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Distinct Signalling Pathways Mediate Insulin and Phorbol Ester-stimulated Eukaryotic Initiation Factor 4F Assembly and Protein Synthesis in HEK 293 Cells*

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Stimulation of serum-starved human embryonic kidney (HEK) 293 cells with either the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or insulin resulted in increases in the phosphorylation of 4E-BP1 and p70 S6 kinase, eIF4F assembly, and protein synthesis. All these effects were blocked by rapamycin, a specific inhibitor of mTOR. Phosphatidylinositol 3-kinase and protein kinase B were activated by insulin but not by TPA. Therefore TPA can induce eIF4F assembly, protein synthesis, and the phosphorylation of p70 S6 kinase and 4E-BP1 independently of both phosphatidylinositol 3-kinase and protein kinase B. Using two structurally unrelated inhibitors of MEK (PD098059 and U0126), we provide evidence that Erk activation is important in TPA stimulation of eIF4F assembly and the phosphorylation of p70 S6 kinase and 4E-BP1 and that basal MEK activity is important for basal, insulin, and TPA-stimulated protein synthesis. Transient transfection of constitutively active mitogen-activated protein kinase interacting kinase 1 (the eIF4E kinase) indicated that inhibition of protein synthesis and eIF4F assembly by PD098059 is not through inhibition of eIF4E phosphorylation but of other signals emanating from MEK. This report also provides evidence that increased eIF4E phosphorylation alone does not affect the assembly of the eIF4F complex or general protein synthesis.

Protein synthesis is regulated by a variety of stimuli including insulin (1) and phorbol esters (2–6). A key step in the regulation of protein synthesis is the assembly of the initiation complex eIF4F,[1] containing the initiation factors eIF4G, a large scaffolding protein, eIF4E, the protein which binds to the 5′ cap structure (7-methylguanosine triphosphate) and eIF4A, a bidirectional RNA helicase (reviewed by Sonenberg (7)). The formation of the eIF4F complex (usually defined operationally as the association of eIF4G with eIF4E) is regulated by a variety of stimuli through multiple phosphorylation events. Two principal mechanisms have been suggested to play an important role in regulating eIF4F assembly. First, it has been proposed that the phosphorylation of eIF4E may be important in the formation of the eIF4F complex, as eIF4E associated with eIF4G is more highly phosphorylated than free eIF4E (8, 9) and since increased phosphorylation of eIF4E often parallels increases in total protein synthesis (reviewed by Kleijn *et al.* (10)). Recent data have provided evidence that eIF4E is phosphorylated by MAP kinase interacting kinase 1 (Mnk1), which is itself phosphorylated by both p38-MAPK and Erk (11–15). The second mechanism regulating eIF4F assembly involves a family of eIF4E-binding proteins (4E-BPs, archetype 4E-BP1) whose affinity for eIF4E is regulated through their phosphorylation (16–20). 4E-BP1 binds to a site on eIF4E which overlaps the binding site for eIF4G (18, 21, 22). 4E-BP1 thus competes with eIF4G for binding to eIF4E and can therefore inhibit eIF4F complex formation. Phosphorylation of 4E-BP1 leads to a reduction in its affinity for eIF4E and its dissociation, making eIF4E available to bind eIF4G, potentially leading to an increase in mRNA translation (18–20).

Most studies on the signaling pathways involved in the regulation of protein synthesis have focused on insulin (reviewed by Proud and Denton (1)). It has been demonstrated that insulin can lead to increased eIF4F assembly, activation of protein synthesis, and the phosphorylation of 4E-BP1. Insulin also induces the phosphorylation of p70 S6 kinase which phosphorylates ribosomal protein S6 and is linked to an increase in the translation of a specific subset of mRNAs which contain polypyrimidine tracts within their 5′-untranslated regions (5′-terminal oligopyrimidine tract mRNAs) (23–25). Phosphorylation of 4E-BP1 and p70 S6 kinase in response to insulin is inhibited both by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors wortmannin and LY294002 (26–30) and by the inhibitor of mTOR, rapamycin, indicating that PI 3-kinase and mTOR play a role in their regulation (19, 31–34). PKB, a kinase which lies downstream of PI 3-kinase and is activated by phosphorylation by PDK1 and 2 in response to insulin (35), is thought to be important in the signal transduction pathway which leads to the phosphorylation of both p70 S6 kinase and 4E-BP1 (27, 36).

It has been demonstrated that phorbol esters can also lead to increases in protein synthesis and the phosphorylation status of a number of initiation factors, including members of the eIF4F complex (2–6). However, the roles of these phosphorylation events in the activation of protein synthesis by phorbol ester and the nature of the signaling pathways which lead to these increases are unknown. Phorbol esters offer a paradigm for stimuli acting via phospholipase C which generate diacylglycerol (37) and therefore use a proximal signaling pathway

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[1] The abbreviations used are: eIF(4F, 4A, 4E, 4G), eukaryotic initiation factor (4F, 4A, 4E, 4G); HEK, human embryonic kidney; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 4E-BP1, eIF4E-binding protein 1; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/Erk kinase; Erk, extracellular signal-regulated kinase; Mnk, MAP kinase interacting kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; GFP, green fluorescent protein.

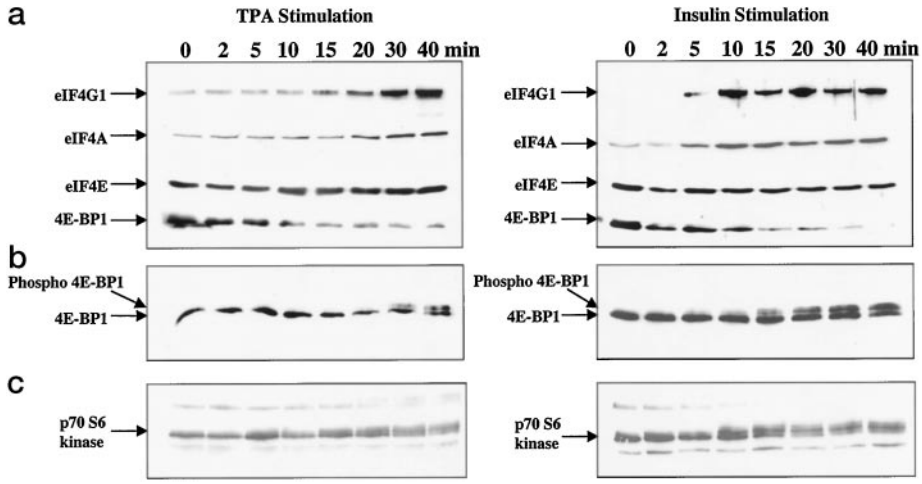

FIG. 1. **Insulin and TPA stimulate eIF4F assembly concomitantly with increased phosphorylation of 4E-BP1 and p70 S6 kinase.** HEK 293 cells were serum starved for 16 h prior to the addition of either 1 μM TPA or 100 nM insulin for the time periods indicated. *a,* analysis of proteins isolated by m^{7}GTP-Sepharose pull-down separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, eIF4A, and 4E-BP1. *b,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-4E-BP1 antiserum. Phospho-4E-BP1 represents the more phosphorylated forms of 4E-BP1. *c,* 10 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-p70 S6 kinase antiserum. These results are representative of three separate experiments.

which may be different to that utilized by insulin.

In this report we characterize in parallel the signaling pathways by which both insulin and phorbol esters increase eIF4F assembly and total protein synthesis in HEK 293 cells. We demonstrate that the phorbol ester, TPA, and insulin stimulate eIF4F assembly and protein synthesis. The stimulation of eIF4F assembly and protein synthesis by insulin and TPA is inhibited by rapamycin. However, TPA stimulates eIF4F assembly and protein synthesis through a mechanism that is independent of PI 3-kinase and PKB but dependent on Erk, whereas insulin stimulates the eIF4F assembly and protein synthesis through a mechanism that requires PI 3-kinase and may involve PKB. We also demonstrate that increased eIF4E phosphorylation is not required for stimulation of eIF4F assembly. The signaling pathways and the roles of individual initiation factors in eIF4F assembly and protein synthesis are discussed.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—[^{35}S]Methionine and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech. Microcystin, wortmannin, rapamycin, PD098059 and CHAPS were from Calbiochem. Materials for tissue culture were from Life Technologies, Inc. m^{7}GTP-Sepharose was from Amersham Pharmacia Biotech. U0126 was obtained from Promega. All other chemicals (unless stated) were obtained from Sigma.

Cell Culture and Treatment of Cells—HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Prior to treatment, cells were grown to 80% confluence before being serum starved for 16 h. Details of treatments are provided in the figure legends. After treatment, cells were washed in phosphate-buffered saline and lysed in extraction buffer (0.1% Triton X-100, 50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 1 mM benzamidine-HCl, 0.5 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of pepstatin A, antipain, and leupeptin). The lysates were then centrifuged for 10 min at 16,000 $\times$ *g*. The supernatants were removed and used for further analysis.

SDS-Polyacrylamide Gel Electrophoresis (PAGE), Isoelectric Focusing, and Immunoblotting—SDS-PAGE and Western blotting were performed as described previously (38, 39). Anti-human eIF4E antiserum was raised against a synthetic peptide corresponding to residues 5–23 of the protein. The anti-4E-BP1 antibody was raised against a peptide corresponding to residues 101–113 of human 4E-BP1. Anti-eIF4G1 and -eIF4A were generous gifts from Drs S. Morley (University of Sussex) and H. Trachsel (Bern University), respectively. Anti-phospho-PKB Ser^{473} and anti-phospho-Erk antibodies were supplied by New England Biolabs. Isoelectric focusing of eIF4E was performed as described previously (6) using Ampholines (Amersham Pharmacia Biotech) in the pH range 3.5–10.

m^{7}GTP-Sepharose Chromatography—m^{7}GTP-Sepharose chromatography was performed as described previously (6). 25 μl of a 50/50 slurry of m^{7}GTP-Sepharose CL-6B (Amersham Pharmacia Biotech) was added to 0.2–0.5 mg of protein. The lysates were then rotated for 1 h at 4 °C. The m^{7}GTP-Sepharose was pelleted by centrifugation at 1000 $\times$ *g* for 2 min. The beads were then washed three times in extraction buffer. For SDS-PAGE, proteins were removed from the m^{7}GTP matrix by boiling it in SDS loading buffer.

In Vitro Kinase Assays—PKB assays were performed as described by Cross *et al.* (40). Briefly, lysates were subjected to immunoprecipitation with antibodies directed against PKB α, β, and γ (a generous gift from Dr. D. Alessi, Dundee University). The immunoprecipitates were incubated with kinase buffer (50 mM Tris, pH 7.4, 5 μM protein kinase A inhibitor, 100 μM EGTA, 10 mM $Mg(Ac)_2$, 1 μM microcystin, 50 μM ATP, 1 μCi of [γ-^{32}P]ATP) and 30 μM of the peptide substrate "Crosstide," (GRPRTSSFAEG) at 30 °C for 15 min. The kinase reactions were then spotted onto DE81 paper, washed with 1% phosphoric acid, and radioactivity was determined by the Cerenkov method. For *in vitro* PI 3-kinase assays, cell lysates (~0.5 mg of protein) were mixed with anti-phosphotyrosine (clone PY20, generously given by Drs. A. N. Carter, J. Miesenhelder and S. Simon, The MBVL, Salk Institute, La Jolla, CA). Immunoprecipitates were collected by brief centrifugation and washed twice with 1 ml each of: (i) 25 mM Hepes, 120 mM NaCl, 5 mM β-glycerophosphate, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM EDTA, 50 mM sodium fluoride, 1%(v/v) Triton X-100, pH 7.6; (ii) as i without Triton X-100 to remove detergent; (iii) 100 mM Tris, 500 mM LiCl, pH 8.0; (iv) 25 mM Hepes, 120 mM NaCl, 5 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM EDTA, pH 7.6, to remove high salt, each wash was supplemented with 1 mM Na_3VO_4, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 0.02 mM leupeptin. The detergent and high salt washes were to remove possible contaminants. The washed beads were re-suspended in 0.04 ml of the final buffer additionally supplemented with 0.01 mM [γ-^{32}P]ATP (10–25 Ci/mmol) and phosphatidylinositol/phosphatidylserine vesicles to give final concentrations of 0.1 mM with respect to each lipid. Vesicles were prepared as a 4-fold concentrate by sonicating dried phospholipid films in 25 mM Hepes, 100 mM NaCl, 1 mM EGTA, pH 7.6, using 3 $\times$ 20-s bursts at approximately 50% maximum power from a probe tip equipped with a micro-cup horn. Kinase reactions were stopped by addition of 0.6 ml of methanol, chloroform, 12 M HCl (80:40:1, v:v) followed by 0.2 ml of chloroform and 0.12 ml of 0.1 M HCl. Samples were mixed and briefly spun and the lower phases removed to fresh tubes and washed with 0.78 ml of synthetic upper phase. The washed lower phases were dried in vacuum, re-suspended in 0.02 ml of chloroform/methanol (2:1, v/v) and [^{32}P]PtdIns(3)P present separated from contaminating materials by thin layer chromatography (TLC) on Silica Gel 60 plates, pre-dipped in 1% (w/v) potassium oxalate, 5 mM EDTA, pH 8.5 (in methanol:water, 1:1 v/v), and dried prior to use, developed in methanol, chloroform, water, 35% ammonia solution (20:15:5:3, v/v). Bands corresponding to [^{32}P]phosphatidylinositol 3-phosphate were located by autoradiography.

Protein Synthesis Measurements—15 min after stimulation with either insulin or TPA the cells were incubated with [^{35}S]methionine at 10 μCi/ml for 30 min. The cells were then washed in phosphate-buffered saline and lysed in extraction buffer. 20 μg of protein was spotted onto 3MM paper (Whatman). The filters were then washed by boiling them for 1 min in 10% trichloroacetic acid containing 0.1 g/liter methionine. This was repeated three times. The filters were then dried and immersed in scintillant before determining radioactivity by scintillation counting.

^{32}P in Vivo Labeling—Cells were serum starved in Dulbecco's mod-

ified Eagle's medium minus phosphate medium for 16 h prior to addition of 50 μCi/ml [^{32}P]orthophosphate. Cells were then treated with inhibitors or agonists as indicated in the text.

Transient Transfections—Transient transfections were performed as described by Alessi *et al.* (41). Plasmid pEBG-T332D expressing constitutively active Mnk1 (Mnk1 T332D) were kindly provided by Dr. A. J. Waskiewicz (Fred Hutchinson Cancer Center, Seattle, WA) (12, 15).

RESULTS

Insulin and TPA Increase eIF4F Assembly and Protein Synthesis—Serum-starved HEK 293 cells were treated with either TPA or insulin and samples were taken over a 40-min time course. eIF4F assembly and the phosphorylation of 4E-BP1 and p70 S6 kinase were monitored over this time period. TPA and insulin each increased the assembly of the eIF4F complex, as characterized by an increase in the association of eIF4G and eIF4A with eIF4E (Fig. 1*a*) concomitantly with the dissociation of 4E-BP1 from eIF4E. These effects occurred in parallel with the phosphorylation of 4E-BP1 and p70 S6 kinase as indicated by shifts in their migrations on SDS-PAGE (Fig. 1, *b* and *c*). However, differences were observed in the time taken to reach maximal p70 S6 kinase phosphorylation, 4E-BP1 phosphorylation, and eIF4F assembly for the two stimuli, with insulin treatment resulting in a maximal effect within 15–20 min as compared with 30–40 min in response to TPA (the response to TPA does not significantly increase from 40 to 60 min (data not shown)). Stimulation with either insulin or TPA does not always result in the complete dissociation of 4E-BP1 (Fig. 1*a*) indicating maximal stimulation has not been reached. However, the response to TPA levels out by approximately 40 min as little additional stimulation was observed by 60 min (results not shown). The apparent association of eIF4A with eIF4E in the absence of eIF4G seen in Fig. 1*a* is due to the greater sensitivity of the anti-eIF4A antibody as compared with that of the anti-eIF4G1 antibody, and reflects a low basal level of eIF4F complexes in untreated cells.

Measurements of the incorporation of [^{35}S]methionine into protein revealed that insulin or TPA treatment of the cells each induced a substantial increase in protein synthesis of approximately 40–50% over untreated cells (see Figs. 2*d* and 3*e*). Taken together, these data show that treatment of HEK 293 cells with TPA or insulin leads to the phosphorylation of p70 S6 kinase and 4E-BP1 in parallel with increases in eIF4F assembly and the rate of total protein synthesis.

Stimulation by TPA and Insulin of eIF4F Assembly and Protein Synthesis Is Dependent upon mTOR—mTOR plays an important role in the regulation of the phosphorylation of both p70 S6 kinase and 4E-BP1 in response to many stimuli (32–34), although the signal transduction pathways leading to the activation of mTOR are essentially unknown. We therefore investigated the role of mTOR in TPA- or insulin- stimulated eIF4F assembly, protein synthesis, and 4E-BP1 and p70 S6 kinase phosphorylation using a specific inhibitor of mTOR, rapamycin. Cells were incubated with rapamycin for 45 min prior to treatment with insulin or TPA. Rapamycin blocked the stimulation of eIF4F assembly by either insulin or TPA and also inhibited the phosphorylation of p70 S6 kinase and 4E-BP1 (Fig. 2, *a-c*). The stimulation of protein synthesis by insulin or TPA was also partially inhibited by rapamycin when compared with rapamycin-treated control cells (Fig. 2*d*). The data show that mTOR plays a central role in the stimulation of eIF4F assembly and the phosphorylation of 4E-BP1 and p70 S6 kinase in response to TPA or insulin.

Stimulation by Insulin, but Not TPA, of eIF4F Assembly and Protein Synthesis Is Dependent on the Activation of PI 3-Kinase—It has previously been shown, in several cell types, that the activation of both p70 S6 kinase and 4E-BP1 by insulin occurs by a mechanism involving PI 3-kinase (see *e.g.* Refs. 36 and 42). We therefore wished to determine whether TPA and insulin activated PI 3-kinase in HEK 293 cells.

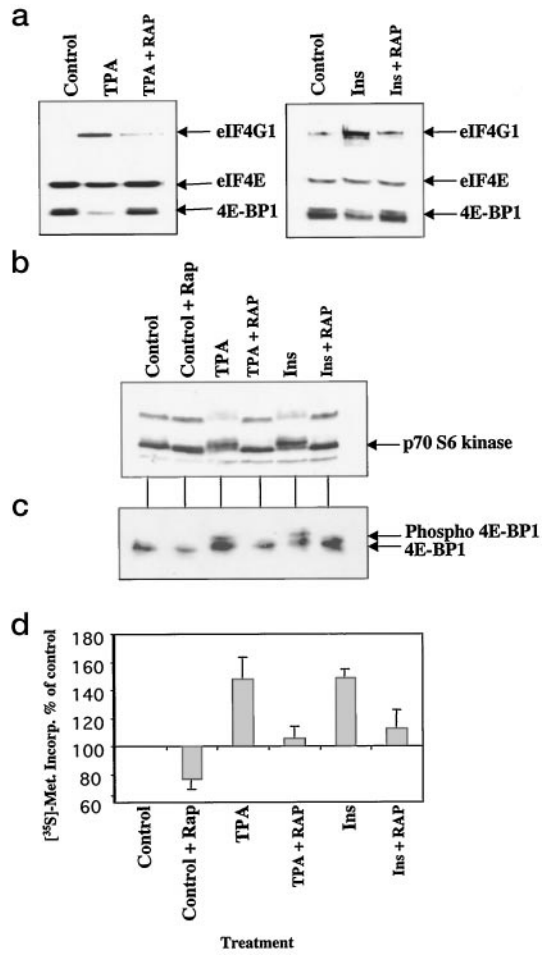

FIG. 2. **The mTOR inhibitor rapamycin inhibits TPA or insulin-stimulated eIF4F assembly and protein synthesis.** HEK 293 cells were serum starved for 16 h. Cells were pretreated with 200 nM rapamycin (*RAP*) for 40 min prior to the addition of 1 μM TPA or 100 nM insulin for 45 min. *a,* analysis of proteins isolated by m^{7}GTP-Sepharose pull-down separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, and 4E-BP1. *b,* 10 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti p70 S6 kinase antiserum. *c,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-4E-BP1 antiserum. Phospho-4E-BP1 represents the more phosphorylated forms of 4E-BP1. *d,* incorporation of [^{35}S]methionine into protein expressed as % of control (n = 4: ±S.E.). These results are representative of three separate experiments, except where stated.

Antibodies directed against phosphotyrosine were used to pull-down phosphotyrosine-associated PI 3-kinase from insulin or TPA-treated cell lysates. The activity of PI 3-kinase was then determined by an *in vitro* assay (Fig. 3*a*). Insulin treatment led to a large increase in PI 3-kinase activity as indicated by the generation of phosphatidylinositol 3-phosphate (Fig. 3*a*). TPA did not activate PI 3-kinase at any of the time points tested (Fig. 3*a*). However, TPA might activate PI 3-kinase via a phosphotyrosine independent mechanism. Therefore, in order to investigate further the possible role of PI 3-kinase in insulin and TPA-stimulated eIF4F assembly, protein synthesis and the phosphorylation of both p70 S6 kinase and 4E-BP1, serum-starved cells were pretreated with wortmannin, an inhibitor of PI 3-kinase, before addition of either insulin or TPA. As shown in Fig. 1, TPA and insulin increased eIF4F assembly (Fig. 3*b*) and the phosphorylation of p70 S6 kinase and 4E-BP1 (Fig. 3, *c* and *d*), as well as protein synthesis (Fig. 3*e*). In the presence

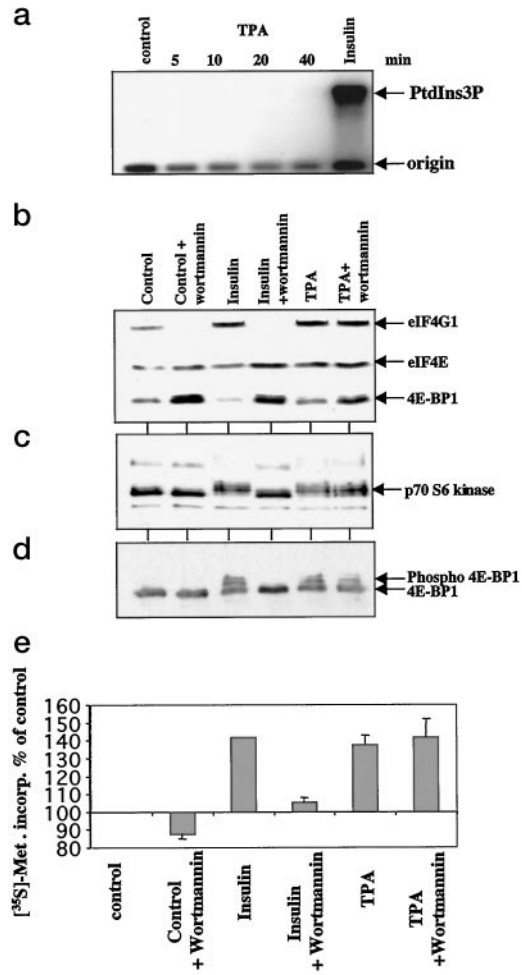

FIG. 3. **TPA does not activate PI 3-kinase.** *a, in vitro* PI 3-kinase assay of lysates from HEK 293 cells. Cells were serum starved for 16 h prior to the addition of 100 nM insulin or 1 μM TPA for the time period indicated: *control*, unstimulated; *insulin*, cells stimulated with insulin for 5 min. Representative of two separate experiments. *b-e,* HEK 293 cells were serum starved for 16 h, 100 nM wortmannin was added 15 min prior to and after the addition of 100 nM insulin or 1 μM TPA for 45 min. *b,* analysis of proteins isolated by m^7GTP-Sepharose pull-down separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, and 4E-BP1. *c,* 10 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-p70 S6 kinase antiserum. *d,* 20 μg of cell lysates were run on a SDS-polyacrylamide gel followed by Western blotting using anti-4E-BP1 antiserum. Phospho-4E-BP1 represents the more phosphorylated forms of 4E-BP1. Results in *b, c*, and *d* are representative blots from three separate experiments. *e,* incorporation of [^{35}S]methionine into protein expressed as % of control (n = 3: ±S.E.).

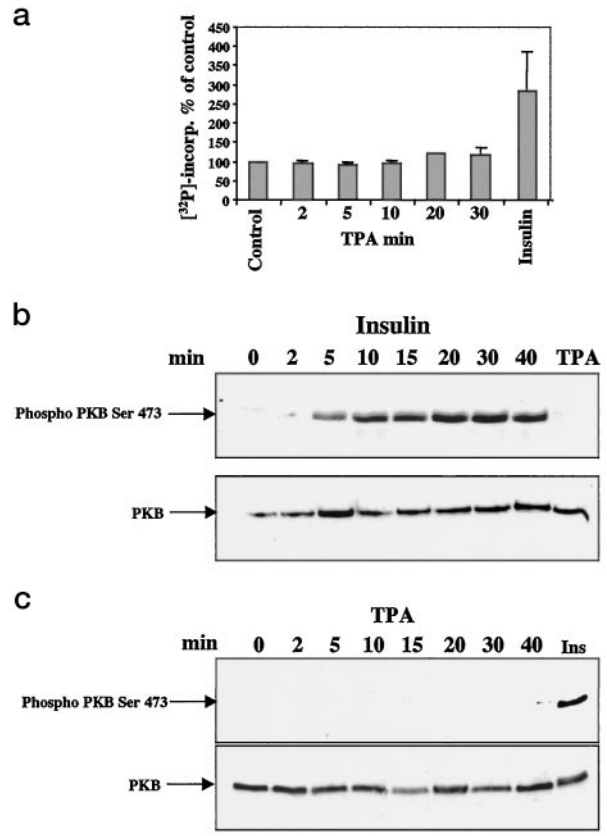

FIG. 4. **Insulin but not TPA activates PKB.** *a,* activation of PKB assessed by *in vitro* kinase assay. HEK 293 cells were serum starved for 16 h prior to the addition of either 100 nM insulin for 5 min or 1 μM TPA for the time periods indicated. PKB was then precipitated from the lysates for an *in vitro* PKB assay (see "Experimental Procedures"). Results are expressed as ^{32}P incorporation into substrate as a % of control, *i.e.* unstimulated cells (n = 3: ±S.E.). *b* and *c*, HEK 293 cells were serum starved for 16 h prior to the addition of either 100 nM insulin or 1 μM TPA for the time periods indicated. 10 μg of cell lysates were run on a SDS-polyacrylamide gel and Western blotted using anti-PKB Ser^{473} phospho-specific antiserum (*upper panel*) or as loading control, anti-PKB antiserum (*lower panel*). Results are representative of three separate experiments.

of wortmannin, insulin was unable to stimulate eIF4F assembly, the phosphorylation of p70 S6 kinase and 4E-BP1 or protein synthesis (Fig. 3, *b-e*). However, the stimulation by TPA of eIF4F assembly, the phosphorylation of p70 S6 kinase, and 4E-BP1 and increases in protein synthesis were unaffected by wortmannin (Fig. 3, *b-e*). These data show that the stimulation of eIF4F assembly and protein synthesis by TPA occurs through a pathway which is independent of PI 3-kinase, whereas PI 3-kinase activity is required for insulin-stimulated eIF4F assembly and protein synthesis.

PKB Is Activated by Insulin but Not by TPA—Several lines of evidence suggest that PKB plays an important role in the regulation of both p70 S6 kinase and 4E-BP1 and that activation of PKB through phosphorylation by PDK1 and -2 is dependent upon PI 3-kinase (35, 36, 42–46). A number of stimuli lead to the activation of PKB including insulin (41). There is also evidence that mTOR is a target for signaling via PKB (42). As the activation of eIF4F assembly, protein synthesis and phosphorylation of 4E-BP1 and p70 S6 kinase by TPA did not require PI 3-kinase, unlike their control by insulin, it was important to examine how TPA induced these effects. We therefore investigated whether PKB was activated by TPA and also in parallel, investigated the activation of PKB by insulin. Insulin stimulation led to a rapid activation of endogenous PKB in HEK293 cells (within 5 min, Fig. 4*a*). Indeed, it has previously been reported that insulin rapidly activates PKB expressed from constructs transiently transfected into HEK 293 cells (41). However, TPA did not elicit any detectable activation of PKB (Fig. 4*a*). Phosphorylation of PKB at Thr^{308} and Ser^{473} is essential for full activation of PKB (41, 47). As an adjunct to direct measurements of its activity, the phosphorylation status of PKB following both insulin and TPA treatment of cells was therefore monitored using a phospho-specific antibody against Ser^{473}. Insulin treatment resulted in a rapid increase in the phosphorylation of PKB at Ser^{473}, which was sustained throughout the period of the experiment (40 min) (Fig. 4*b*). However, TPA treatment of cells did not result in any detectable phosphorylation at Ser^{473} (Fig. 4*c*), consistent with the absence of a change in activity.

These data reveal a clear difference in the signaling pathways through which insulin and TPA regulate protein synthesis, eIF4F assembly, and the phosphorylation of p70 S6 kinase

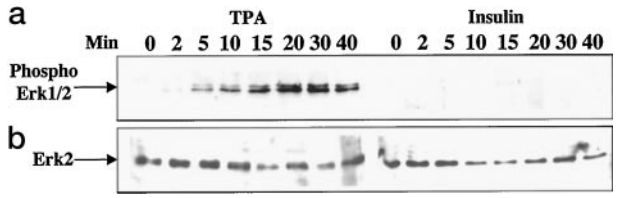

FIG. 5. **TPA, but not insulin, stimulates the phosphorylation of Erk1 and -2.** HEK 293 cells were serum starved for 16 h prior to the addition of either 100 nM insulin or 1 μM TPA for the time periods indicated. Western blot analysis of proteins separated by SDS-polyacrylamide gel using: *a,* phospho-specific antibody against Erk1 and -2; and *b,* anti-Erk2 antibody as loading control. These results are representative of three experiments.

and 4E-BP1. The effects of insulin require PI 3-kinase (and may involve activation of PKB) while the effects of TPA are independent of both.

Role of the MAP Kinase Pathway in TPA and Insulin Stimulated eIF4F Assembly and Protein Synthesis—It has been shown that phorbol esters activate Erk in many cell types. It has also been suggested that Erk may play a role in the phosphorylation of both p70 S6 kinase (48) and 4E-BP1 (17, 49–51). We therefore explored the role of Erk in the stimulation by insulin or TPA of eIF4F assembly, protein synthesis, and the phosphorylation of 4E-BP1 and p70 S6 kinase.

The activation state of Erk was examined using a phospho-specific antibody to Erk, which detects the activated forms of Erk1 and -2. TPA treatment of cells resulted in the phosphorylation of Erk (Fig. 5*a*) concomitant with the phosphorylation of both p70 S6 kinase and 4E-BP1 observed in Fig. 1. This suggests that Erk might be involved in the phosphorylation of p70 S6 kinase and 4E-BP1 in response to TPA. In marked contrast, insulin treatment did not result in any detectable phosphorylation of Erk (Fig. 5*a*).

To study further the role of Erk in TPA-stimulated eIF4F assembly and protein synthesis, serum-starved cells were pretreated with PD098059 (an inhibitor of MEK, the upstream activator of Erk) (52, 53). As expected, this resulted in a dose-dependent inhibition of the ability of TPA to induce Erk phosphorylation as assessed using the phospho-specific antibody against Erk (Fig. 6*a*). The addition of PD098059 also resulted in a dose-dependent inhibition of TPA-stimulated eIF4F assembly, whereas PD098059, even at higher concentrations, only had a partial effect on insulin-stimulated eIF4F assembly (Fig. 6*b*). Indeed, in many experiments, 50 μM PD098059 had no effect on insulin-activated eIF4F assembly (results not shown). PD098059 also caused the dose-dependent inhibition of the phosphorylation of both 4E-BP1 and p70 S6 kinase in response to TPA (Fig. 6*c*). It also inhibited the insulin-induced phosphorylation of p70 S6 kinase and (partially) of 4E-BP1, even though phosphorylation of Erk was not observed upon insulin treatment (Figs. 5 and 6). Inhibition by PD098059 of insulin-stimulated phosphorylation of p70 S6 kinase and 4E-BP1 has previously been reported (see "Discussion") (50).

50 μM PD098059 severely inhibited the rates of basal, TPA- and insulin-stimulated protein synthesis (Fig. 6*d*). This does not appear to be related to the extent of eIF4F assembly (Fig. 6*b*), since insulin was still able to promote eIF4F assembly in the presence of PD098059. In order to confirm that these effects were through the inhibition of MEK, these experiments were repeated using a structurally unrelated inhibitor of MEK, U0126 (54). The effects of this compound on TPA or insulin stimulated phosphorylation of Erk1/2, 4E-BP1, and p70 S6 kinase and eIF4F assembly (Fig. 7, *a* and *b*) were very similar to those observed using PD098059 (Fig. 6). This therefore strongly suggests that the effects of PD098059 are due to its ability to inhibit MEK and thus Erk activation.

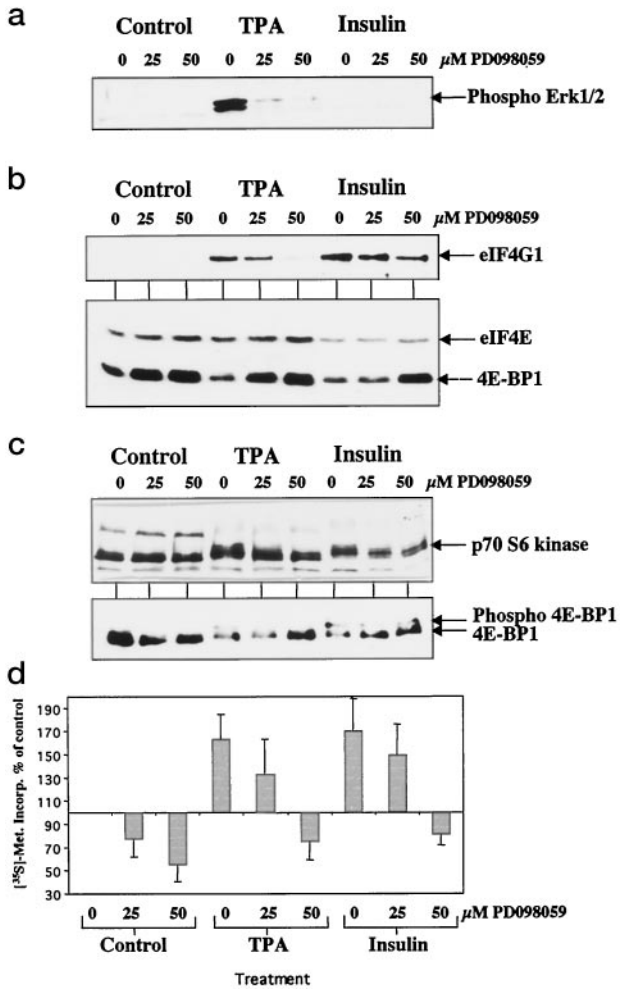

FIG. 6. **Effect of PD098059 on eIF4F assembly and protein synthesis.** HEK 293 cells were serum starved for 16 h. The cells were then preincubated in either 25 or 50 μM PD098059 for 45 min before addition of either 100 nM insulin or 1 μM TPA for an additional 45 min. *a,* Western blot analysis, using a phospho-specific antibody against Erk1 and -2. *b,* analysis of proteins isolated by m^{7}GTP-Sepharose pull-down separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, and 4E-BP1. *c,* Western blot analysis using antibodies against either p70 S6 kinase or 4E-BP1. Phospho-4E-BP1 represents the more phosphorylated forms of 4E-BP1. *d,* incorporation of [^{35}S]methionine into protein expressed as percentage of control (n = 5: ±S.E.). All these results are representative of at least three separate experiments, except where stated.

These results provide strong evidence that the MAP kinase pathway (specifically MEK) plays an important role in the stimulation of eIF4F assembly and the phosphorylation of p70 S6 kinase and 4E-BP1 induced by TPA. They also suggest that this pathway plays a role in maintaining basal translation rates.

Role of eIF4E Phosphorylation in Basal, TPA, and Insulin-stimulated eIF4F Assembly and Protein Synthesis—It has been suggested that phosphorylation of eIF4E may play an important role in the regulation of both protein synthesis and eIF4F assembly (8, 9, 10). Mnk1, the kinase believed to phosphorylate eIF4E *in vivo*, lies downstream of Erk and p38 MAP kinase (12, 13, 15), and provides the only established link between Erk signaling and the translational machinery. Insulin treatment did not cause phosphorylation of Erk suggesting that increased eIF4E phosphorylation may not be an essential element in the stimulation of eIF4F assembly and protein synthesis by insulin. The effect of insulin or TPA treatment on the phosphorylation of eIF4E was therefore investigated using two different

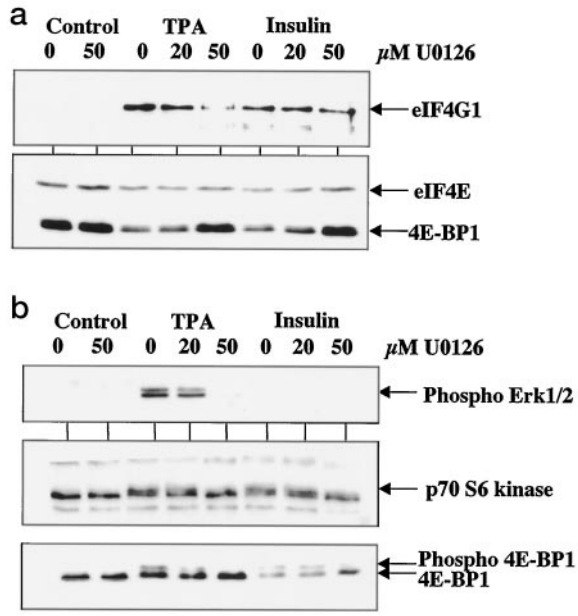

FIG. 7. **Effect of U0126 on eIF4F assembly.** HEK 293 cells were serum starved for 16 h. The cells were then preincubated in either 20 or 50 μM U0126 for 45 min before addition of either 100 nM insulin or 1 μM TPA for a further 45 min. *a,* analysis of proteins isolated by m^7GTP-Sepharose pull-down separated on a SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, and 4E-BP1. *b,* Western blot analysis using antibodies against either phospho-Erk1 and -2, p70 S6 kinase, or 4E-BP1. Phospho-4E-BP1 represents the more phosphorylated forms of 4E-BP1. All these results are representative of three separate experiments.

but complementary techniques, isoelectric focusing and *in vivo* radiolabeling (Fig. 8*a*). TPA treatment resulted in a large increase in the level of phosphorylation of eIF4E and this effect was inhibited by PD098059, whereas insulin stimulation did not alter the levels of phosphorylation of eIF4E (Fig. 8*a, i* and *ii*) (phosphate starvation had no significant effect on the rate of protein synthesis, results not shown). This demonstrates that increased phosphorylation of eIF4E is not required for the stimulation of eIF4F assembly and protein synthesis by insulin in HEK 293 cells. However, as discussed above, inhibitors of MEK do inhibit TPA-stimulated eIF4F assembly and protein synthesis (Figs. 6 and 7) and this could be through the inhibition of eIF4E phosphorylation. It was also possible that the observed inhibition of both basal and insulin-stimulated protein synthesis by the MEK inhibitors was a consequence of the inhibition of basal eIF4E phosphorylation. eIF4E is indeed phosphorylated (albeit at a low level) in serum-starved HEK 293 cells (Fig. 8, *a* and *b*) and in other cell types (6, 13). However, PD098059 did not affect the phosphorylation state of eIF4E in untreated cells (Fig. 8*b*) indicating that inhibition by PD098059 of basal rates of translation (Fig. 6) is not due to decreased eIF4E phosphorylation but rather to the inhibition of other signals emanating from MEK. However, the inhibition by PD098059 and U0126 of TPA-stimulated eIF4F assembly and protein synthesis observed in Figs. 6 and 7 might be related to inhibition of TPA-stimulated eIF4E phosphorylation (Fig. 8*a*). To explore this possibility, HEK 293 cells were transiently transfected with constructs expressing a constitutively active form of Mnk1 (Mnk1 T322D) (15). Controls were also performed in which cells were transfected with a plasmid expressing green fluorescent protein (GFP). Transfection efficiencies of over 90% were routinely obtained. Transient transfection of HEK 293 cells with Mnk1 T332D resulted in over 95% phosphorylation of eIF4E in serum-starved, insulin- or TPA-treated cells (Fig. 8*b*). As expected, in these cells, the phosphorylation state of eIF4E was unaffected by the addition of PD098059. However, in the cells expressing Mnk1 T322D, PD098059 still inhibited TPA-stimulated eIF4F assembly (Fig. 8*c*) and protein synthesis (Fig. 8*d*) as well as the basal rate of protein synthesis (Fig. 8*d*), effects similar to those seen in non-transfected cells (Fig. 6) (no difference in the effect of the inhibitor or activators were seen on eIF4F assembly, between the Mnk1 T332D transfected cells and controls, *i.e.* untransfected or GFP transfected cells, data not shown). These results indicate that the inhibition of TPA stimulated eIF4F assembly and protein synthesis caused by PD098059 is not primarily due to inhibition of eIF4E phosphorylation but through some other mechanism, presumably through another target of MEK-dependent signaling. Interestingly, markedly enhancing the phosphorylation of eIF4E (to 95%) by expressing constitutively active Mnk1 did not increase basal or insulin-/TPA-stimulated eIF4F assembly or protein synthesis (Fig. 8, *c* and *d*). Thus, elevated levels of eIF4E phosphorylation are themselves not sufficient to enhance either eIF4F assembly or protein synthesis.

DISCUSSION

In this report, we demonstrate that treatment of HEK 293 cells with TPA or insulin leads to increases in both eIF4F assembly and protein synthesis. TPA or insulin treatment also led to the phosphorylation of 4E-BP1 and p70 S6 kinase, two proteins that are important in the regulation of protein synthesis. However, the signaling pathways by which the stimuli elicit these effects are distinct as TPA stimulation occurs via a mechanism which does not involve the activation of PI 3-kinase or PKB whereas insulin stimulation requires PI 3-kinase and may involve the activation of PKB. Stimulation of eIF4F assembly, protein synthesis, and the phosphorylation of 4E-BP1 and p70 S6 kinase by both agents is inhibited by rapamycin. TPA activation of MEK appears to be important in increases in protein synthesis, eIF4F assembly, and the phosphorylation of p70 S6 kinase and 4E-BP1. The data also suggest that MEK plays an important role in basal and insulin-stimulated protein synthesis. Insulin does not stimulate eIF4E phosphorylation but nevertheless increases eIF4F assembly and protein synthesis. These results indicate that increased eIF4E phosphorylation is not required for eIF4F assembly or the activation of general protein synthesis. It should be noted that insulin at the concentrations used in this report (10^{-7} M), can activate insulin receptors and those of insulin-like growth factor-1 (55).

Phorbol esters are believed to mediate their effects through the activation of the diacylglycerol-dependent conventional PKCs (cPKCs; α, β_1, β_2, and γ) and the novel PKCs (nPKCs; δ, ϵ, η, θ, and μ) (37) and can activate PI 3-kinase in a number of cell types, including Swiss 3T3 cells (56), 3T3-L1 adipocytes (57, 58), and JB6 cells (59). Interestingly, however, in this report, we do not detect any activation of PI 3-kinase or of a downstream effector, PKB. The role of PKB in the activation of p70 S6 kinase and the phosphorylation of 4E-BP1 has been the subject of several studies. Evidence has mainly relied on the overexpression of PKB or mutants of PKB within cells (27, 36, 42, 44, 60). Expression of constitutively active mutants of PKB results in the activation of p70 S6 kinase and the phosphorylation of 4E-BP1 (44). However, recent evidence suggests that PKB may not play a critical role in p70 S6 kinase activation, but does play an important role in the phosphorylation of 4E-BP1 (46). Here we provide evidence that the activation of PKB is not an essential requirement for the phosphorylation of either p70 S6 kinase or 4E-BP1, at least upon stimulation with phorbol ester. Therefore, TPA stimulates the phosphorylation of p70 S6 kinase and 4E-BP1, eIF4F assembly, and protein synthesis through a mechanism which is independent of PI 3-kinase and PKB, presumably involving the activation of one

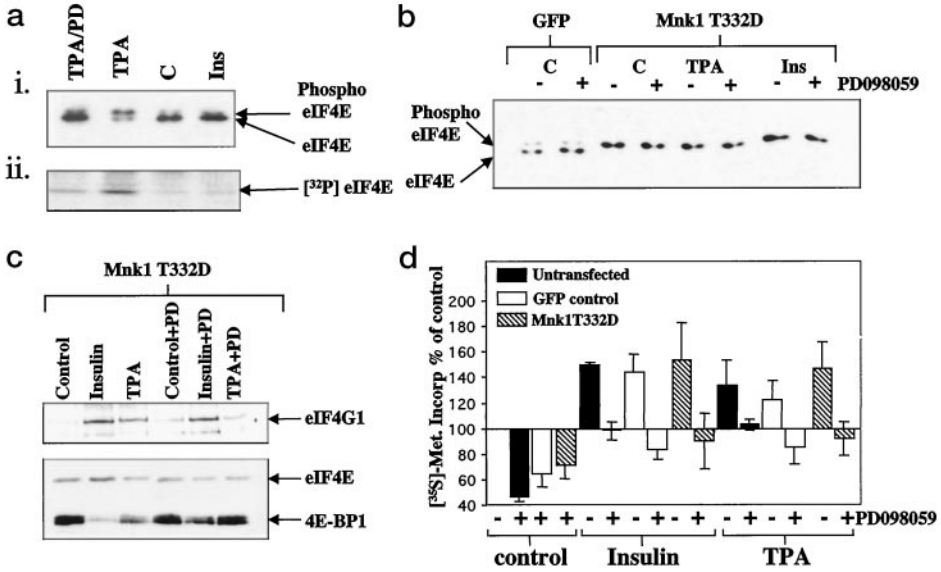

FIG. 8. **Role of eIF4E phosphorylation in eIF4F assembly and translation.** *a,* HEK 293 cells were serum starved for 16 h (for *in vitro* labeling cells were additionally starved of phosphate). Where indicated the cells were pretreated with 50 μM PD098059 for 45 min prior to the addition of 100 nM insulin or 1 μM TPA. (i) Proteins isolated by m^{7}GTP-Sepharose pull-down were separated on an isoelectric focusing gel followed by Western blot analysis using anti-eIF4E antiserum. Representative blot from three experiments. (ii) ^{32}P *in vivo* labeled proteins were isolated by m^{7}GTP-Sepharose pull-down and then separated by SDS-polyacrylamide gel. Radiolabeled eIF4E was visualized by autoradiography. *b-d,* HEK 293 cells were either transiently transfected with constructs expressing constitutively active Mnk1 T332D or GFP for 24 h before being serum starved for 16 h. Where indicated the cells were pretreated with 50 μM PD098059 for 45 min prior to the addition of 100 nM insulin or 1 μM TPA for an additional 45 min. *b,* analysis of proteins isolated by m^{7}GTP-Sepharose pull-down separated on an isoelectric focusing gel followed by Western blot analysis using anti-eIF4E antiserum (*GFP*, serum starved cells transfected with GFP). This experiment was repeated three times. *c,* analysis of proteins from Mnk1 T332D-transfected cells isolated by m^{7}GTP-Sepharose pull-down separated on SDS-polyacrylamide gel followed by Western blotting using antisera against eIF4E, eIF4G1, and 4E-BP1. (*Control,* untreated cells). Representative results from three separate experiments. *d,* incorporation of [^{35}S]methionine into protein expressed as percentage of control (n = 3: ±S.E.). In all cases, differences in the rates of protein synthesis between control cells and cells transfected with Mnk1 T332D proved statistically insignificant as judged using Student's t test (p = 0.05).

or more members of the cPKC or nPKC families. Consistent with our data, treatment of interleukin 2-responsive lymphoid cells with phorbol ester also leads to the activation of p70 S6 kinase via a rapamycin-sensitive, wortmannin-insensitive pathway (61). These data imply the existence of an alternative pathway leading to the activation of p70 S6 kinase. However, the links between PKC and the regulation of p70 S6 kinase and 4E-BP1 remain to be established.

It has been demonstrated that both eIF4F assembly and protein synthesis can be inhibited by rapamycin probably through inhibition of the phosphorylation of 4E-BP1 (18–20). Rapamycin inhibited both insulin- and TPA-induced 4E-BP1 phosphorylation and also inhibited eIF4F assembly, evidence of the important role 4E-BP1 phosphorylation plays in regulating both TPA and insulin-stimulated eIF4F assembly and translation. These data also indicate that TPA and insulin share commonality in signaling through mTOR and that therefore the signaling pathways activated by these stimuli converge, even though their upstream signaling events are distinct.

Activation of Erk in response to phorbol esters is believed to be mediated by PKC through the activation of Raf (c-Raf1), an upstream activator of MEK (62). The activation of Erk by TPA and its inhibition by inhibitors of MEK correlated well with changes in protein synthesis, eIF4F assembly, p70 S6 kinase, and 4E-BP1 phosphorylation, evidence that signaling through Erk may provide an important input into these events. Indeed, the MEK inhibitors had a much greater inhibitory effect on TPA- than insulin-induced eIF4F assembly and the phosphorylation of p70 S6 kinase and 4E-BP1. However, the role of Erk in the stimulation of translation by TPA is difficult to assess given the strong inhibitory effect of PD098059 on protein synthesis in untreated cells.

In many cell types previously studied insulin treatment results in the activation of Erk. However, in HEK 293 cells, Erk is not activated by insulin and therefore Erk activation cannot play a role in insulin-stimulated protein synthesis or eIF4F assembly. Surprisingly, pretreatment of cells with inhibitors of MEK, PD098059, and the structurally unrelated compound U0126, resulted in an inhibition of insulin-stimulated protein synthesis as well as basal protein synthesis. This implies that a basal input from MEK or a kinase downstream of MEK is required for general protein synthesis. However, this is not due to the inhibition of the basal level of eIF4E phosphorylation as PD098059 had no effect on basal eIF4E phosphorylation and inhibition by PD098059 was not relieved by the overexpression of constitutively active Mnk1. The insulin induced phosphorylation of 4E-BP1 and p70 S6 kinase was also partially inhibited by the MEK inhibitors. The inhibition by PD098059 of insulin-stimulated p70 S6 kinase and 4E-BP1 phosphorylation has previously been reported in Chinese hamster ovary cells (50) but in these cells insulin does activate Erk. However, those authors show that there was not a clear correlation between inhibition of Erk activation by PD098059 and the inhibition of 4E-BP1 and p70 S6 kinase phosphorylation. MAP kinase-like kinase(s) may play an important role in the phosphorylation of p70 S6 kinase as a number of proline-rich phosphorylation sites have been identified within the C-terminal pseudosubstrate domain of p70 S6 kinase (48). It has also been suggested that Erk/Erk-activated kinases may be involved in the phosphorylation of 4E-BP1 (17, 49, 50). It is therefore possible that a low basal activity of MEK is required for both protein synthesis and the basal phosphorylation of p70 S6 kinase and 4E-BP1 which accounts for their sensitivity to PD098059.

It has been suggested that eIF4E phosphorylation may also play an important role in eIF4F assembly and general protein

synthesis (8, 9). Since insulin treatment of HEK293 cells results in no detectable phosphorylation of Erk (an upstream kinase of Mnk1; an eIF4E kinase) or increase in eIF4E phosphorylation, stimulation of eIF4E phosphorylation is clearly not a critical requirement for eIF4F assembly and the activation of general protein synthesis in response to insulin. However, eIF4E phosphorylation may play other roles in the upregulation of the translation of specific transcripts with highly structured 5′-untranslated regions (63, 64). Interestingly, increased phosphorylation of eIF4E by Mnk1 T332D did not affect basal, insulin-, or TPA-stimulated eIF4F assembly or rates of general protein synthesis. This indicates that eIF4E phosphorylation is not sufficient to increase eIF4F assembly or general protein synthesis.

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Distinct Signalling Pathways Mediate Insulin and Phorbol Ester-stimulated Eukaryotic Initiation Factor 4F Assembly and Protein Synthesis in HEK 293 Cells

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