II–Not I fragment containing TK and *hyg B* sequences from pRBK (Invitrogen); we then added Bgl II–Kpn I–Xba I–Not I sites to make pRBL. For pRLCAR, the Sph I–Kpn I insert of #254 {3-kb upstream sequences of *Xenopus* cardiac actin gene–CAT reporter CAT reporter [T. Mohun, N. Garrett, J. Gurdon, *EMBO J.* **5**, 3185 (1986)]} was cloned into pRBL cut with Sph I and Kpn I. For pRLgal, a 4.8-kb Xho I fragment (A. Hemmati-Brivanlou and R. Harland, unpublished material) that contains *X. laevis hsp 70* [M. Bienz, *EMBO J.* **3**, 2477 (1984)] and nuclear localized β-galactosidase [W. Smith and R. Harland, *Cell* **67**, 753 (1991)] sequences was inserted into pRBL.

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9. Transfected cells were trypsinized, pelleted in a clinical centrifuge (~1000 rpm for 3 to 4 min), and incubated in 0.4 mM FDG (Molecular Probes, stock in dH_2O) in L-15 medium for 20 to 40 min. After repelleting, cells were resuspended in saline or L-15 medium, and fluorescence assayed at 40 to 50 min.
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12. To make wells to hold eggs for injection, we laid a template made of Dow Corning Sylgard 184 silicone elastomer (with protruding egg-sized knobs) face down in a 35-mm plastic petri dish on a layer of melted 2.5% agarose (Sigma type V) in 25% modified Ringer solution (MR), pH 7.0. MR contains 100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes. Needles for transplantations were hand-made from 30-μl Drummond micropipettes and had a needle bore of ~300 μm and a tip diameter of 20 to 40 μm (2 to 3 times the cell diameter to prevent clogging and cell lysis). Tips were clipped to a beveled shape [J. Gurdon, *Methods Cell Biol.* **16**, 125 (1977)]. A dissecting microscope with ×20 eyepieces (~×60 magnification) was used for transplantation; cells were visible against a dark background throughout. For injection, an air-filled 20- to 40-ml glass Hamilton syringe (VWR Scientific) was mounted vertically (tip-upward) and connected to the needle by a length of tubing; a Syringe Microburet (Micro-metric Instrument, Cleveland, OH) was used to control the movement of the glass syringe plunger. The needle was held in a micromanipulator (Narishige, Japan). For preparation of the donor nuclei, we used cell monolayers at 50 to 75% confluence to assure that cells were actively dividing. Cells from one to two 25-cm^2 flasks were trypsinized, pelleted in a clinical centrifuge (3 to 4 min at ~500 rpm), and resuspended in 0.5 ml of transplantation buffer (TB) {0.25 M sucrose, .75 mM NaCl, 0.5 mM spermidine trihydrochloride, and 0.15 mM spermine tetrahydrochloride, pH 7.0 [J. Gurdon, *J. Emb. Exp. Morph.* **36**, 523 (1976)]}. We added 10 μl of lysolecithin (L-α-lysophosphatidylcholine, Sigma, Type I: a stock of 1 mg/ml in dH_2O), and cells were incubated 15 min in this solution. During this incubation, unfertilized eggs were dejellied in 2.5% cysteine hydrochloride, pH 8.0, and then maintained in 100% MR, pH 7.0. After incubation with lysolecithin, two volumes of TB containing 3% bovine serum albumin was added to the cell suspension, and cells were collected by centrifugation as before. Cells were resuspended in about 250 μl of transplantation buffer. Higher lysolecithin doses and longer times of exposure retard development after the gastrula stage promoted by the treated cells. Transplantations were done within 60 to 90 min after preparing X-C cells and unfertilized eggs. The cell suspension, pipetted onto a flat surface, settled into a one- to two-cell-deep carpet. The needle was loaded at the proper density by sweeping it slowly just above this carpet. For injection, a slow outward flow was generated such that single cells entered the needle tip every 1 to 2 s and could be injected in a minimal amount of buffer. The unfertilized egg membrane was pierced in the animal hemisphere with a single, sharp motion, then drawn back to deliver a cell below the membrane. After transplantation, eggs were left undisturbed in 25% MR (pH 7.0) until first cleavage. Cleaving embryos were incubated in 25% MR (pH 7.0) containing 5% Ficoll. After gastrulation, embryos were moved into individual wells of 24-well plates and raised in 25% MR (pH 7.0) containing gentamicin sulfate (50 μg/ml); the vitelline envelope was removed from embryos showing cell leakage after gastrulation, as this improved survival.
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17. A Mineralight lamp (model UVGL-25 from UVP, San Gabriel, CA) with the blue filter removed was used for egg enucleation. Eggs were dejellied and placed animal hemisphere-up in wells in agarose-covered dishes. The lamp was mounted 31 mm above the dish; eggs were submerged in 25% MR to 2-mm depth (from the well bottom) for irradiation. The ultraviolet dosage (short-wave setting) for our lamp at this distance was 3400 μW/cm^2. Using the method of J. Gurdon [*Q. J. Microsc. Sci.* **101**, 299 (1960)], we found an irradiation time of 45 to 60 s effectively destroyed egg chromosomes without affecting normal cleavage and development.
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PHAS-I as a Link Between Mitogen-Activated Protein Kinase and Translation Initiation

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PHAS-I is a heat-stable protein (relative molecular mass ≈12,400) found in many tissues. It is rapidly phosphorylated in rat adipocytes incubated with insulin or growth factors. Nonphosphorylated PHAS-I bound to initiation factor 4E (eIF-4E) and inhibited protein synthesis. Serine-64 in PHAS-I was rapidly phosphorylated by mitogen-activated (MAP) kinase, the major insulin-stimulated PHAS-I kinase in adipocyte extracts. Results obtained with antibodies, immobilized PHAS-I, and a messenger RNA cap affinity resin indicated that PHAS-I did not bind eIF-4E when serine-64 was phosphorylated. Thus, PHAS-I may be a key mediator of the stimulation of protein synthesis by the diverse group of agents and stimuli that activate MAP kinase.

PHAS-I (*1*) was identified in rat adipocytes as one of several proteins that were phosphorylated in response to insulin (*2*). Rat PHAS-I is 93% identical to 4E-BP1, an eIF-4E binding protein cloned from a human placenta complementary DNA library (*3*). The mRNA cap-binding protein eIF-4E, which forms part of the larger eIF-4F complex, is limiting for the initiation step of translation, which is in turn usually rate-limiting for translation (*4*). PHAS-I (4E-BP1) inhibits eIF-4E function as it decreases translation of capped mRNA, both in vitro and when expressed in cultured osteosarcoma cells (*3*). Moreover, increased phosphorylation of PHAS-I in adipocytes with insulin is associated with decreased binding of PHAS-I to eIF-4E, indicating that the stimulation of translation by insulin may result from the release of eIF-4E from inhibition by PHAS-I (*3*). This mechanism would explain earlier findings that were suggestive of a stimulatory effect of insulin on eIF-4F activity (*5*). We found that PHAS-I was regulated by phosphorylation by MAP kinase. Thus, PHAS-I appears to mediate the regulation of protein synthesis by the large and diverse group of hormones, growth factors, and other stimuli that signal through the MAP kinase pathway (*6*).

The association of PHAS-I with eIF-4E was regulated by insulin in murine 3T3-L1 adipocytes. Insulin had no effect on the amount of eIF-4E in extracts of these cells,

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but decreased by approximately 75% the amount of eIF-4E coimmunoprecipitated with antibody to PHAS-I (Fig. 1A). To investigate further the interaction between eIF-4E and PHAS-I, we partially purified eIF-4E by using an affinity resin of the cap homolog 7-methyl–guanosine triphosphate (m^7GTP) (Fig. 1B). The eIF-4E from both control and insulin-treated cells quantitatively bound to the resin. Some PHAS-I also bound (Fig. 1B), as would be expected from its association with eIF-4E (*3*); in addition, insulin markedly decreased the amount of PHAS-I recovered, which is consistent with the dissociation of PHAS-I from eIF-4E (Fig. 1B). In immunoblots of control cells, PHAS-I appeared as a triplet, with the more slowly migrating species representing more highly phosphorylated forms (Fig. 1B). Insulin-stimulated phosphorylation decreased the electrophoretic mobility of PHAS-I, resulting in conversion to the lower mobility species. Only the two high-mobility forms were recovered with the affinity resin (Fig. 1B), as would be expected if insulin-stimulated phosphorylation of PHAS-I promoted dissociation of the PHAS-I–eIF-4E complex (*3*). Because eIF-4E is also phosphorylated in response to insulin (*5*), we determined whether phosphorylation of eIF-4E prevented binding to PHAS-I. We accomplished this by using a PHAS-I affinity resin (Fig. 1C). Almost no eIF-4E from control cells bound to the PHAS-I resin, which indicates that the factor was complexed and unavailable to bind exogenous PHAS-I. Insulin increased the binding of eIF-4E to the resin (Fig. 1C), indicating both that insulin increased the amount of free eIF-4E and that the hormone did not induce a modification of eIF-4E that prevented binding to PHAS-I.

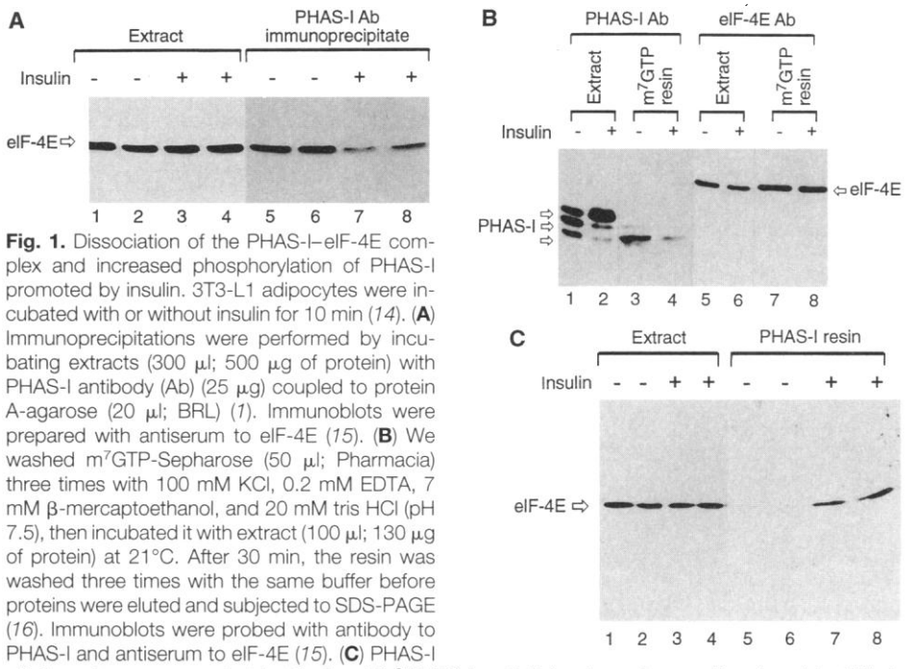

Fig. 1. Dissociation of the PHAS-I–eIF-4E complex and increased phosphorylation of PHAS-I promoted by insulin. 3T3-L1 adipocytes were incubated with or without insulin for 10 min (*14*). (**A**) Immunoprecipitations were performed by incubating extracts (300 μl; 500 μg of protein) with PHAS-I antibody (Ab) (25 μg) coupled to protein A-agarose (20 μl; BRL) (*1*). Immunoblots were prepared with antiserum to eIF-4E (*15*). (**B**) We washed m^7GTP-Sepharose (50 μl; Pharmacia) three times with 100 mM KCl, 0.2 mM EDTA, 7 mM β-mercaptoethanol, and 20 mM tris HCl (pH 7.5), then incubated it with extract (100 μl; 130 μg of protein) at 21°C. After 30 min, the resin was washed three times with the same buffer before proteins were eluted and subjected to SDS-PAGE (*16*). Immunoblots were probed with antibody to PHAS-I and antiserum to eIF-4E (*15*). (**C**) PHAS-I affinity resin was generated by binding (His^6)PHAS-I, a histidine-tagged recombinant protein (*10*), to immobilized Ni^{2+} resin (HisBind, Novagen) [5 μg of (His^6)PHAS-I per 50 μl of resin]. After binding, the resin was washed three times with buffer [50 mM NaF, 450 mM NaCl, and 20 mM tris HCl (pH 7.9)] containing 5 mM imidazole and then incubated with extract (300 μl; 500 μg of protein) at 21°C for 15 min. The resin was then washed five times before proteins were eluted with 1 M imidazole, 0.5 M NaCl, and 20 mM tris HCl (pH 7.9). Immunoblots were prepared with antiserum to eIF-4E (*15*).

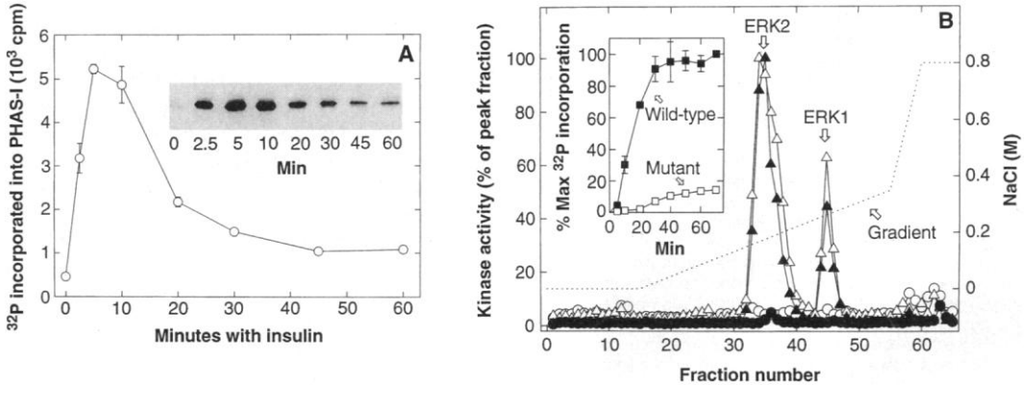

Fig. 2. Phosphorylation of PHAS-I by MAP kinase. Samples (10 μl) of extract or column fractions were mixed with 10 μl of reaction mixture containing 20 mM Hepes (pH 7.4), 100 mM β-glycerolphosphate, 3 mM EGTA, 0.2 mM Na_3VO_4, 2 mM dithiothreitol, 20 mM $MgCl_2$, 200 μM [γ-^{32}P]adenosine triphosphate (ATP) (~1,200,000 cpm), and 0.2 mg/ml of recombinant PHAS-I (*10*). After 20 min at 30°C, SDS sample buffer was added, and ^{32}P incorporated into PHAS-I was determined after SDS-PAGE (*16*). MAP kinase activity was measured with MBP as substrate (*11*, *13*). (**A**) Cells were incubated for various times with insulin before extracts were prepared (*13*) and PHAS-I kinase activity was measured. Mean values ± half of the range in two experiments are presented. The inset shows the ^{32}P-labeled PHAS-I. (**B**) Cells were incubated without (circles) or with (triangles) EGF for 10 min (*14*). Extracts (5 ml) were applied at a rate of 1 ml/min to a Mono-Q column (*17*). Proteins were eluted with the indicated gradient of NaCl, and 1-ml fractions were collected. PHAS-I (solid symbols) and MBP kinase (open symbols) activities were measured. The inset depicts time courses of phosphorylation of recombinant wild-type and mutant PHAS-I by recombinant MAP kinase. In the mutant, Ser^{64} was changed to Ala by oligonucleotide-directed mutagenesis. PHAS-I proteins were expressed in bacteria, purified, and phosphorylated as described (*10*). After 1 hour, the stoichiometry of phosphorylation of PHAS-I by MAP kinase was ≈1.2 mol/mol (*10*).

Experiments were done to identify the kinases mediating the insulin-stimulated phosphorylation of PHAS-I. Phosphorylation of recombinant PHAS-I was increased in extracts of insulin-treated adipocytes (Fig. 2A). The stimulated activity reached a peak after 5 min that was 12 times higher than that in the control extracts, then declined over the next 40 min to an activity approximately 2.5 times greater than that in nonstimulated cells. This time course closely resembled that of MAP kinase activation (*7*). A role for MAP kinase was implied also by experiments showing that the phosphorylation of PHAS-I was increased by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (*8*), two

growth factors known to activate MAP kinase (6). Like insulin, EGF increased PHAS-I kinase activity in adipocytes. After chromatography of cell extracts on a Mono-Q column, EGF-stimulated activity appeared in two peaks that corresponded exactly to the peaks of the MAP kinase isoforms, ERK1 and ERK2, measured with myelin basic protein (MBP) as substrate (Fig. 2B). All of the insulin-stimulated PHAS-I kinase that eluted from Mono-Q was associated with the two peaks of MAP kinase activity (9).

The rate of phosphorylation of PHAS-I by recombinant ERK2 is almost identical to the rate of phosphorylation of MBP (*10*), one of the best substrates for the kinase (*11*). Peptide mapping experiments indicate that Ser^{64} is the primary site of phosphorylation of PHAS-I by recombinant MAP kinase in vitro and in response to insulin in adipocytes (*10*). Changing Ser^{64} to Ala decreased phosphorylation of PHAS-I by MAP kinase (Fig. 2B), confirming the assignment of Ser^{64} as the major MAP kinase phosphorylation site.

To determine whether phosphorylation by MAP kinase affected the association of PHAS-I with eIF-4E, we prepared affinity resins by binding nonphosphorylated and MAP kinase–phosphorylated histidine-tagged PHAS-I to protein A-Sepharose with an antibody to PHAS-I. With control extracts, very little eIF-4E bound to either the phosphorylated or the nonphosphorylated PHAS-I (Fig. 3A), again suggesting that most of the eIF-4E in control cells is bound to endogenous protein. Treatment of cells with insulin increased the amount of eIF-4E that bound immune complexes containing nonphosphorylated PHAS-I (Fig. 3A). However, in neither the control nor the insulin-treated extracts did eIF-4E bind MAP kinase–phosphorylated PHAS-I (Fig. 3A). In another approach, eIF-4E was partially purified with m^7GTP resin from extracts of insulin-treated adipocytes and incubated with ^{125}I-labeled PHAS-I proteins (Fig. 3B). Nonphosphorylated, ^{125}I-labeled PHAS-I bound to resin containing eIF-4E. In contrast, little if any MAP kinase–phosphorylated PHAS-I bound to the resin. Binding was restored when the MAP kinase–phosphorylated PHAS-I was dephosphorylated by protein phosphatase 2A (PP2A) (Fig. 3B), confirming that phosphorylation was responsible for the loss of binding activity.

Our results indicate that activation of MAP kinase in response to insulin, EGF, or other stimuli results in increased phosphorylation of Ser^{64} in PHAS-I, which then dissociates from eIF-4E and thus allows the initiation of translation (*3*). Investigations of the regulation of PHAS-I phosphorylation have been confined to adipocytes, where amounts of the protein are greatest. However, with the exception of kidney tissue, every tissue that we analyzed contained an immunoreactive protein that appeared similar, if not identical, to PHAS-I (Fig. 4). The immunoblot pattern indicates that phosphorylated and nonphosphorylated forms of PHAS-I are present in most tissues (Fig. 4). MAP kinase is also found in these tissues, where it is stimulated by a variety of growth factors or hormones (6). Our results suggest that the stimulation of protein synthesis by such agents is mediated by phosphorylation of PHAS-I.

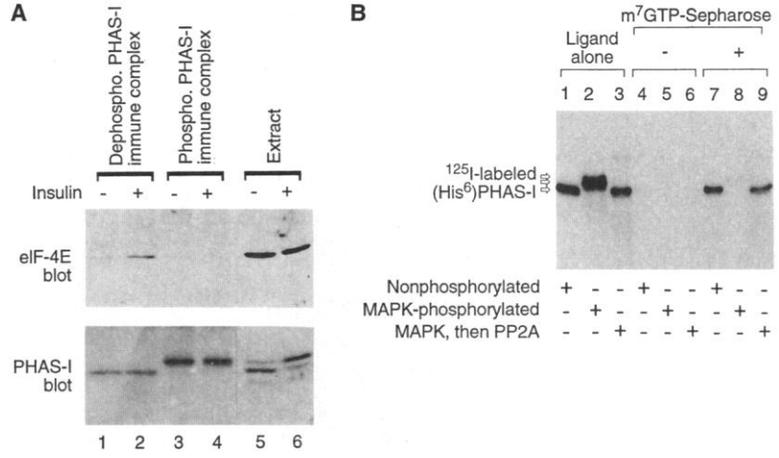

Fig. 3. Failure of PHAS-I phosphorylated by MAP kinase to associate with eIF-4E. (His^6)PHAS-I (*10*) was incubated in buffer containing 20 mM $MgCl_2$ and 1 mM ATP, either without (dephospho.) or with (phospho.) recombinant MAP kinase for 1 hour. (**A**) We generated PHAS-I affinity resins by incubating the PHAS-I samples (5 μg) with antibody to PHAS-I (5 μg) coupled to protein A-agarose (25 μl). To prevent binding to endogenous PHAS-I, we saturated the antibody by incubation with the PHAS-I peptide antigen (1 mM) (*1*). The immune complexes were washed three times in homogenization buffer, then incubated with cell extracts (300 μl; 500 μg of protein) for 20 min at 21°C. The resins were washed three times with homogenization buffer before proteins were eluted and subjected to SDS-PAGE (*16*). After blotting with antiserum to eIF-4E, the blot was stripped and incubated with antibody to PHAS-I. Antibody binding was detected with protein A coupled to horseradish peroxidase. (**B**) (His^6)PHAS-I that had been phosphorylated by MAP kinase (MAPK) was dephosphorylated by incubation with the catalytic subunit of bovine heart PP2A (600 mU/ml) for 30 min at 30°C. Samples (20 μg) of nonphosphorylated, MAP kinase–phosphorylated, and PP2A-dephosphorylated PHAS-I proteins were labeled with ^{125}I with the use of carrier-free $Na^{125}I$ (200 μCi) and chloramine T (*17*). The m^7GTP-Sepharose was incubated with (+) or without (−) extracts from insulin-treated adipocytes as described in Fig. 1B. The washed resins were incubated with the ^{125}I-labeled PHAS-I (~50,000 cpm). After 20 min at 21°C, the resin was washed four times before proteins were eluted and subjected to SDS-PAGE.

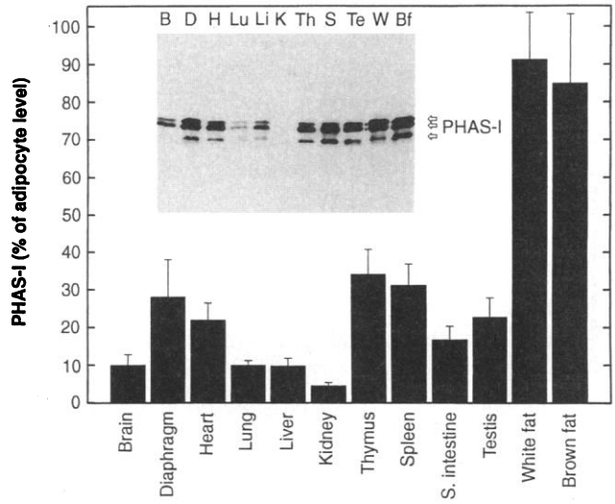

Fig. 4. Tissue distribution of PHAS-I. Isolated rat adipocytes and the following tissues were homogenized: brain (B), diaphragm (D), heart (H), lung (Lu), liver (Li), thymus (Th), spleen (S), small intestine, testis (Te), epididymal white fat (W), and intrascapular brown fat (Bf). Homogenates were centrifuged at 10,000*g* for 20 min. Extract protein concentrations were adjusted to 4 mg/ml before samples were incubated at 100°C for 10 min. After centrifugation at 10,000*g* for 30 min, samples (50 μl) were subjected to SDS-PAGE and an immunoblot was prepared with antibody to PHAS-I (inset). The arrows denote the three mobility forms of PHAS-I detected in most tissues. Relative amounts of PHAS-I were determined by optical density scanning and are expressed as percentages (± SD of three experiments) of the amount found in isolated adipocytes.

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14. 3T3-L1 adipocytes were cultured as described (*7*). The growth medium was replaced with Krebs' Ringer phosphate buffer (KRP) containing 2% bovine serum albumin before cells were incubated at 37°C with 20 nM insulin (porcine; 27 units/mg; Eli Lily) or 100 nM EGF (UBI). Cells were rinsed with KRP, scraped from dishes (10 cm), homogenized (1 ml of buffer per dish), and centrifuged at 10,000*g* for 20 min. Protein was measured with bicinchoninic acid (*12*). When immunoprecipitations were done or when Ni^{2+} chelate resin was used, cells were homogenized in 50 mM NaF, 450 mM NaCl, and 20 mM tris HCl (pH 7.9). When m^7GTP-Sepharose was used, homogenization buffer was 50 mM NaF and 80 mM β-glycerol phosphate (pH 7.3).
15. For immunoblotting, samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*13*) before proteins were electrophoretically transferred to nylon sheets, which were then immersed in phosphate-buffered saline (PBS) containing 5% powdered milk (Carnation). Sheets were incubated with the eIF-4E antiserum (*3*) (diluted 2000 times) or affinity-purified PHAS-I antibody (*1*) (2 μg/ml) and washed four times with PBS. Unless otherwise stated, antibody binding was detected by enhanced chemiluminescence with horseradish peroxidase conjugated to donkey antibody to rabbit immunoglobulin G (Amersham).
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