Involvement of Stress-activated Protein Kinase and p38/RK Mitogen-activated Protein Kinase Signaling Pathways in the Enhanced Phosphorylation of Initiation Factor 4E in NIH 3T3 Cells*

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The initiation factor (eIF) 4E is regulated by modulating both the phosphorylation and the availability of the protein to participate in the initiation process. Here we show that either serum treatment or activation of the stress-activated protein kinase (JNK/SAPK) led to enhanced phosphorylation of eIF4E in quiescent NIH 3T3 cells. Although the immunosuppressant, rapamycin, was found to stabilize the association of eIF4E with its negative regulator, 4E-BP1, this drug did not prevent the early effects of serum stimulation on the overall rate of translation, polyribosome formation, the phosphorylation status of eIF4E, or the recruitment of eIF4E into the eIF4F complex. However, the rapid enhancement of eIF4E phosphorylation in response to serum was largely prevented by the inhibitor of mitogen-activated protein (MAP) kinase activation, PD98059. Activation of the JNK/SAPK signaling pathway with anisomycin resulted in enhanced phosphorylation of eIF4E, which was prevented by either rapamycin or the highly specific p38 MAP kinase inhibitor, SB203580. These data illustrate that multiple signaling pathways, including those of distinct members of the MAP kinase family, mediate the phosphorylation of eIF4E and that the association of eIF4E with 4E-BP1 does not necessarily prevent phosphorylation of eIF4E in vivo.

Control of polypeptide synthesis plays an important role in cell proliferation, with physiological regulation of protein synthesis almost always exerted at the level of polypeptide chain initiation (reviewed in Refs. 1 and 2). This phase is regulated, in part, by the phosphorylation of initiation factors involved in binding mRNA to the 40 S ribosomal subunit, a step which appears to be rate-limiting in many cell systems (1–6). The cap structure present at the 5′ end of mRNA facilitates its binding to the ribosome, a process mediated by at least three initiation factors (eIF4A,1–4B, and -4F) and ATP hydrolysis (1–4, 7, 8). eIF4F is a cap-binding protein complex composed of three subunits; eIF4E, which specifically recognizes the cap structure (9); eIF4A, an ATP-dependent, single strand RNA-binding protein with helicase activity (4, 8); and eIF4G, which acts as a bridging molecule between eIF4E and the 40 S ribosome, probably via eIF3 (10, 11). It is believed that eIF4F functions to unwind secondary structure in the mRNA 5′-untranslated region to facilitate binding to the 40 S ribosomal subunit (1–6).

Consistent with its proposed regulatory role, eIF4E exists in both phosphorylated and non-phosphorylated forms. Although increased levels of eIF4E phosphorylation have been directly correlated with enhanced rates of translation in a variety of cell types (reviewed in Ref. 1), it is still not clear how phosphorylation of eIF4E modulates its activity. While there may be a direct effect of phosphorylation on cap structure recognition in vitro (12), phosphorylation of eIF4E in vivo can also be correlated with enhanced interaction with other components of the eIF4F complex (13, 14).

Two additional proteins (4E-BP1 and 4E-BP2), which interact with eIF4E and inhibit cap structure-dependent translation, have been identified as downstream signaling targets (15, 16). Phosphorylation of 4E-BP1 disrupts its interaction with eIF4E, liberating eIF4E to interact with a conserved hydrophobic region of eIF4G. A similar sequence found in 4E-BP1 is involved in binding to eIF4E and competes with eIF4G for eIF4E binding (17). It is believed that the phosphorylation of 4E-BP1 and consequent liberation of eIF4E lead to the up-regulation of translation (1, 15). It has also been claimed that association with 4E-BP1 prevents the phosphorylation of eIF4E by protein kinase C in vitro (18). In several cell types, the phosphorylation of 4E-BP1 is inhibited by the immunosuppressant, rapamycin, which prevents the activation of the p70src signaling pathway and stabilizes the interaction between eIF4E and 4E-BP1 (see Refs. 1, 4, and 16, and references therein). However, rapamycin does not prevent the phosphorylation of eIF4E in primary pig T cells (19), Xenopus oocytes (20), CHO.T cells in response to insulin (21), or NIH 3T3 cells in response to serum (22).

We have examined the signal transduction pathways that are involved in the enhanced phosphorylation of eIF4E and its recruitment to ribosomes. Our data indicate that, in NIH 3T3 cells, eIF4E phosphorylation is enhanced in response to either serum or to activation of the JNK/SAPK and p38/RK signaling pathways with anisomycin. In response to serum stimulation, eIF4E phosphorylation is largely mediated via the classical MAP kinase pathway, and is independent of both p70src signaling and association of eIF4E with 4E-BP1. However, following anisomycin treatment, phosphorylation of eIF4E via JNK/SAPK is dependent upon p70src and the p38/RK signaling pathways. These data suggest that the phosphorylation of eIF4E and 4E-BP1 can be regulated independently and that each may play a direct role in regulating translational initiation in vivo.

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1 The abbreviations used are: eIF, eukaryotic initiation factor; m7GTP, 7-methyl guanosine triphosphate; PMA, phorbol 12-myristate 13-acetate; Mops, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; VSIEF, vertical-slab isoelectric focusing; GST, glutathione-S-transferase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; FCS, fetal calf serum.

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EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—Materials for tissue culture were from Life Technologies, Inc. [35S] methionine was from ICN, Immobilon polyvinylidene difluoride was from Millipore, and mGTP-Sepharose was from Pharmacia Biotech Inc. Microcystin was from Calbiochem; unless otherwise stated, all other chemicals were from Sigma. Antisera to 4E-BP1 (THAS-I) was kindly provided by Dr. J. Lawrence, Jr. (Washington University School of Medicine, St. Louis, MO) and Prof. R. Denton (Department of Biochemistry, Bristol, UK) and the plasmid encoding the glutathione-S-transferase fusion protein GST-T-c-Jun(1–79) was provided by Dr. M. Karrin (University of California at San Diego, San Diego, CA). Rapamycin was a kind gift from Dr. J. Kay (Department of Biochemistry, Sussex, UK). P098059 was from Parke-Davis, RO31-8220 was from the Roche Research Center, UK, and SB203580 was a gift from SmithKline Beecham (King of Prussia, PA).

Tissue Culture—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium with Glutamax™ supplemented with 10% fetal calf serum (FCS). Prior to treatment with rapamycin, RO31-8220, PD98059, or SB203580, cells were grown to 80% confluence and then serum-starved in 0.5% FCS for 48 h. Treatments with agonists/inhibitors were as described in individual figure legends. Cells were then washed and extracted as described below.

Preparation of Cell Extracts—Following treatment, the medium was removed and plates of cells were transferred to ice. Cells were scraped into 0.5 ml of Buffer A (50 mM Mops-KOH, pH 7.4, 2.5 mM EGTA, 1 mM EDTA, 40 mM β-glycerophosphate, 1 μM microcystin, 120 mM NaCl, 7 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4), incubated by centrifugation, and washed in 0.5 ml of the same buffer. Cells were resuspended in 0.1 ml of Buffer A/10-cm plate and lysed by the addition of 0.5% (v/v) Nonidet P-40, 0.5% (v/v) deoxycholate, and 0.1% (v/v) Triton X-100 and vortexing. Cell debris was removed by centrifugation in a microcentrifuge for 5 min at 4 °C, and the resultant supernatant was frozen in liquid N2.

Measurement of Protein Synthesis—Cells were grown in six-well plates and starved, as described above, prior to activation in the presence of 25 μCi/ml [35S]methionine, for the times described in the figure legends. The medium was removed and cells washed in Buffer B (20 mM Tris-HCl, pH 7.4, 0.134 mM NaCl, 1 μM microcystin, 2 mM benzamidine) containing 5 mM unlabeled methionine, prior to lysis with 0.3 M NaOH. Incorporation of radioactivity into protein was determined by precipitation with trichloroacetic acid.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Vertical Slab Iso-electric Focusing (VSIEF), and Immunoblotting—One-dimensional polyacrylamide gels and vertical slab iso-electric focusing gels were run as described (19), and proteins transferred to polyvinylidene difluoride membrane. eIF4E, eIF4G, and 4E-BP1 were detected with specific rabbit anti-peptide antisera as described previously (20) and in the individual figure legends.

mGTP-Sepharose Chromatography—For the isolation of eIF4E and associated proteins, cell extracts of equal protein concentration were subjected to mGTP-Sepharose chromatography as described (19, 20). The beads were washed three times in Buffer A and bound protein eluted with either SDS-PAGE or VSIEF sample buffer, as indicated.

Immunoprecipitation of eIF4G—Immunoprecipitation of eIF4G and associated eIF4E from cell extracts of equal protein concentration was as described previously (20).

JNK/SAPK Kinase Assays—Solid-state protein kinase assays using the GST-c-Jun(1–79) fusion protein were performed as described (23). Briefly, samples of cell extracts (40 μg) were mixed with 10 μg of GST-c-Jun(1–79) prebound to GSH-Sepharose. After 20 min at 30 °C, the beads were washed twice in kinase assay buffer (50 mM Mops-KOH, pH 7.4, 20 mM MgCl2, 40 mM β-glycerophosphate, 1 μM microcystin, 7 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM phenethylmethylsulfonyl fluoride, 2 mM Na3VO4), and resuspended in 20 μl of the same buffer containing 100 μM [γ-32P]ATP (1500 cpm/pmol). After 20 min at 30 °C, reactions were terminated with SDS-PAGE sample buffer, followed by analysis by SDS-PAGE and autoradiography.

RESULTS AND DISCUSSION

Enhanced eIF4E Complex Formation in Vivo Can Occur Independently of Enhanced 4E-BP1 Phosphorylation—eIF4E plays a central role in the regulation of translation, with a strong correlation between the phosphorylation of eIF4E, eIF4F complex formation, and the rate of protein synthesis and cell growth (reviewed in Ref. 1). However, the mechanism by which phosphorylation of eIF4E at Ser-209 (25, 26) enhances its activity is not understood. In addition to phosphorylation, the activity of eIF4E can also be modulated by its availability to participate in the initiation process, mediated by its interaction with specific binding proteins, 4E-BP1 and 4E-BP2 (4, 15).

These studies have been facilitated by the use of rapamycin, an inhibitor of the p70S6K signaling pathway, which blocks cell cycle progression (27), prevents the phosphorylation of 4E-BP1 in response to growth factors (1, 4, 16, 28), and inhibits cap structure-dependent initiation of translation by the subsequent inactivation of eIF4E (4, 15, 22). However, this may be a simplistic view, as studies by Beretta et al. (22) have shown a lack of temporal correlation between the inhibition of phosphorylation of 4E-BP1 and the inhibition of translation. In addition, stabilization of the eIF4E/4E-BP1 complex did not prevent the serum-stimulated phosphorylation of eIF4E in vivo.

To examine this further, we have analyzed the correlation between the phosphorylation of 4E-BP1, polysome formation, eIF4F complex formation, and the activation of protein synthesis in NIH 3T3 cells. At 4 h following serum stimulation (Fig. 1A), rapamycin had only a small inhibitory effect (20–25%) on the rate of total protein synthesis, under conditions where the activation of p70S6K was completely prevented (data not shown). This inhibition was further increased to 30–35% by 20 h. At early times after serum stimulation, rapamycin had little effect on polysome formation (Fig. 1B), even if the cells had been preincubated with rapamycin prior to activation; further incubation in the presence of rapamycin resulted in a 25–30% decrease in polysome formation (data not shown). We have also examined the phosphorylation of 4E-BP1 and its association with eIF4E. Multiple forms of 4E-BP1 can be distinguished on SDS-PAGE gels (Fig. 1C), with the γ form identified as more highly phosphorylated than the α and β forms (28). Serum stimulation resulted in the characteristic mobility shift from the predominant α and β forms to the γ form (lane 2 versus lane 1), an effect that was largely prevented by the co-addition of rapamycin (lane 3). Isolation of eIF4E from the unstimulated cells by mGTP-Sepharose (lane 4) indicated, as expected (1, 4) that the factor was mainly associated with the less phosphorylated (α and β) forms of 4E-BP1. As predicted from the current models, enhanced phosphorylation of 4E-BP1 was concomitant with its release from eIF4E (lane 5 versus lane 4), an effect largely prevented by rapamycin (lane 6). Preincubation of cells with rapamycin for 30 min prior to activation caused a greater accumulation of the α form of 4E-BP1, but did not increase the level of 4E-BP1 associated with eIF4E above that presented in lane 6.2 To determine the effect of rapamycin on eIF4F complex formation, extracts were prepared following serum stimulation for 4 h in the absence or presence of rapamycin prior to immunoprecipitation of eIF4G. The level of associated eIF4E was then assessed by SDS-PAGE and immunoblotting; the results of quantification of these data are presented in the figure legend. As shown in Fig. 1D, serum treatment enhanced the recovery of eIF4E associated with eIF4G by 2.5-fold (lane 1 versus lane 3 and figure legend), indicative of a stimulation of eIF4F complex assembly. Similar observations have been reported for other cell types following activation of protein synthesis (reviewed in Ref. 1). Although rapamycin prevented the phosphorylation of 4E-BP1 and stabilized its association with eIF4E (panel C, lane 4 versus lane 6), the recovery of eIF4E associated with eIF4G was enhanced by 2.3-fold. These data suggest that rapamycin did not prevent the assembly of the eIF4F complex at 4 h following serum stimulation, suggesting that enhanced eIF4F complex formation in

2 S. J. Morley, unpublished data.
FIG. 1. Rapamycin does not affect eIF4F complex formation in serum-stimulated NIH 3T3 cells. Panel A, NIH 3T3 cells were serum-starved for 48 h prior to the addition of either 50 nM rapamycin (+rapa) or vehicle alone (−rapa), 25 μCi/ml [35S] methionine, and serum (10%). Cells were harvested at the times indicated, and the incorporation of radioactive methionine into trichloroacetic acid-precipitable material was determined. The right-hand panel shows the effect of rapamycin expressed as the percentage of the control. These data are representative of those obtained in three separate experiments, and the error bars indicate the standard deviation from the mean. 

Panel B, serum-starved cells were incubated for 4 h in the absence or presence of FCS (10%), and in the absence or presence of 50 nM rapamycin, as indicated. Extracts were prepared in the presence of detergents, and equal amounts of protein were fractionated on sucrose density gradients, as described under “Experimental Procedures.” Sedimentation was from left to right, and an arrow indicates the sedimentation of the 40S ribosome. 

Panel C, aliquots of extracts (40 μg) prepared as in panel B were either analyzed directly or subjected to m7GTP-Sepharose to isolate eIF4E and associated proteins, prior to SDS-PAGE and immunoblotting with antiserum specific to 4E-BP1 (a kind gift from J. Lawrence). The α-form of 4E-BP1 is the least phosphorylated and the γ-form is the most highly phosphorylated form of 4E-BP1. These data are representative of those obtained in three separate experiments. Panel D, serum-starved cells were incubated in the absence or presence of 50 nM rapamycin or FCS (10%) for 4 h prior to preparation of extracts. Aliquots containing equal protein (50 μg) were then subjected to immunoprecipitation with anti-eIF4G antiserum as described under “Experimental Procedures.” Isolated proteins were resolved by SDS-PAGE, and the recovery of eIF4G (upper panel) and associated eIF4E (lower panel) was determined by immunoblotting and quantified by densitometric scanning. When expressed as the amount of eIF4G/eIF4E for each variable, they yielded the following: lane 1, 0.3 ± 0.1 (S.D., n = 3); lane 2, 0.4 ± 0.1 (S.D., n = 3); lane 3, 1.1 ± 0.2 (S.D., n = 3); lane 4, 0.9 ± 0.1 (S.D., n = 3).
These findings are not consistent with the in vitro studies of Haghighat et al. (17), who reported that the interaction of 4E-BP1 with eIF4E prevents the interaction of eIF4E with eIF4G. Possible explanations for this are that either 4E-BP1 is not present in excess of eIF4E in these cells or that there is a population of eIF4E that is inaccessible to 4E-BP1 and is sufficient to support enhanced protein synthesis at early times of activation. Indeed, even prolonged incubation with rapamycin for 20 h resulted in only a 30–40% decrease in the amount of eIF4E associated with eIF4G in this system (data not shown).

As suggested by Beretta et al. (22), these data may reflect a slow rate of exchange of eIF4E through the eIF4F complex, such that eIF4E is only able to interact with 4E-BP1 after it is released from eIF4F.

The MAP Kinase Inhibitor, PD98059, but Not Rapamycin Prevents the Serum-induced Phosphorylation of eIF4E—We have also examined the intracellular signaling pathways modulating the enhanced phosphorylation of 4E-BP1 and eIF4E, the association of 4E-BP1 with eIF4E, and eIF4F complex formation in response to serum stimulation. In addition to rapamycin, we have used the following well characterized inhibitors: PD98059, to prevent activation of the classical MAP kinase pathway (29); RO31-8220, which functions as a general inhibitor of protein kinase C (30) but also stimulates the JNK/SAPK signaling pathway (31); and SB203580, which is a specific inhibitor of the p38/RK MAP kinase (32). Fig. 2A shows that there was a moderate increase in the rate of protein synthesis over the initial 30 min following serum addition. As shown in Western blots in Fig. 2B (lane 2), enhanced translation rates could be correlated with increased phosphorylation of 4E-BP1 and the dissociation of 4E-BP1 from eIF4E. The concomitant shift of immunoreactive eIF4E to the upper form on V5IF4E indicated enhanced phosphorylation (19, 20). Quantification of these data by densitometric scanning (see figure legend) indicated that the percentage of total eIF4E in the phosphorylated form was increased from 8% in the unstimulated cells to 40% following serum stimulation. In addition, co-immunoprecipitation of eIF4E with an antiserum recognizing eIF4G, indicated a 2-fold increase in eIF4F complex formation (see figure legend for quantification). In agreement with published data, rapamycin did not suppress the effect of serum on the rate of translation (Fig. 2A, lane 4) or the phosphorylation of eIF4E (19–22), but largely prevented the phosphorylation of 4E-BP1 and stabilized its association with eIF4E (Fig. 2B, lane 4). These data suggest that the association of eIF4E with 4E-BP1 does not necessarily prevent phosphorylation of eIF4E in vivo. PD98059, which had little effect on the rate of translation when added alone (Fig. 2A, lane 8) or following serum stimulation (Fig. 2A, lane 5), prevented activation of classical ERK2 MAP kinases (Ref. 29 and data not shown) and attenuated the phosphorylation of eIF4E (Fig. 2B, lane 5 and legend). However, it did not prevent the phosphorylation of 4E-BP1 or the dissociation of this protein from eIF4E. Serum-stimulated association of eIF4E with eIF4G was also unaffected by this inhibitor. These data are consistent with published work, which shows that MAP kinase activation is not required for increased phosphorylation of 4E-BP1 (16).

In addition, as shown for CHO.T cells (21), these data indicate that signaling through the MAP kinase pathway is in part responsible for mediating the phosphorylation of eIF4E. Consistent with these findings is the observation that NIH 3T3 cells overexpressing MAP kinase kinase show an elevated basal level of eIF4E phosphorylation.2

RO31-8220 Augments the Phosphorylation of eIF4E in Response to Serum—A large number of studies have implicated protein kinase C in regulation of eIF4E phosphorylation and activity (reviewed in Ref. 1). Our data (Fig. 2B, lane 6) confirms other reports showing enhanced phosphorylation of eIF4E in many cell types following treatment with the phorbol ester, PMA. In NIH 3T3 cells, PMA caused a small but reproducible increase in protein synthesis (Fig. 2A, lane 6). PMA treatment also enhanced association of eIF4E with eIF4G by 3.3-fold (see figure legend) but did not result in release of the binding protein from association with eIF4E (Fig. 2B, lane 6). The widely used protein kinase C inhibitor, RO31-8220, was detrimental to basal rates of protein synthesis (data not shown), and severely inhibited the response to serum and PMA (Fig. 2A, lanes 3 and 7). However, treatment of cells with RO31-8220 alone increased phosphorylation of eIF4E (data not shown), and in combination with serum, augmented the enhanced phosphorylation of eIF4E (61% of total eIF4E in the phosphorylated form; see legend for details) observed with serum alone (40% of eIF4E in the phosphorylated form; Fig. 2B, lane 3 versus lane 2). This probably reflects the recent finding that RO31-8220 inhibits the expression of MAP kinase phosphatase, prolonging the activation of MAP kinase (31), a condition that could be expected to enhance the phosphorylation of eIF4E. However, since RO31-8220 is now known to activate the c-Jun N-terminal kinase (JNK/SAPK) (31), our data also suggest the possibility that this signaling pathway may have a role in the enhanced phosphorylation of eIF4E.

Activation of JNK/SAPK by Anisomycin Enhances eIF4E Phosphorylation—The three known groups of the MAP kinase family include the classical MAP kinases (ERKs), stress-activated kinases (JNK/SAPK) and p38 MAP kinase (p38/RK). They are at the center of three distinct but closely related phosphorylation cascades, which play a critical role in transducing extracellular signals into intracellular responses. The archetypal MAP kinase pathway, activated by serum, growth factors and mitogens, is stimulated in response to ras-GTP loading, activation of raf proto-oncogene, phosphorylation of mitogen-activated protein kinase kinase, which in turn phosphorylates and activates the MAP kinases, ERK1 and ERK2. In addition, this pathway is activated by G-protein signaling and phosphatidylinositol turnover (33–35). On the other hand, cells respond to cellular stress agents by induction of two structurally related but distinct pathways, JNK/SAPK and p38/RK (34). JNK/SAPK is activated by a mitogen-activated protein kinase-like kinase, SEK1 (MKK4, JNKK), while p38/RK is phosphorylated by related kinases MKK3 and MKK6, which are themselves part of an ill-defined, overlapping signaling cascade (33–35). Study of these signaling cascades is complicated by cross-talk between them, but it is clear that JNK/SAPK and p38/RK activation culminates in the activation of transcription factors, enhanced expression of the immediate-early genes c-fos and c-jun and hence in the regulation of cell growth and differentiation (33–36).

To investigate the potential role of the JNK/SAPK and p38/RK MAP kinase signaling pathways in the phosphorylation of eIF4E and its association with 4E-BP1, we have used anisomycin, which strongly activates JNK/SAPK in numerous cell types (33–37) in conjunction with serum and the inhibitors listed above. As shown in Fig. 3A, serum enhanced the amount of total eIF4E in the phosphorylated form from 10% in control cells to 47% (see legend for details), promoted the dissociation of 4E-BP1 from eIF4E, but did not greatly increase JNK/SAPK activity in NIH 3T3 cells (lane 2 versus lane 1). The apparent less complete serum-stimulated dissociation of 4E-BP1 from eIF4E than presented in Fig. 2 reflects the use of a more sensitive antiserum in this experiment (see figure legend). Serum-stimulated phosphorylation of eIF4E was insensitive to
In the presence of SB203580 (lane 4); however, treatment with SB203580 alone resulted in the complete dephosphorylation of eIF4E (lane 3; quantified in figure legend). These data suggest that either p38/RK MAP kinase is involved in the negative regulation of an eIF4E phosphatase or else it is partially activated in serum-starved cells. Anisomycin, which activated the JNK/SAPK signaling pathway (Fig. 3A, lane 5), enhanced the level of total eIF4E in the phosphorylated form to 50% and promoted the dissociation of the 4E-BP1/eIF4E complex. Similar data were obtained when levels of anisomycin insufficient to inhibit the elongation phase of translation were employed (data not shown). Interestingly, in contrast to serum, anisomycin-induced phosphorylation of eIF4E was sensitive to rapamy-
cin (lane 6) and SB203580 (lane 7), but not PD98059 (lane 9; see legend for quantification). These data indicate that more than one family of MAP kinase is involved in regulating the phosphorylation status of eIF4E in NIH 3T3 cells.

Previously, we have shown that in primary T cells and following meiotic maturation of Xenopus oocytes, the activation of protein synthesis can be correlated with enhanced recruitment of eIF4E to the ribosome (19, 20). To analyze the potential role for increased phosphorylation of eIF4E in promoting interaction of the factor with ribosomes, serum-starved cells were stimulated with serum in the absence or presence of the inhibitors described above, and the level of ribosome-associated eIF4E visualized by immunoblotting. Fig. 3B shows that, relative to the unstimulated cells (lane 1, 7.7% of eIF4E in the phosphorylated form), both serum (lane 2, 47% of eIF4E in the phosphorylated form) and PMA (lane 7, 45% of eIF4E in the phosphorylated form) enhanced the phosphorylation of total eIF4E (upper panel) and increased the recovery of eIF4E on ribosomes (lower panel). Rapamycin (lane 5) and PD98059 (lane 6) had little effect on the recruitment of eIF4E to the ribosome in response to serum stimulation, although PD98059 did prevent the enhancement of phosphorylation of eIF4E.

Further fractionation of ribosomes by sucrose density gradient analysis showed that serum treatment enhanced binding of eIF4E to the 40 S ribosomal subunit; VSV eIF4E and immunoblot analysis of this population of eIF4E indicated that, as seen with the reticulocyte lysate (38), it consisted of a population including both phosphorylated and non-phosphorylated forms (data not shown). In contrast, anisomycin treatment enhanced the level of eIF4E in the phosphorylated form to 45% without recruiting the factor to the ribosome (lane 3). Interestingly, while rapamycin prevented the anisomycin-induced phosphorylation of eIF4E (lane 4; see legend for details), it resulted in recruitment of eIF4E to the ribosome, albeit to a lower level than observed with serum (lane 2). The reasons for this are unclear at this time.

The simplest explanation for these observations would be that serum and/or anisomycin activates an eIF4E kinase via a MAP kinase signaling pathway. Anisomycin, but not serum, induced the activation of p38 MAP kinase, the activation of a phosphorylation of a peptide substrate designed after a phosphorylation site in HSP 27 (39) (data not shown). A role for MAP kinases in mediating the enhanced phosphorylation of eIF4E have been proposed during insulin (40–42), but not PD98059 (lane 3). Therefore, as described for human cells (48), our studies indicate that the phosphorylation of eIF4E and 4E-BP1 can be regulated independently and that each may play a direct role in translational initiation in vivo.

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