

branch distances to M33 and M31 will be measured, and main-sequence fitting undertaken for LMC clusters, allowing further checks on the zero point of the Cepheid $P-L$ relation. These data will provide a direct means of assessing the systematic errors in the current extragalactic distance scale at each stage of its

application, and allow a determination of the Hubble constant to unprecedented accuracy. The results of this paper suggest that a Hubble constant, accurate to 10%, and measured through secondary techniques out to scales of hundreds of Mpc, is now a realizable goal. □

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Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function

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The cloning is described of two related human complementary DNAs encoding polypeptides that interact specifically with the translation initiation factor eIF-4E, which binds to the messenger RNA 5'-cap structure. Interaction of these proteins with eIF-4E inhibits translation but treatment of cells with insulin causes one of them to become hyperphosphorylated and dissociate from eIF-4E, thereby relieving the translational inhibition. The action of this new regulator of protein synthesis is therefore modulated by insulin, which acts to stimulate the overall rate of translation and promote cell growth.

ALL eukaryotic mRNAs (except organellar) bear a 'cap' structure (m⁷GpppN, where N is any nucleotide and m is a methyl group) at their 5' terminus¹, which is recognized by a cap-binding protein, eIF-4E (ref. 2). This polypeptide exists as a subunit of a complex, termed eIF-4F, which consists of eIF-4E, eIF-4A (a bidirectional RNA helicase) and p220 (for review, see ref. 3). Interaction of the cap-binding complex with the mRNA, followed by unwinding of the mRNA 5' secondary structure, is

thought to facilitate attachment of the smaller 40S ribosomal subunit to mRNA (see ref. 4 for review). The process of initiation of protein synthesis is the rate-limiting step in translation and the point of extensive regulation. Phosphorylation of initiation factors is important in the regulation of translation initiation (reviewed in ref. 5). Addition of the peptide hormone insulin to adipocytes or muscle cells stimulates protein synthesis at the initiation level^{6,8}, and concomitantly increases eIF-4E phosphorylation^{9,10}. This modification enhances the activity of eIF-4E (ref. 11) and partly explains the increased translation rates after treatment of cells with insulin. eIF-4E plays a central

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eIF-4E-binding proteins

A DNA database search revealed a high degree of identity (93 and 97% similarity) between the protein sequence of human 4E-BP1 and a cDNA encoding a rat protein termed PHAS-I (ref. 18; Fig. 1b), indicating that 4E-BP1 is the human homologue of PHAS-I. The PHAS-I protein is unusual in that it is soluble after boiling and in 2% trichloroacetic acid^{13, 15, 21}. It was initially identified as a protein phosphorylated in response to insulin in rat adipose tissue and in murine 3T3-L1 adipocytes^{13, 16, 21}. Also, it is phosphorylated in response to growth factors and phorbol esters^{21, 22}.

To confirm that 4E-BP1 and 4E-BP2, as well as rat PHAS-I, interact with eIF-4E, recombinant glutathione *S*-transferase fusion proteins (GST-4E-BP1, GST-4E-BP2) and His-tagged PHAS-I were analysed for interaction with eIF-4E by far-western blotting. After SDS-PAGE, purified proteins were transferred to nitrocellulose and probed with ³²P-labelled HMK-eIF-4E. The HMK-eIF-4E probe did not interact with GST alone (Fig. 2, lane 1), but reacted equally with GST-4E-BP1 and GST-4E-BP2 (lanes 2 and 3; GST-4E-BP2 migrates more slowly than GST-4E-BP1 because it contains the HMK sequence). eIF-4E also bound to rat PHAS-I (lane 4, PHAS-I, indicated by a triangle; the slower polypeptide, presumably an aggregate, appears after storage). PHAS-I migrated at a position corresponding to *M_r* 22,000 (22K) on SDS-PAGE, although the size of the protein calculated from its sequence is only ~12K. This also happens with 4E-BPs synthesized *in vitro* (Fig. 3), in agreement with earlier results¹⁸.

We confirmed that 4E-BP1 interacts with eIF-4E by co-immunoprecipitation (using only 4E-BP1[PHAS-I] as no antibody is available against 4E-BP2). RNA transcripts generated *in vitro* were translated together with eIF-4E mRNA in a wheat-

germ translation extract (wheat-germ rather than reticulocyte lysate was used as the latter contained endogenous 4E-BP1 (4E-BP1:eIF-4E ~ 1:20), whereas 4E-BP1 did not bind to endogenous wheat-germ eIF-4E; data not shown) and the translation products immunoprecipitated with anti-PHAS-I antiserum. This antiserum was specific for 4E-BP1 because it did not precipitate eIF-4E synthesized *in vitro* (Fig. 3a; compare lanes 4 and 1). Translation of 4E-BP1 in the wheat-germ extract yielded a polypeptide of ~20K on SDS-PAGE (lane 3), as shown for the rat recombinant protein. This translation product was immunoprecipitated by anti-PHAS-I antiserum (compare lanes 6 and 3). PHAS-I antibody immunoprecipitated eIF-4E and 4E-BP1 together following co-translation of their mRNAs (compare lanes 5 and 2). Similar results were obtained using anti-eIF-4E antiserum; neither protein was immunoprecipitated by preimmune serum (data not shown).

We also demonstrated an interaction between 4E-BP1 and eIF-4E by synthesizing them in wheat-germ translation extract and then incubating them with a m⁷GDP-coupled agarose resin²⁰: eIF-4E, which recognizes the cap structure, bound to the resin, as expected (Fig. 3b, lane 1), but 4E-BP1 did not (lane 2). However, 4E-BP1 was retained by the resin in the presence of eIF-4E (lane 3). This shows that the interaction of 4E-BP1 with eIF-4E does not preclude the binding of eIF-4E to the cap structure.

Insulin and affinity of 4E-BP1 for eIF-4E

As insulin triggers the phosphorylation of PHAS-I (4E-BP1), we tested whether this phosphorylation could modulate the interaction of 4E-BP1 with eIF-4E. Rat adipose tissue was incubated with or without insulin and extracts were heat-treated to enrich for the heat-stable 4E-BP1 protein¹⁴. Soluble proteins

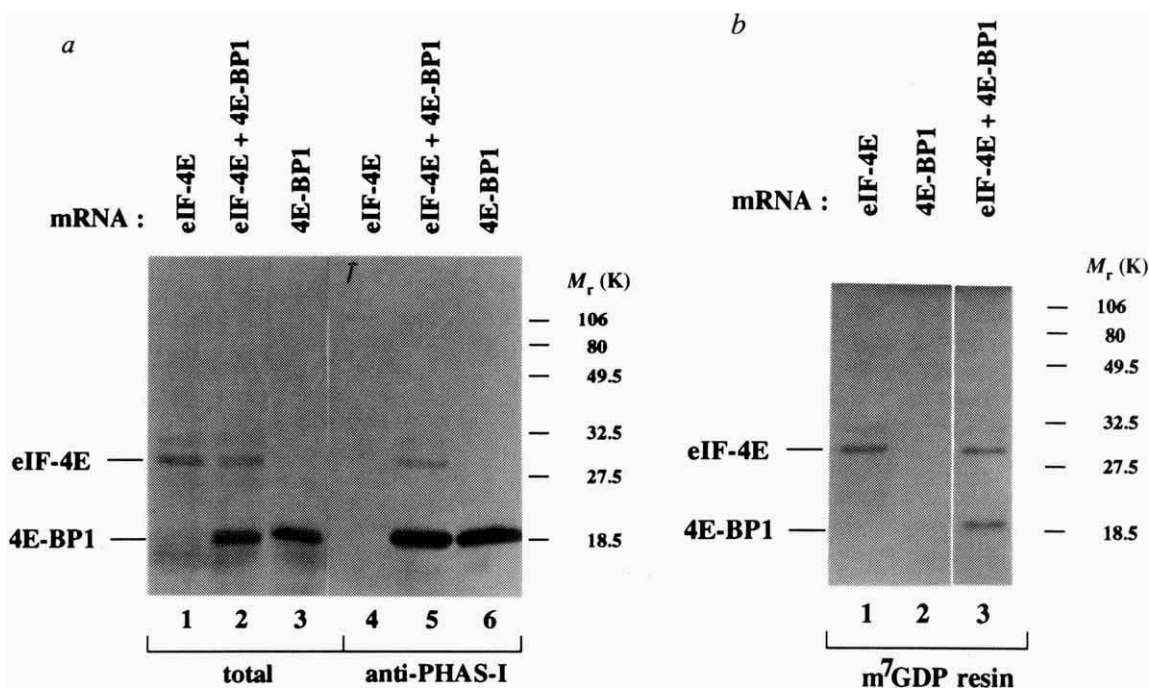


FIG. 3 Complex formation *in vitro* between eIF-4E-BP1. *In vitro* translation products of eIF-4E and 4E-BP1 were analysed by immunoprecipitation with anti-PHAS-I antiserum (a) or by binding to a m⁷GDP (cap) affinity resin (b).

METHODS. Plasmids pKS-eIF-4E and pKS-4E-BP1 were linearized with *Sall* and *Bam*HI respectively, transcribed with T7 and T3 RNA polymerase, respectively, in the presence of m⁷GpppG to yield capped transcripts as described³¹. For a, mRNAs (10 µg ml⁻¹) encoding eIF-4E, 4E-BP1 or both were translated using wheat-germ extract (Promega) with ³⁵S-methionine for 1 h at 25 °C. Samples were then analysed directly or incubated with rabbit anti-PHAS-I antiserum (2 µl) and 300 µl

3% protein A-Sepharose (Pharmacia) suspension in Tris-buffered saline for 1 h at 4 °C, the resin was washed (3 × 1 ml) with 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1% NP-40, 1 mM EDTA, and eluted proteins were separated on a SDS-15% polyacrylamide gel, fixed and treated with EN³Hance (DuPont), and exposed to X-ray film. For b, mRNAs (4 µg ml⁻¹) encoding eIF-4E (lane 1), 4E-BP1 (lane 2) or both (lane 3) were translated as in a. Translation products were incubated with m⁷GDP affinity resin in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 2 mM EDTA), the resin was washed (3 × 1 ml) in buffer A, and bound proteins were eluted in SDS sample buffer and analysed as in a.

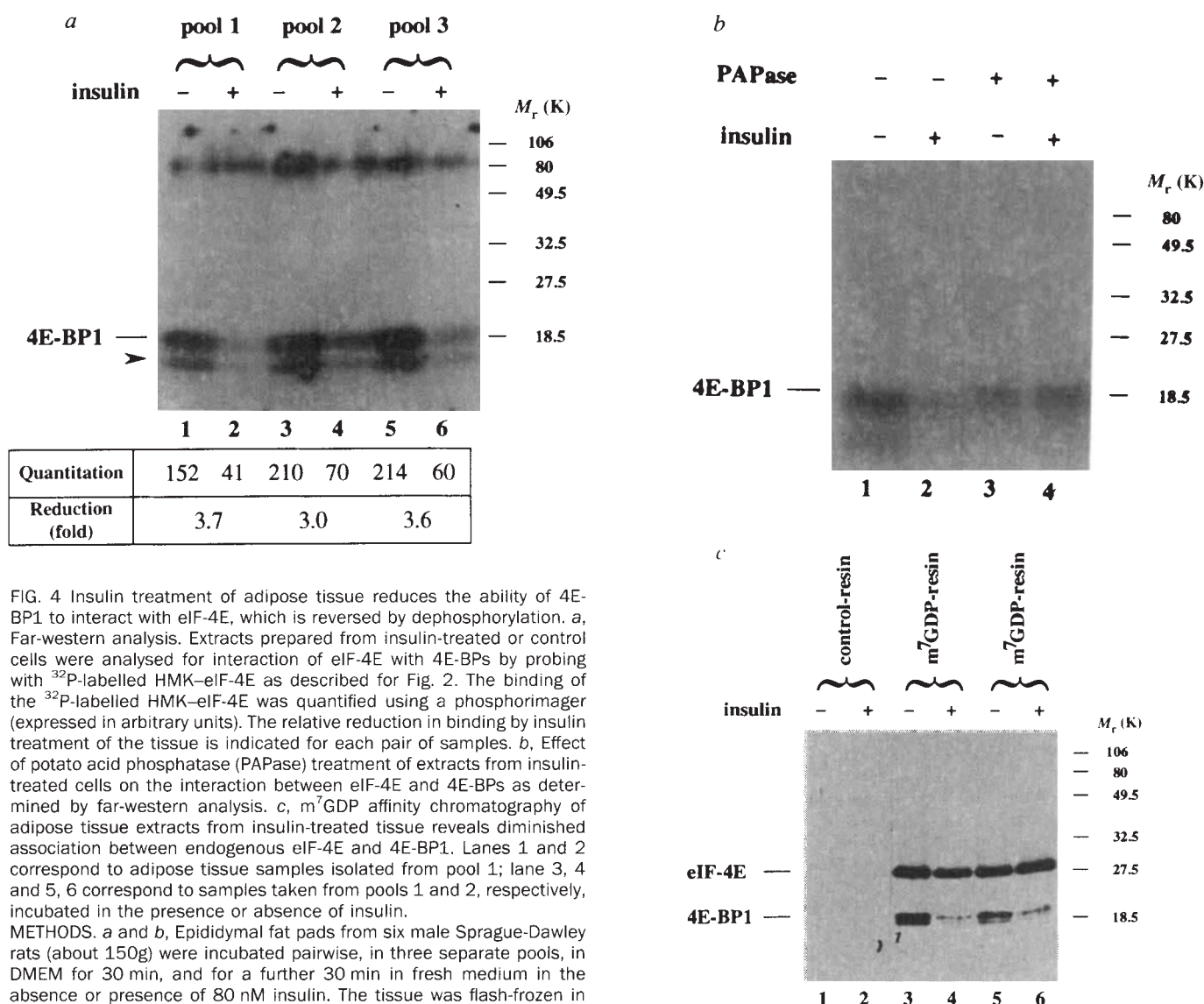


FIG. 4 Insulin treatment of adipose tissue reduces the ability of 4E-BP1 to interact with eIF-4E, which is reversed by dephosphorylation. **a**, Far-western analysis. Extracts prepared from insulin-treated or control cells were analysed for interaction of eIF-4E with 4E-BPs by probing with 32 P-labelled HMK-eIF-4E as described for Fig. 2. The binding of the 32 P-labelled HMK-eIF-4E was quantified using a phosphorimager (expressed in arbitrary units). The relative reduction in binding by insulin treatment of the tissue is indicated for each pair of samples. **b**, Effect of potato acid phosphatase (PAPase) treatment of extracts from insulin-treated cells on the interaction between eIF-4E and 4E-BPs as determined by far-western analysis. **c**, m^7 GDP affinity chromatography of adipose tissue extracts from insulin-treated tissue reveals diminished association between endogenous eIF-4E and 4E-BP1. Lanes 1 and 2 correspond to adipose tissue samples isolated from pool 1; lane 3, 4 and 5, 6 correspond to samples taken from pools 1 and 2, respectively, incubated in the presence or absence of insulin.

METHODS. **a** and **b**, Epididymal fat pads from six male Sprague-Dawley rats (about 150g) were incubated pairwise, in three separate pools, in DMEM for 30 min, and for a further 30 min in fresh medium in the absence or presence of 80 nM insulin. The tissue was flash-frozen in liquid nitrogen and extracted in 3 ml cold buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA, using a Brinkman PT-10 polytron. The homogenate was centrifuged at 8,000 r.p.m. for 5 min, and the infranant collected. Aliquots were treated at 100 °C for 7 min, the precipitated protein removed by centrifugation, and the remaining protein precipitated with trichloroacetic acid (15%). Samples were analysed by SDS-PAGE, transferred to nitrocellulose and processed for far-western analysis as for Fig. 2. For **b**, extracts were analysed directly or following incubation with potato acid phosphatase (6U) in 100 mM MES, pH 6.0, for 30 min at 37 °C, and repetition of the heat treatment. **c**, Rat adipose tissue was incubated in the absence or

presence of insulin (80 nM) for 30 min and flash-frozen. Tissue extracts were prepared and the infranant was incubated with m^7 GDP-coupled agarose (cap column) (lanes 3–6) or unmodified agarose (lanes 1 and 2), washed (3×1 ml) with buffer A (Fig. 3 legend), and bound proteins eluted with SDS sample buffer and analysed by SDS-PAGE. The proteins were transferred to nitrocellulose, incubated in TBS/5% milk/0.1% Tween-20 and then simultaneously with rabbit anti-eIF-4E (1:2,000) and rabbit anti-PHAS-I antibody ($2 \mu\text{g ml}^{-1}$), followed by horseradish-peroxidase-conjugated anti-rabbit IgG and detected using an ECL kit (Amersham).

were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with 32 P-labelled HMK-eIF-4E. Two polypeptides migrating at ~ 20 K interact with eIF-4E in extracts from adipose tissue in the absence of insulin. The slower-migrating polypeptide probably corresponds to 4E-BP1, whereas the faster species (indicated by an arrow) may be 4E-BP2, as the protein synthesized *in vitro* in a reticulocyte lysate migrates faster than 4E-BP1 (data not shown; an additional unknown polypeptide of $M_r \sim 85$ K also interacted with eIF-4E). Treatment with insulin led to a marked reduction (about 3–4-fold in three separate pools of adipose tissue) in the interaction of the two ~ 20 K polypeptides with eIF-4E (Fig. 4a; compare lanes 2 and 4, 6 and 1, 3 and 5). Analysis of the same extracts using an antiserum raised against the C terminus of PHAS-I showed that treatment with insulin did not alter the total amount of 4E-BP1 (data not

shown). These results demonstrate that the interaction between exogenous eIF-4E and 4E-BP1 is decreased in response to insulin.

To show directly that phosphorylation of 4E-BP1 upon insulin treatment is responsible for the dissociation of 4E-BP1 from eIF-4E, extracts were treated with acid phosphatase. Insulin treatment resulted in decreased interaction between eIF-4E and 4E-BP1 (Fig. 4b; compare lanes 2 and 1). But when samples were treated with phosphatase, the binding of eIF-4E to 4E-BP1 in insulin-treated extracts was increased to a level comparable to control extracts (compare lanes 4 and 3; the amount of protein recovered after phosphatase treatment and re-boiling was reduced relative to untreated extracts, which explains the weaker signal in lane 3 compared to lane 1; number of experiments, $n=3$).

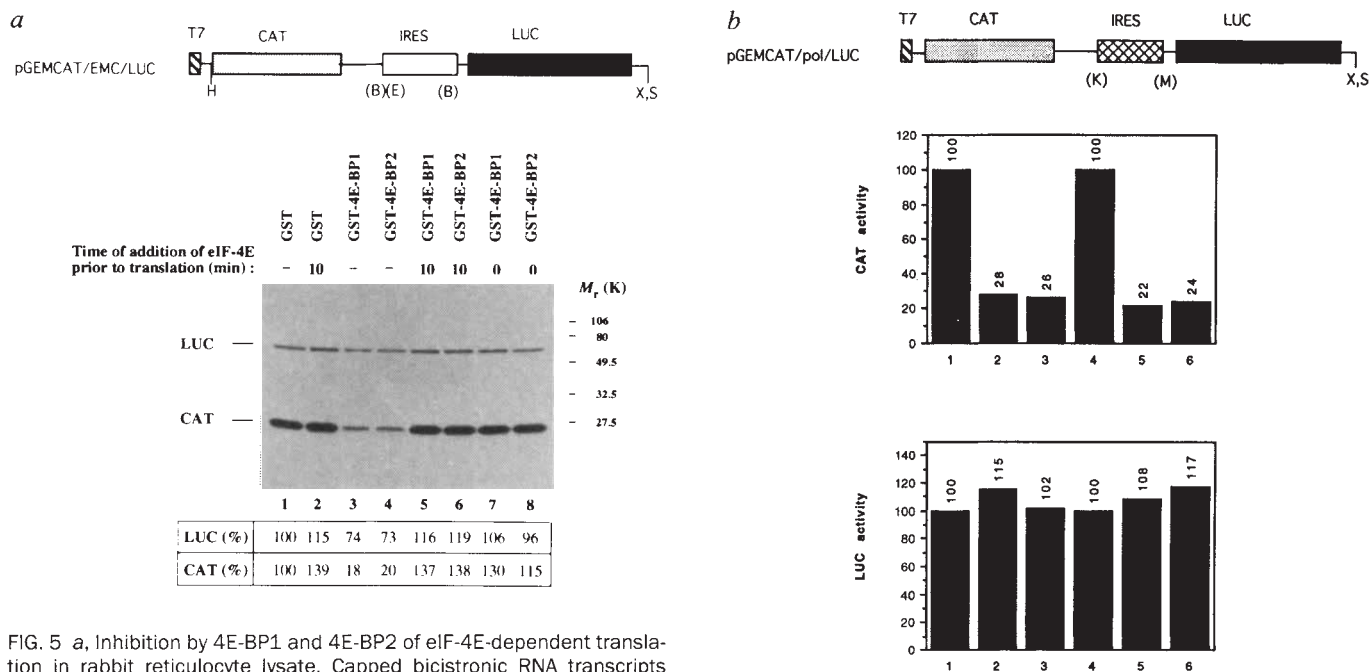


FIG. 5 **a**, Inhibition by 4E-BP1 and 4E-BP2 of eIF-4E-dependent translation in rabbit reticulocyte lysate. Capped bicistronic RNA transcripts encoding both chloramphenicol acetyltransferase (CAT) and luciferase (LUC), were translated in rabbit reticulocyte lysate (Promega). The lysate was preincubated for 10 min with purified GST (400 ng) (lanes 1, 2), GST-4E-BP1 (600 ng) (lanes 3, 5, 7) or GST-4E-BP2 (600 ng) (lanes 4, 6, 8). Recombinant eIF-4E (400 ng) was added where indicated, either at the beginning of the preincubation (lanes 7, 8) or at the start of translation (lanes 2, 5, 6). **b**, Inhibition of cap-dependent protein synthesis *in vivo* by expression of 4E-binding proteins.

METHODS. **a**, RNA was produced using T7 RNA polymerase from *Xho*I-linearized pGEMCAT/EMC/LUC (top). The IRES (internal ribosome entry site) is derived from the 5' untranslated region of encephalomyocarditis virus (EMCV). The indicated restriction enzyme sites are as follows: H, *Hind*III; B, *Bam*HI; E, *Eco*RI; S, *Sac*I, X, *Xho*I. Translation was carried out according to the manufacturer's instructions with $7 \mu\text{g ml}^{-1}$ mRNA in $15 \mu\text{l}$ for 30 °C before analysis by SDS-PAGE. Translation of CAT and LUC was quantified using a phosphorimager, and expressed as arbitrary units. Incorporation into CAT and LUC in the presence of GST alone (lane 1) was set at 100%. **b**, Human TK⁻ 143B cells (ATCC CRL 8303)

were infected with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase. Cells were transfected with plasmids pGEMCAT/pol/LUC (top) (2 μg ; lanes 1–3) or pGEMCAT/EMC/LUC (2 μg ; lanes 4–6), encoding bicistronic mRNAs (top). pGEM/pol/LUC contains the poliovirus type-II Lansing IRES. Restriction enzyme sites as in **a**; K, *Kpn*I, and M, *Msc*I. Plasmids (5 μg) derived from pRC/CMV (Invitrogen) and containing the coding sequence for 4E-BP1 (lanes 2, 5) or 4E-BP2 (lanes 3, 6) under control of the T7 promoter were cotransfected with the reporter constructs using lipofectin (15 μg) (BRL). After 20 h, cell extracts were prepared and analysed for CAT expression by enzyme-linked immunosorbent assay (Boehringer) and LUC activity was measured in millivolts in a luminometer (BIOORBIT) using a luciferase assay system (Promega). Expression of each reporter protein assayed from the reporter vector alone was set at 100% in each case. The experiment was carried out twice, with <10% variation between the experiments.

To determine whether insulin affects the interaction of 4E-BP1 with eIF-4E *in vivo*, we used fat-cell extracts from untreated and insulin-treated tissue to assay for the ability of 4E-BP1 to bind through eIF-4E to a $m^7\text{GDP}$ affinity column. Bound proteins were eluted with SDS-sample buffer, resolved by SDS-PAGE, then immunoblotted with anti-PHAS-I and anti-eIF-4E antibodies. Neither eIF-4E nor PHAS-I bound to the resin not containing the cap (Fig. 4c, lanes 1 and 2). Both eIF-4E and PHAS-I bound to the $m^7\text{GDP}$ resin when extracts from untreated control cells were used (two pools of tissue were analysed; lanes 3 and 5). But although the amount of PHAS-I from insulin-treated cells that bound to the cap column was significantly reduced, similar levels of eIF-4E were bound in each case (lanes 3–6). Consistent with these results, in 3T3-L1 cells incubated with $^{32}\text{P}_i$, PHAS-I antiserum immunoprecipitated ^{32}P -labelled PHAS-I and eIF-4E. Insulin treatment enhanced the phosphorylation of PHAS-I and reduced the amount of eIF-4E precipitating with PHAS-I (data not shown). We therefore conclude that the phosphorylation of 4E-BP1 in response to insulin exposure results in 4E-BP1 dissociation from eIF-4E, indicating that this may account for the enhanced translation activity in adipose tissue upon insulin treatment.

Inhibition of cap-dependent translation

To investigate the possibility that interaction of the 4E-BPs with eIF-4E interferes with cap-dependent but not cap-independent

translation, we made use of artificial bicistronic mRNAs containing the internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) (ref. 23; Fig. 5a). The translation of chloramphenicol acetyltransferase (CAT) is cap- and eIF-4E-dependent, but the translation of luciferase (LUC) directed by the EMCV IRES is cap-independent²³. Translation of this RNA transcript in rabbit reticulocyte lysate produced both CAT and luciferase efficiently (Fig. 5a). Preincubation of the lysate for 10 min before addition of RNA with purified GST-4E-BP1 or GST-4E-BP2 specifically inhibited translation of CAT by ~5-fold (lanes 3 and 4, respectively). This inhibition could be relieved by addition of recombinant eIF-4E, regardless of whether it was present during (lanes 5 and 6), or after (lanes 7 and 8) the preincubation of 4E-BPs with reticulocyte lysate. This suggests that the formation of the 4E-BP-eIF-4E complex does not irreversibly inhibit the translational machinery. In contrast to the deleterious effect of 4E-BPs on cap-dependent translation, there was no significant effect (1.3-fold inhibition) on IRES-directed luciferase translation following addition of GST-4E-BPs (compare lanes 3 and 4 to lane 1).

To investigate whether 4E-BPs inhibit cap-dependent translation *in vivo*, the same bicistronic reporter system was analysed using a transient expression assay in cells infected with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase^{24,25}. Expression of CAT and luciferase was efficient from pGEM-CAT/pol/LUC or pGEM-CAT/EMC/LUC

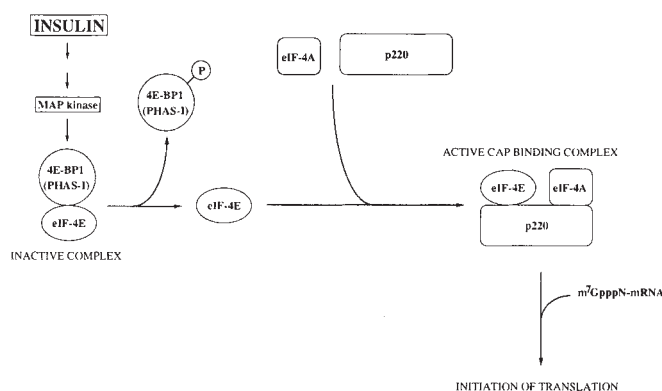


FIG. 6 A model for the mechanism of stimulation of translation by insulin. The model presumes that binding of 4E-BP1 to eIF-4E prevents the interaction of the latter with p220. Insulin-dependent phosphorylation of 4E-BP1 by MAP kinase releases eIF-4E, and makes it available to form an active cap-binding complex (eIF-4F). Although not indicated in the model, insulin stimulates the phosphorylation of eIF-4E, possibly through a protein kinase C-dependent pathway⁹, which is also likely to enhance translation initiation.

mRNAs when they were assayed alone. When plasmids encoding 4E-BP1 or 4E-BP2 were cotransfected with either of the reporter plasmids, there was specific inhibition of CAT expression (4–5-fold) but little effect on luciferase expression (Fig. 5b). Hence, 4E-BP1 and 4E-BP2 are specific inhibitors of cap-dependent translation both *in vivo* and *in vitro*. These results also demonstrate that the requirement for eIF-4E differs for cap-dependent and IRES-directed translation.

Discussion

We have isolated cDNA clones from a human placenta cDNA library encoding two previously unknown eIF-4E-binding proteins. The sequence of human 4E-BP1 is almost identical to that of PHAS-I from rat adipose tissue and skeletal muscle. In adipose tissue stimulated by insulin, PHAS-I is one of the major phosphorylation targets, together with ribosomal protein S6 and ATP citrate lyase¹⁶. We have shown that purified PHAS-I has eIF-4E-binding activity. The insulin-dependent increase in the phosphorylation of 4E-BP1 (PHAS-I) in adipose tissue dramatically diminishes the ability of the protein to interact with eIF-4E. Interaction of 4E-BP1 with eIF-4E inhibits cap-dependent protein synthesis both *in vitro* and *in vivo*, but has only a small effect on cap-independent protein synthesis. Thus, 4E-BP1 must be specifically affecting the process of cap-dependent translation initiation. The data support a model (Fig. 6), in which the phosphorylation of 4E-BP1 by MAP kinase, in response to insulin treatment, results in its dissociation from eIF-4E. This presum-

ably allows eIF-4E to interact with the p220 subunit to form a functional eIF-4F complex, which is required for translation initiation^{1–4}. The finding that PHAS-I (4E-BP1) is phosphorylated by MAP kinase both *in vivo* and *in vitro*¹⁷ is highly significant as it directly links the insulin signal transduction pathway to translation initiation. Insulin treatment of 3T3-L1 cells, or NIH 3T3 cells overexpressing the insulin receptor also leads to an increase in the phosphorylation of eIF-4E (refs 9, 10), and phosphorylation of eIF-4E is thought to be required for its activity¹¹. The enhanced phosphorylation of eIF-4E in insulin-treated tissue may therefore also contribute to the decreased interaction between eIF-4E and 4E-BPs. But phosphorylation of 4E-BP1 alone is sufficient to modulate the interaction, because the eIF-4E probe used to detect the interaction was made in *E. coli* and was not phosphorylated at an authentic site.

Our model provides an explanation for data demonstrating that insulin enhances translation initiation in adipocytes and in muscle cells^{6–8} about 2–3-fold. Insulin specifically stimulates the translation of capped, but not uncapped, mRNAs in 3T3-L1 cells²⁶, paralleling the discriminatory inhibition of 4E-BPs on cap-dependent translation: these experiments involved transfection of capped or uncapped luciferase mRNAs into 3T3-L1 cells followed by insulin treatment, suggesting that insulin enhances the function of the cap-binding complex²⁶. The changes in the binding activity of 4E-BP1 described here would explain these observations.

Because two closely related proteins, each capable of efficiently binding eIF-4E, could be isolated from the placental cDNA library, it is possible that there is a family of such related proteins in different cell types or even within the same cell. Different tissues gave only a weak northern blot signal with a PHAS-I cDNA probe, except for muscle and adipose tissue, for which the signal was strong¹⁸. Inasmuch as this analysis would not be expected to detect the related 4E-BP2 mRNA, it could be present in other tissues, so the two (and possibly more) members of the 4E-BP family could be expressed differentially in a tissue-specific manner.

Overexpression of eIF-4E results in cell transformation¹². It has been proposed that overexpression of eIF-4E increases the amount of eIF-4F complex and enhances translation initiation of mRNAs encoding growth-promoting proteins. Indeed, translation of cyclin D1 (ref. 27) and ornithine decarboxylase is stimulated in cells overexpressing eIF-4E (ref. 28). Also, insulin preferentially stimulates translation of ornithine decarboxylase transcripts in fibroblasts overexpressing the insulin receptor¹⁰. Our findings are consistent with this model. Hyperphosphorylation of 4E-BP1 (PHAS-I), which enhances eIF-4E activity, also occurs in response to growth-promoting factors like EGF and PDGF²¹, which activate the MAP kinase pathway. 4E-BP1, 4E-BP2 and related proteins may therefore also play a key role in regulating translation rates and cell growth in response to treatment with growth-promoting factors. □

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