinant eIF4E/4E-BP1 complex (equivalent to 0.5 mg/ml 4E-BP1), 5 mM MgCl_2 and 0.1 mM [γ - ^{32}P]ATP (typically 1000 c.p.m./pmol). Reactions were stopped by the addition of Laemmli sample buffer; phosphoproteins were separated by SDS/PAGE and detected by autoradiography with pre-flashed Amersham Hyperfilm in cassettes with intensifying screens. The ability of column fractions to phosphorylate other proteins and peptides was determined by replacing the eIF4E/4E-BP1 complex in the above assay with the protein or peptide at a final concentration of 1 mg/ml. After incubation at 30 °C for 15 min, samples (40 μl) were removed from the assay mixture and spotted on P81 phosphocellulose paper squares (Whatman, Maidstone, Kent, U.K.) and immersed in 150 mM H_3PO_4 . The papers were rinsed three times with fresh H_3PO_4 and once in ethanol, then dried and counted for radioactivity in 10 ml of water by the Čerenkov method.

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

Radiolabelled 4E-BP1, generated by phosphorylation *in vitro*, was separated by SDS/PAGE and blotted on PVDF membranes [11]. 4E-BP1 bands (detected by radioautography) were cut into small (1 mm²) pieces and proteins were digested with 10 μg of sequencing-grade modified trypsin to approx. 1 μg of 4E-BP1 in 200 μl of 50 mM NH_4HCO_3 , pH 8.0, containing 5 % (v/v) acetonitrile. Digestion was at 37 °C for 12 h followed by the addition of the same amount of trypsin and incubation for a further 5 h. 4E-BP1 purified from ^{32}P -labelled fat cells was separated by HPLC. Fractions containing 4E-BP1 were pooled, dried and washed extensively with water before digestion with trypsin.

In all cases, digests were dried to 100 μl and acidified by adding trifluoroacetic acid to 0.1 % (v/v) and subjected to reverse-phase HPLC with a C_8 column as described below. Further digestions (of approx. 200 pmol of purified tryptic peptide) were for 12 h at 37 °C with 5 μg of sequencing-grade chymotrypsin (in 100 mM Tris/HCl, pH 7.8, containing 10 mM

CaCl_2), with 2 μg of sequencing-grade endopeptidase Asp-N (in 50 mM NaH_2PO_4 , pH 7.8), or with 5 μg of sequencing-grade endopeptidase Glu-C [in 25 mM $(\text{NH}_4)_2\text{CO}_3$, pH 7.8, containing 5 % (v/v) acetonitrile]. Aliquots of digested material were mapped by TLC with a pH 3.5 buffer [pyridine/acetic acid/water (1:10:189, by vol.)] for the electrophoresis dimension and chromatography with pyridine/acetic acid/butanol/water (10:3:15:12, by vol.) buffer in the second dimension [27].

Radioactive peptides to be analysed for their phospho amino acid content were scraped from TLC plates and eluted with 60 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid. Peptides in the dried eluate were digested for 95 min at 110 °C in 6 M HCl and amino acids were separated by TLC with pH 1.9 buffer [formic acid/acetic acid/water (25:78:897, by vol.)]. Phospho amino acid standards (1 μg of each) were run and located by staining with ninhydrin.

Purification by HPLC

Proteins and peptides were separated on a C_8 column (Brownlee Aquapore RP-300, 2.1 mm diam., 7 μm particle size, attached to a Applied Biosystems 172 HPLC system) with a 2–72 % (v/v) acetonitrile gradient over 35 min at a flow rate of 100 $\mu\text{l/min}$; 100 μl fractions were collected. Absorbance was measured at 212 nm and ^{32}P radioactivity was measured by the Čerenkov method.

RESULTS AND DISCUSSION

Effects of insulin and rapamycin on the phosphorylation of 4E-BP1 in rat fat cells explored by two-dimensional thin-layer analysis, HPLC separation and phospho amino acid determination of peptides released by tryptic digestion

Treatment of rat epididymal fat cells with insulin greatly increased the incorporation of ^{32}P into 4E-BP1, as found previously [11]. This phosphorylation was associated with a complete dissociation of 4E-BP1 from eIF4E (results not shown). Pretreatment of fat cells with rapamycin blocked this insulin-induced dissociation and resulted in a marked decrease in the overall phosphorylation of the 4E-BP1 not bound to eIF4E. However, as shown previously [11], under these conditions there was a large increase in the phosphorylation of the 4E-BP1 that remained bound to eIF4E (Figure 1d).

The unbound or eIF4E-bound radiolabelled 4E-BP1 was digested with trypsin and the resultant peptides were separated by the two-dimensional thin-layer analysis as described previously [14] except that in the present studies longer periods of electrophoresis were employed (Figure 1). The resulting phosphopeptide maps showed that the 4E-BP1 not bound to eIF4E (which accounted for at least 80 % of the total 4E-BP1, even in the absence of insulin) gave rise to three major radiolabelled phosphopeptides, which we have designated A, B and C (Figures 1a–1c). The amounts of all three phosphopeptides were increased substantially in cells treated with insulin; these increases were blocked essentially completely by rapamycin. Similar effects of insulin were seen in our earlier study [14], but phosphopeptides B and C were not fully resolved in the electrophoresis dimension. In contrast, 4E-BP1, which remains bound to eIF4E in cells incubated with insulin and rapamycin, was only phosphorylated on phosphopeptide C (Figure 1e).

Figure 2(a) shows the corresponding separation by reverse-phase HPLC of the radiolabelled tryptic peptides from unbound 4E-BP1 in cells incubated with insulin compared with that from eIF4E-bound 4E-BP1 in cells incubated with insulin plus rapamycin. In agreement with the two-dimensional thin-layer analysis

Figure 1 Autoradiographs of phosphopeptide maps derived from tryptic digests of 4E-BP1 (a–c, e) or SDS/PAGE separation of eIF4E-bound 4E-BP1 complexes (d)

Fat cells were labelled with medium containing [32 P], and then incubated for 30 min with or without rapamycin (20 nM). The cells were then incubated for a further 10 min with or without insulin (83 nM), extracted in buffer A and treated with m⁷GTP–Sepharose to isolate eIF4E-bound 4E-BP1 complexes (d, e). After this, immunoprecipitation with antiserum to 4E-BP1 was performed to purify unbound 4E-BP1 (a–c). Proteins were separated by SDS/PAGE, the radiolabelled 4E-BP1 was eluted and digested with trypsin. (a–c, e) The resulting phosphopeptides were subjected to two-dimensional thin-layer analysis. (d) An autoradiograph of the labelled proteins purified with m⁷GTP–Sepharose and separated by SDS/PAGE. Additions to fat-cell incubation media were: CON, none; INS, insulin (83 nM); RAP, rapamycin (20 nM). Further details are given in the Experimental section. Results are typical of at least four separate experiments. (f) A key showing details of the two-dimensional thin-layer analysis and migration of the two markers xylene cyanol (xyl-c) and dinitrophenol lysine (dnp-k), as well as the peptides denoted A, B and C.

(Figures 1b and 1e), three phosphopeptides corresponding to A, B and C were separated from the former; one phosphopeptide, corresponding to C, was separated from the latter. It should be noted that phosphopeptide A was eluted in the flow-through (with the P_i), whereas phosphopeptides B and C were eluted close together at approx. 36 and 33 min retention time [58 % and 52 % (v/v) acetonitrile] respectively (Figures 2a–2e).

Figure 2(f) shows the phospho amino acid analysis of phosphopeptides A and B derived from unbound 4E-BP1 (from cells incubated with insulin) or phosphopeptide C from bound 4E-BP1 (from cells incubated with insulin plus rapamycin). In each case the phosphopeptides were separated by two-dimensional electrophoresis before analysis of their phospho amino acid content. It is clear that peptide A contained both phosphoserine and phosphothreonine as shown previously [14]; peptide B contained only phosphothreonine and peptide C mainly phosphoserine. The small amount of phosphothreonine identified in peptide C was probably due to contamination with another minor phosphopeptide (in fact, because phosphoserine is less stable than phosphothreonine, the relative amount of phosphoserine in peptide C was much greater than that of phosphothreonine). Similar results were obtained when the peptides were resolved by HPLC before phospho amino acid analysis (results not shown). Phosphopeptide C derived from unbound 4E-BP1 in cells incubated with insulin was also found to contain only phosphoserine.

Studies on the identity of phosphopeptides A, B and C

Attempts to use microsequencing techniques to identify the phosphopeptides were not successful in these experiments, largely because of the small amounts of material available. However,

studies on the properties of the phosphopeptides did allow them to be identified with reasonable confidence when taken in conjunction with the studies of Fadden et al. [15]. They concluded that insulin increases the phosphorylation of Thr-36, Thr-45, Ser-64, Thr-69 and Ser-82.

Table 1 lists peptides that might be derived from rat 4E-BP1 after digestion with trypsin, together with their expected chargees at pH 3.5 (the pH used in the electrophoresis dimension of the thin-layer analysis) and whether the peptides were likely to be substrates for further proteases.

Phosphopeptide A

This phosphopeptide contained phosphoserine and phosphothreonine, had a small positive charge at pH 3.5 and was eluted in the flow-through of HPLC (Figure 2).

Phosphorylation of recombinant 4E-BP1 with ERK-1 was observed only when the 4E-BP1 was not bound to eIF4E (Figure 3a), in agreement with earlier work [11,12]. Digestion of the product with trypsin gave rise to a single phosphopeptide that behaved in the two-dimensional thin-layer analysis (and HPLC; results not shown) as peptide A, but contained much more phosphoserine than phosphothreonine (Figures 3b and 3c). Other studies [15,16] have indicated that unbound 4E-BP1 is phosphorylated preferentially by ERK-1 on Ser-64 and that the further phosphorylation of Thr-36, Thr-45 and Thr-69 occurs much more slowly. The phosphopeptide running as peptide A after phosphorylation by ERK-1 is likely to be NS⁶⁴(p)PVAK. In intact cells, phosphopeptide A is probably a mixture of this peptide plus T⁶⁹(p)PPK, as the two peptides would be expected to have very similar net charges at pH 3.5 and to be rather hydrophilic; they would therefore not be well separated in the

Figure 2 HPLC, two-dimensional thin-layer and phospho amino acid analyses of radiolabelled phosphopeptides derived from 4E-BP1

Fat cells were incubated as in Figure 1 with either insulin or rapamycin plus insulin. (a) HPLC of radiolabelled tryptic phosphopeptides derived from eIF4E-bound 4E-BP1 separated from fat cells after treatment with rapamycin plus insulin (○) or unbound 4E-BP1 purified from cells treated with insulin alone (●). (b–e) Autoradiographs after two-dimensional thin-layer analysis of the peptides separated by HPLC from unbound 4E-BP1 from cells treated with insulin alone (b–d) or from eIF4E-bound 4E-BP1 from cells treated with rapamycin plus insulin (e). (f), Phospho amino acid analysis of peptides A, B and C separated by two-dimensional thin-layer analysis. Abbreviation: P_i, inorganic phosphate. Results are typical of at least five separate experiments.

systems used. Fadden et al. [15] have shown that this latter phosphopeptide is eluted in the flow-through of an HPLC system similar to that of the present study, as does phosphopeptide A.

Table 1 Tryptic peptides that might be derived from 4E-BP1

Expected charge was calculated assuming the following charges at pH 3.5: +1 (N-terminal NH₃⁺ group and R groups of lysine, arginine and histidine); −0.5 (C-terminal CO₂[−] group); −1 (phosphoserine and phosphothreonine) [37]. Peptides 2, 4 and 8 contain adjacent lysine/arginine residues and hence potentially give rise to two products. In each case the values for the expected charges of the larger peptides are given in parentheses.

Tryptic peptide	Sequence	Expected charge at pH 3.5 for peptide with		Predicted substrate for			Comments
		One phosphate	Two phosphates	Chymotrypsin	Glu-C	Asp-N	
1	MSAGSSCSQTPSR	+0.5	−0.5	No	No	No	
2(2a)	AIPTR(R)	+0.5 (+1.5)	−	No	No	No	
3	VALGDGVQLPPGDYSTT ³⁶ PGGTLFSTT ⁴⁵ PGGTR	+0.5	−0.5	Yes	No	No	Peptide B (two phosphates) No S or T
4(4a)	IYYDR(K)						No S or T
5	FLMECR						
6	NS ⁶⁴ PVAK	+0.5	−	No	No	No	Peptide A (one phosphate)
7	T ⁶⁹ PPK	+0.5	−	No	No	No	Peptide A (one phosphate)
8(8a)	DLPTIPGVTSPTSDEPPMQASQSHLHSSPEDK(R)	+2.5 (+3.5)	+1.5 (+2.5)	No	Yes	Yes	
9	AGGEES ¹¹¹ QFEMDI	−0.5	−	Yes	Yes	Yes	Peptide C (one phosphate)

Figure 3 Phosphorylation of 4E-BP1 by ERK-1 and casein kinase 2 *in vitro*

(a) Unbound 4E-BP1 or the eIF4E-bound 4E-BP1 complex were phosphorylated with [γ -³²P]ATP and either ERK-1 or casein kinase 2. Proteins were separated by SDS/PAGE and revealed by autoradiography. (b) Two-dimensional thin-layer analysis and (c) phospho amino acid analysis of radiolabelled tryptic peptides derived from unbound 4E-BP1 phosphorylated *in vitro*.

Phosphopeptide B

This phosphopeptide contained phosphothreonine, had a slightly negative net charge and seemed to be relatively hydrophobic (Figure 2). These properties are consistent with this peptide's being tryptic peptide 3 phosphorylated on both Thr-36 and Thr-

Figure 4 Separation of rat epididymal fat-cell extracts by using Mono-S chromatography

Rat epididymal fat cells (200 mg dry weight) were incubated in the presence of 83 nM insulin for 10 min before extraction in 0.5 ml of extraction buffer C. The resulting extracts were filtered and applied to a Mono-S column attached to a Pharmacia SMART system previously equilibrated in buffer D. The solid line represents the absorbance at 280 nm. Samples (20 μ l) of the resulting fractions were assayed for their ability to phosphorylate purified recombinant eIF4E-bound 4E-BP1, as described in the Experimental section. The results of 4E-BP1 phosphorylation (■) and eIF4E phosphorylation (□) are expressed in arbitrary units resulting from densitometric scanning of autoradiographs with a Hewlett Packard Scanjet-4c. Further samples (20 μ l) of the fractions were assayed for their ability to phosphorylate the 32-mer ribosomal protein S6-derived peptide KEAKEKRQEIQAKKRLSSLRASSTKSESSQK (1 mg/ml); the results are expressed as c.p.m. incorporated in 15 min by 20 μ l of the fraction (●).

45 (Table 1). There is no evidence for 4E-BP1 phosphorylated on only Thr-36 or Thr-45 alone in fat cells, as this would give rise to a more hydrophobic peptide with a net charge of +0.5 at pH 3.5; such a peptide was not observed.

Phosphopeptide C

This phosphopeptide contained phosphoserine, had a charge of approx. -0.5 at pH 3.5 and a hydrophobicity similar to that of peptide B (Figure 2). Inspection of the possible tryptic phosphoserine-containing peptides (Table 1) suggests that the phosphopeptide might be that from the C-terminus of 4E-BP1, which contains Ser-111. In particular, peptides 8 and 8a, which contain Ser-82 as well as Ser-99 and Ser-100, are excluded because these peptides would carry a substantial positive charge of +2.5 or +3.5 and such peptides were not observed. Further studies on the identity of peptide C are described below.

Separation of an insulin-stimulated 4E-BP1 kinase by Mono-S chromatography

To identify potential kinases responsible for the phosphorylation of 4E-BP1, rat epididymal fat-cell extracts were separated by Mono-S chromatography and fractions were assayed for kinase activity with complexes of recombinant 4E-BP1 with recombinant eIF4E. A single peak of 4E-BP1 kinase activity was identified, which was eluted by 200–300 mM NaCl at pH 7.4 by using the discontinuous gradient indicated in Figure 4. The 4E-BP1 kinase activity eluted from the Mono-S column was found to be markedly increased if the epididymal fat cells had previously been incubated in the presence of insulin. The mean ($\pm$ S.E.M.)

Figure 5 Effects of insulin, rapamycin and wortmannin on the 4E-BP1 kinase activity

(a) Rat fat cells were incubated at 37 °C for 30 min with DMSO vehicle (lanes a and b) or in the presence of 20 nM rapamycin (lanes c and d) or 100 nM wortmannin (lanes e and f). The cells were then incubated for a further 10 min without addition (lanes a, c and e) or after the addition of 83 nM insulin (lanes b, d and f). The fat cells were then extracted in buffer C and the extracts were separated by Mono-S chromatography. Fractions containing 4E-BP1 kinase activity were pooled and assayed for their ability to phosphorylate recombinant eIF4E-bound 4E-BP1 as described in the legend to Figure 4. (b) The ability of the pooled kinase fraction from insulin-treated tissue to phosphorylate unbound 4E-BP1 (lane a) was compared with its ability to phosphorylate the purified eIF4E-bound 4E-BP1 complex containing an equal amount of 4E-BP1 (lane b).

Figure 6 Elution of the 4E-BP1 kinase activity during Superose 12 size-exclusion chromatography

Fractions containing 4E-BP1 kinase activity (and co-eluting eIF4E kinase and S6 peptide kinase activities) from the Mono-S column were pooled, concentrated to 50 μ l and applied to a Superose 12 column connected to a Pharmacia SMART system that had been previously equilibrated in buffer D containing 200 mM NaCl. The resulting fractions were assayed for their ability to phosphorylate 4E-BP1 (■) and eIF4E (□) using eIF4E-bound 4E-BP1; and to phosphorylate the 32-mer ribosomal S6-derived peptide (●) (as described in the legend to Figure 4). The elution positions of various molecular-mass markers (in kDa) are shown.

stimulation in six separate experiments was 13.8 ± 5.4 -fold (Figure 5a, lanes a and b). The insulin-stimulated kinase phosphorylated 4E-BP1 with a stoichiometry of 0.65 ± 0.03 ($n = 4$) mol of phosphate incorporated per mol of 4E-BP1 after incubation for 60 min at 30 °C. Under these conditions, there was no detectable change in the migration of the 4E-BP1 in SDS/PAGE. In addition, 4E-BP1 complexed to eIF4E was a much better substrate than unbound 4E-BP1 for the kinase (6.9 ± 1.6 -fold for six separate experiments) (Figure 5b).

Preincubation of the fat cells with wortmannin (100 nM), an inhibitor of phosphatidylinositol 3-kinase, abolished the stimulation of the 4E-BP1 kinase activity by insulin (Figure 5a, lanes e and f). In contrast, preincubation with rapamycin (20 nM), which blocked the activation of p70 ribosomal S6 protein insulin (results not shown), had no detectable effect on the ability of

Figure 7 Further analysis of the tryptic phosphopeptide derived from recombinant 4E-BP1 phosphorylated by the fat-cell kinase (b, d, f) and comparison with phosphopeptide C from eIF4E-bound 4E-BP1 from fat cells treated with rapamycin plus insulin (a, c, e)

Radiolabelled tryptic phosphopeptide C was purified as described in the legend to Figure 2 and analysed by two-dimensional TLC (a). In (c), tryptic phosphopeptide C was analysed together with the product of its further digestion with chymotrypsin on the same TLC plate. (e) Both radiolabelled tryptic peptide C (●) and its chymotrypsin digest (○) were also analysed separately by HPLC. (b, d) The tryptic peptide phosphorylated by the fat-cell kinase was analysed by two-dimensional TLC before (b) or after (d) its further digestion with chymotrypsin. (f) The tryptic peptide (●) and its chymotryptic digest (○) were also separated by HPLC.

insulin to stimulate the 4E-BP1 insulin activity (Figure 5a, lanes c and d).

Mono-S chromatography also reveals the presence of an insulin-stimulated eIF4E kinase

The 4E-BP1 kinase activity was co-eluted from the Mono-S column with an insulin-stimulated eIF4E kinase (Figure 5a) and an S6 peptide kinase activity (Figure 4). However, when the 4E-BP1 kinase was further purified with a Superose 12 gel-filtration column attached to a Pharmacia SMART system, these activities were completely separated, with the 4E-BP1 kinase eluting earlier than both the eIF4E kinase and S6 peptide kinase activities (Figure 6). The eIF4E kinase was stimulated by insulin (2.1 ± 0.2 -fold, mean $\pm$ S.E.M. for six separate experiments). The effects of insulin were blocked by wortmannin (100 nM) but were unaffected by rapamycin (20 nM) (Figure 5a).

Identification of the site phosphorylated by the fat-cell 4E-BP1 kinase

Tryptic digestion of 4E-BP1 that had been phosphorylated by the fat-cell kinase, and the separation of the resulting phosphopeptides in two dimensions by TLC, produced the pattern of labelled peptides seen in Figure 7(b). The major tryptic phospho-

peptide was purified by HPLC and sequenced by using a standard automated Edman degradation on an ABI 477A Automated Sequencer. The N-terminal sequence was found to be AGGEES¹¹¹(p)QFEMD. It should be noted that during Edman degradation phosphoserine is converted to dehydroalanine, which can be detected because it has a different HPLC elution position from any of other amino acid. Therefore the position of the phosphoserine can be accurately determined by automated Edman degradation [28]. Identification of Ser-111 as the phosphorylation site was confirmed by the ability of the fat-cell kinase to phosphorylate the synthetic peptide CGRAGGEESQFEMD based on the C-terminal sequence of 4E-BP1. The mean ($\pm$ S.E.M.) incorporation of ³²P into this peptide (0.25 mM) was 10.9 ± 2.0 pmol of ³²P incorporated/min per g dry weight of cells ($n = 3$). In addition, the presence of this peptide (0.75 mM) blocked the ability of the kinase to phosphorylate 4E-BP1 bound to eIF4E by more than 80 % (Figure 8). A similar degree of inhibition was also observed in the presence of an antibody raised against this peptide [25], presumably because the antibody bound close to the phosphorylation site (Figure 8).

Ser-111 is phosphorylated *in vivo* within tryptic peptide C of 4E-BP1

Separation of the tryptic peptide phosphorylated by the fat-cell kinase by TLC indicated that this peptide and tryptic phos-

Figure 8 Phosphorylation of eIF4E-bound 4E-BP1 by the fat-cell kinase in the presence of a synthetic peptide based on the C-terminal sequence of 4E-BP1 or of an antibody raised against this peptide

The ability of the 4E-BP1 kinase separated from insulin-stimulated fat-cell extracts by Mono-S chromatography to phosphorylate eIF4E-bound 4E-BP1 was examined in the absence of further additions (a), in the presence of a synthetic peptide based on the C-terminus of 4E-BP1 (0.75 mM) (b) or an antibody raised against this peptide (300 μ l/ml) (c).

phopeptide C from 4E-BP1 phosphorylated *in vivo* both have a charge of -0.5 at pH 3.5 (Figures 7a and 7b). However, there was a consistent difference in their migrations in the chromatography dimension and in their retention times on HPLC.

Stronger evidence that phosphopeptide C contains Ser-111 came from studies in which the phosphopeptide was further digested with chymotrypsin. This digestion should give rise to AGGEES¹¹¹(p)QF. This product has a predicted charge similar to that of the original phosphopeptide C, but should be more hydrophilic and therefore should not migrate as far in the chromatographic dimension of the thin-layer analysis and should be eluted earlier in HPLC. Figures 7(a), 7(c) and 7(e) show the effects of chymotryptic digestion of the tryptic phosphopeptide C phosphorylated *in vivo*. It should be noted that a mixture of tryptic phosphopeptide C and the product of its further digestion with chymotrypsin were analysed together by TLC (or HPLC) to show clearly the differences in their migrations (Figures 7c and 7e). When recombinant 4E-BP1, phosphorylated by the fat-cell kinase, was treated with trypsin and then chymotrypsin, the resultant chymotryptic product migrated in exactly the same position on TLC as the tryptic phosphopeptide C phosphorylated *in vivo* digested with chymotrypsin (Figures 9a and 9b). This was shown by mixing equal amounts of the radiolabelled products obtained from chymotryptic digestion of the relevant *in vivo* or *in vitro* tryptic phosphopeptides and separating them on the same TLC plate. Only one radiolabelled peptide was evident (Figure 9c). The chymotryptic peptides were also eluted at the same position after separation by HPLC (Figures 7c–7f) (i.e. 30 min retention time). The chymotryptic peptide from recombinant 4E-BP1 phosphorylated with the fat-cell kinase was found to have the sequence AGGEES¹¹¹(p)QF by automated Edman degradation.

In addition, tryptic phosphopeptide C from 4E-BP1 phosphorylated *in vivo* could also be digested with Glu-C and Asp-N because, after digestion, the resultant phosphopeptides were eluted 2 and 4 min respectively before tryptic phosphopeptide C when separated on HPLC (results not shown). As shown in Table 1, no other tryptic peptide of 4E-BP1 was a substrate for cleavage by both Glu-C and Asp-N.

In summary, the fat-cell kinase definitely phosphorylates Ser-111. The general properties of phosphopeptide C strongly suggest that this site is also phosphorylated *in vivo*. The exact co-migration of the two phosphopeptides generated by sequential digestion with trypsin and chymotrypsin is strong confirmatory evidence for the phosphorylation of Ser-111 *in vivo* (Figure 9). The small difference in the behaviour of the tryptic peptides

Figure 9 Demonstration that peptides produced by sequential digestion with trypsin and chymotrypsin of eIF4E-bound 4E-BP1 from fat cells incubated with rapamycin plus insulin (a) and that recombinant eIF4E-bound 4E-BP1 phosphorylated by the fat-cell kinase have identical migrations in two-dimensional TLC (b)

Further details are described in the legend to Figure 7. (c) Results of mixing equal amounts (in terms of incorporated radioactivity) of the peptides generated in (a) and (b).

presumably reflects some difference in the extreme C-terminus of the fat-cell and recombinant 4E-BP1 proteins.

Casein kinase 2 also phosphorylates 4E-BP1 on Ser-111

Casein kinase 2 has been previously shown to phosphorylate recombinant 4E-BP1 when it is bound to eIF4E [11]. In a series of experiments performed as in Figure 3(a), the rate of phosphorylation of bound 4E-BP1 was 2.8 ± 0.3 -fold greater than that of unbound 4E-BP1 (mean $\pm$ S.E.M. for three separate experiments). No change in migration of 4E-BP1 on SDS/PAGE was evident after phosphorylation by casein kinase 2 (Figure 3a). Phosphorylation occurred only on serine (Figure 3c) and, after tryptic digestion, one major (and one minor) phosphopeptide was evident (Figure 3b). The major phosphopeptide represented at least 90% of the phosphorylation of 4E-BP1. It behaved on two-dimensional thin-layer analysis and HPLC in a similar manner to the tryptic phosphopeptide of 4E-BP1 phosphorylated by the fat-cell kinase (Figures 7b and 7f). The tryptic phosphopeptide of 4E-BP1 phosphorylated by casein kinase 2 was purified by HPLC and sequenced by automated Edman degradation. The sequence obtained was AGGES(p)QFEMD, confirming that Ser-111 is also phosphorylated by casein kinase 2 *in vitro*.

Properties of the fat-cell 4E-BP1 kinase and its relationship to casein kinase 2

The fat-cell 4E-BP1 kinase was found to be highly specific for 4E-BP1 as no detectable phosphorylation of myelin basic protein and a number of synthetic peptide substrates was found. The peptides included a peptide based on the sequence around Thr-

Figure 10 Comparison of the effects of heparin and β -glycerophosphate on the activities of casein kinase 2 and the fat-cell 4E-BP1 kinase

The ability of the 4E-BP1 kinase separated from insulin-stimulated fat-cell extracts by Mono-S chromatography to phosphorylate the eIF4E-bound 4E-BP1 was compared with the ability of casein kinase 2 to phosphorylate this complex in the absence or presence of 1 μ M heparin or 50 mM β -glycerophosphate, as indicated.

669 of the epidermal growth-factor receptor (KRELVEPLTPSGEAPNQALLR, a substrate for MAP kinase), a 32-mer ribosomal protein S6-derived peptide (KEAKEKRQEIAK-KRRLSSRLASTSKSESSQK, a substrate for p70 ribosomal S6 protein kinase and p90 rsk), 'kemptide' (LRRASLG, a substrate for protein kinase A and other kinases), 'crosstide' (GRPTSSFAEG, a substrate for protein kinase B), 'MR6', a peptide based on the sequence surrounding Ser-466 of bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PVRMRRNSFT, also a substrate for protein kinase B), glycogen synthase kinase-3 substrate peptide [YRRAAVPPSP-SLSSRHSSPHQS(P)EDEEE] and the casein kinase 2 substrate peptide (RRREEETEEE).

The phosphorylation of 4E-BP1 by the insulin-activated kinase identified in the present study has a number of features that are similar to its phosphorylation by casein kinase 2. Both protein kinases phosphorylate Ser-111 and are more active towards 4E-BP1 bound to eIF4E. However, the fat-cell 4E-BP1 kinase described here is not casein kinase 2 for a number of reasons: (i) the fat-cell kinase binds to a Mono-S cation-exchange column, whereas casein kinase 2 purified from rat liver does not bind to the Mono-S column under the conditions used in the present study (results not shown); (ii) the fat-cell kinase does not phosphorylate the casein kinase 2 substrate peptide; (iii) casein kinase 2 is inhibited by heparin (1 μ M), whereas the fat-cell kinase is unaffected (Figure 10); and (iv) β -glycerophosphate (50 mM) stimulates the activity of casein kinase 2 but inhibits that of the fat-cell 4E-BP1 kinase (Figure 10).

Conclusions

These studies provide confirmation that insulin increases the phosphorylation of four sites within 4E-BP1 that fit a Ser/Thr-Pro motif. These are Ser-64 and Thr-69 (within tryptic phosphopeptide A) plus Thr-36 and Thr-45 (within tryptic phosphopeptide B). Phosphopeptide B seems to be isolated from fat cells as a bis-phosphorylated form under all the conditions that we have studied. This suggests that the phosphorylation of these two sites might occur by an ordered mechanism such that when the first site is phosphorylated the phosphorylation of the other site within the same molecule of 4E-BP1 follows very rapidly. Phosphorylation of the four sites is blocked by rapamycin. We have not observed the differences in effects of rapamycin on the phosphorylation of the individual sites as reported by Fadden et al. [15] because, under our conditions, rapamycin blocks the effects of insulin on the amounts of both phosphopeptides A and B approximately equally. We have also

not found any evidence for the phosphorylation of Ser-82, which was reported by Fadden et al. [15] to be increased by insulin, although to a much smaller extent than that of Thr-36, Thr-45, Ser-64 and Thr-69. This should give rise to a very positively charged phosphopeptide and we have never observed such a phosphopeptide in our studies.

A novel finding of the present study is that insulin increases the phosphorylation of Ser-111 by a rapamycin-insensitive mechanism. This was missed in the study of Fadden et al. [15], possibly because the peptide containing Ser-111 was poorly separated from other radiolabelled phosphopeptides by their techniques, which differed from those of the present study in the choice of proteases and did not distinguish between bound and unbound 4E-BP1. In the present study it is clear that insulin markedly increases the phosphorylation of 4E-BP1 on Ser-111 and that this phosphorylated form of 4E-BP1 is found bound to eIF4E in the presence of rapamycin. In the absence of rapamycin, insulin also initiates the phosphorylation of the four proline-directed sites mentioned above, which are found together with phosphorylated Ser-111 in the unbound protein. These observations are compatible with, but do not prove, the original suggestion that the phosphorylation of Ser-111 of 4E-BP1 bound to eIF4E might act as a priming event that facilitates the phosphorylation of the other sites by a rapamycin-sensitive process [11].

Ser-111 is the only phosphorylation site within 4E-BP1 for casein kinase 2 that fulfils the minimum consensus sequence of -S/T-X (not P)-X-D/E [29]. The present study gives the first published evidence that this is indeed so, although it has also been suggested without published evidence by others [12,15]. Casein kinase 2 might be involved in the effects of insulin on the phosphorylation of Ser-111 because this kinase has been shown to be activated (albeit modestly) by insulin in fat cells [17] and 4E-BP1 bound to eIF4E is a better substrate than unbound 4E-BP1 *in vitro*.

The major finding of this study is the identification of an apparently novel fat-cell kinase that has properties compatible with an important role in the action of insulin on 4E-BP1 in these cells. The kinase is highly specific for Ser-111 of 4E-BP1 and is much more active towards 4E-BP1 bound to eIF4E than towards unbound 4E-BP1. It is also activated by insulin more than 10-fold by a mechanism that is blocked by wortmannin but not by rapamycin. These are all properties that are required of the kinase responsible for the increased phosphorylation of Ser-111 in cells treated with insulin [11].

It is clear from a comparison of the properties of the kinase with casein kinase 2 that they are distinct kinases. It seems likely that the fat-cell 4E-BP1 kinase is more important than casein kinase 2 in the effects of insulin on the phosphorylation of Ser-111 in fat cells as it is activated by insulin more than 10-fold, whereas the effects of insulin on casein kinase 2 are 2-fold at most [17]. It is difficult to make an accurate comparison of the maximum activities of the two kinases in fat cells, as the kinases are extracted and purified before assay by different procedures. However, the observed activity of the fat-cell 4E-BP1 kinase from insulin-treated cells after Mono-S chromatography is equivalent to at least 1 nmol of phosphate incorporated into 4E-BP1/min per g dry weight of fat cells at 30 °C. This seems to be sufficient to account for the increased phosphorylation of Ser-111 observed in fat cells at 37 °C after the addition of insulin, because the increase occurs over approx. 10 min and the total amount of 4E-BP1 in fat-cells is no more than 5 nmol/g dry weight of cells (M. B. Avison, unpublished work).

We find that prior phosphorylation of 4E-BP1 bound to eIF4E by the fat-cell 4E-BP1 kinase does not facilitate the ability of the MAP kinase, ERK-1, to phosphorylate the 4E-BP1 (results not

shown). This is evidence that the phosphorylation of Ser-111 does not result in the dissociation of 4E-BP1 from eIF4E, because free 4E-BP1 is an excellent substrate for ERK-1 [11, 15, 18]. In any case, there is considerable evidence against a role for ERK-1 or ERK-2 in the effects of insulin on the phosphorylation of 4E-BP1. For example, the effect of insulin on ERK-1 and ERK-2 activities is unaffected by rapamycin, whereas the phosphorylation of the proline-directed sites is largely blocked [11, 13, 18] and in a number of cell types the MAP kinase kinase inhibitor PD098059 blocks the activation of ERK-1 and ERK-2 without affecting the phosphorylation of 4E-BP1 [12, 18, 19].

The present studies have also shown for the first time the presence of an insulin-activated eIF4E kinase in extracts of fat cells. The effects of insulin on this kinase are blocked by wortmannin but are insensitive to rapamycin. In other cells, insulin has been shown to increase the phosphorylation of eIF4E on Ser-209 [30–32] and there is evidence that this might increase the affinity of eIF4E for the m⁷GTP cap of mRNA and might enhance the formation of the eIF4E complex [33–35]. Further studies are needed to identify the site phosphorylated by the insulin-stimulated protein kinase Mnk-1, which has recently been shown to phosphorylate eIF4E on Ser-209 [36].

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