Translational regulation during activation of porcine peripheral blood lymphocytes: association and phosphorylation of the α and γ subunits of the initiation factor complex eIF-4F

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Mature peripheral blood lymphocytes exist in a resting state both *in vivo* and when maintained in culture, exhibiting low translation rates consistent with their non-proliferative state. Previously we have shown that activation of these quiescent cells with either phorbol ester or concanavalin A leads to a rapid increase in the rate of protein synthesis and phosphate-labelling of initiation factor eIF-4 α [Morley, Rau, Kay and Pain (1993) Eur. J. Biochem. 218, 39–48]. We now show that neither the early enhanced translation rate nor the early increased phosphate-labelling of eIF-4 α requires the activity of the 70 kDa form of ribosomal protein S6 kinase. In addition, we demonstrate that eIF-4 γ is phosphorylated in response to cell activation, an event which is correlated with phosphorylation of eIF-4 α and enhanced eIF-4F complex formation. In these studies, isoelectric focusing and immunoblot analysis of eIF-4 α indicate that phosphate-

labelling of eIF-4 α following cell activation reflects a modest increase in steady-state phosphorylation, mediated by the enhanced activity of eIF-4 α kinase(s) and inhibition of eIF-4 α phosphatase activity. In the resting cell, eIF-4 α is associated with heat- and acid-stable insulin-responsive protein (PHAS-I; 4E-BP1); following acute stimulation with phorbol ester, there is a 40 % decrease in the amount of PHAS-I associated with eIF-4 α . Incubation of anti-PHAS-I immunoprecipitates with extracts containing activated or immunoprecipitated mitogen-activated protein kinase resulted in a small increase in phosphorylation of recovered PHAS-I and a modest release of eIF-4 α from the PHAS-I–eIF-4 α complex. These data suggest a possible role for PHAS-I in the regulation of eIF-4F complex formation and the rate of translation in primary cells.

INTRODUCTION

Control of polypeptide synthesis plays an important role in cell proliferation (reviewed in [1]), with physiological regulation of protein synthesis almost always exerted at the level of polypeptide chain initiation [2,3]. This phase is regulated, in part, by the phosphorylation of initiation factors involved in binding mRNA to the 40 S ribosomal subunit [1–6], a step which appears to be rate-limiting in many cell systems [1]. The cap structure present on the 5' end of mRNA facilitates its binding to the ribosome, a process mediated by three initiation factors [eukaryotic initiation factor (eIF)-4A, -4B and -4F] and ATP hydrolysis [1–3,7,8]. eIF-4F is a cap binding protein complex composed of three subunits: eIF-4 α (eIF-4E), which specifically recognizes the cap structure [9], eIF-4 β (p46, eIF-4A), an ATP-dependent, single-strand-RNA-binding protein with helicase activity [3,7], and eIF-4 γ (p220, eIF-4G), whose function is unknown but whose integrity is required for eIF-4F complex activity [1–3]. It is believed that eIF-4F functions to unwind secondary structure in the mRNA 5' untranslated region to facilitate binding to the 40 S ribosomal subunit [1,2,7,8].

Consistent with its proposed regulatory role, eIF-4 α exists in both phosphorylated and non-phosphorylated forms [1–3,5] and is believed to be the least abundant of the initiation factors [5,9]. In response to the appropriate stimuli, increased levels of eIF-4 α

phosphorylation have been directly correlated with increased rates of translation in a variety of cell types (reviewed in [1]). More recently, it has been proposed that in adipocytes the regulated phosphorylation of an eIF-4 α -associated protein [heat- and acid-stable insulin responsive protein (PHAS-I; 4E-BP1)] plays a role in modulating the availability of eIF-4 α to enter the initiation pathway [10,11]. However, the role of the phosphorylation of eIF-4 α in this interaction is at present poorly defined and, moreover, it is not known whether such interactions occur in other cell types.

Mature porcine peripheral blood lymphocytes (PBLs) exist in a resting state both *in vivo* and when maintained in culture, with low translation rates consistent with their non-proliferative state [12,13]. Previously we have shown that, in response to phorbol ester or concanavalin A (Con A), there is an increase in the phosphate-labelling of eIF-4 α , which occurs concomitantly with quantitative and qualitative changes in translation [14]. Utilizing cell-free extracts, we now show that this, in part, reflects both a modest increase in eIF-4 α kinase(s) activity and an inhibition of eIF-4 α phosphatase activity. In resting PBLs, eIF-4 α is associated with PHAS-I (4E-BP1); in agreement with findings in adipocytes [10,11], we show that stimulation of PBLs with phorbol ester results in activation of mitogen-activated protein kinase (MAP kinase), phosphorylation of PHAS-I and a concomitant 40 % decrease in the amount of PHAS-I associated with eIF-4 α . We

Abbreviations used: eIF, eukaryotic initiation factor; PHAS-I, insulin-responsive protein, heat- and acid-stable; PBLs, mature porcine peripheral blood lymphocytes; Con A, concanavalin A; MAP kinase, mitogen-activated protein kinase; PVDF, polyvinylidene difluoride; m⁷GTP, 7-methyl-GTP; pNPP, p-nitrophenyl phosphate; VSlEF, vertical slab isoelectric focusing; p90rsk, the 90–92 kDa form of ribosomal S6 kinase; p70s6k, the 70 kDa form of ribosomal protein S6 kinase; PMA, phorbol 12-myristate 13-acetate.

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also show that eIF-4 γ is phosphorylated in response to cell activation, an event correlated with phosphorylation of eIF-4 α and enhanced eIF-4F complex formation. The potential role of these events in the activation of protein synthesis in peripheral blood lymphocytes is discussed.

EXPERIMENTAL

Materials

All materials were from Sigma with the following exceptions: [35 S]methionine and [32 P] P_i were from ICN, Immobilon [polyvinylidene difluoride (PVDF)] was from Millipore and 7-methyl-GTP (m 7 GTP)–Sephacrose was from Pharmacia-LKB.

Tissue culture

PBLs were isolated from defibrinated pig blood and cultured as described previously [14]. The human leukaemic T cell line JURKAT was cultured in RPMI 1640 (GIBCO) supplemented with 10% (v/v) heat-inactivated foetal calf serum and 2 mM glutamine and maintained at 37 °C in 5% CO $_2$.

Labelling of PBLs with [35 S]methionine or [32 P]phosphate

To assess rates of protein synthesis, cell cultures (1×10^7 cells in 5 ml) were incubated for the times indicated, in the absence or presence of mitogen in complete Eagle's basal medium (containing 100 μ M unlabelled methionine), with [35 S]methionine added at a final isotopic concentration of 18 μ Ci/ml. Aliquots ($3 \times 500 \mu$ l) were removed, and cells were isolated by centrifugation and washed in Buffer A (20 mM Tris/HCl, pH 7.4, 0.134 M NaCl) containing 5 mM unlabelled methionine, with incorporation of radioactivity into protein determined by precipitation with trichloroacetic acid.

To investigate the phosphate-labelling of eIF-4 α and eIF-4 γ , PBLs were isolated as described and incubated in phosphate-free Eagle's basal medium supplemented with 5% (v/v) dialysed foetal calf serum and 2 mM glutamine, in the presence of a final isotopic concentration of [32 P] P_i as indicated in the individual figure legends. Following incubation for 18 h at 37 °C, cells were removed from the labelling medium by centrifugation and resuspended in fresh medium containing phosphate, but in the absence of radioactive label. Cells (6×10^7 cells in 30 ml aliquots) were then removed at the times indicated, harvested, washed in Buffer B [50 mM Mops/KOH, pH 7.2, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 5 mM EGTA, 2 mM benzamidine, 40 μ g/ml leupeptin, 0.5 mM PMSF, 80 mM β -glycerophosphate, 50 mM NaF, 10 mM *p*-nitrophenyl phosphate (*p*NPP), 0.1 mM GTP], resuspended in 250 μ l of Buffer B, adjusted to 0.5% (v/v) Nonidet P-40, and vortexed to effect lysis. Cell debris was removed by centrifugation and cell extracts were frozen at –70 °C.

SDS/PAGE and immunoblotting

One-dimensional polyacrylamide gels were run as described [14], and proteins were either stained with Coomassie Blue and radioactivity visualized by autoradiography, or transferred to a PVDF membrane. eIF-4 α and eIF-4 γ were detected with specific rabbit anti-peptide antisera, raised against the following sequences: eIF-4 α , WALWFFKNDKSKTWQANL; eIF-4 γ , KKEAVGDLDDAFKEVN. Prior to use, antisera were affinity-purified using the respective peptide coupled to Affi-Gel 10 (Bio-Rad), according to the manufacturer's instructions. The 90–92 kDa form of ribosomal S6 kinase (p90rsk) and MAP kinase

were decorated with a specific anti-peptide antiserum kindly provided by Dr. J. Blenis, and anti-PHAS-I antiserum was provided by Dr. J. Lawrence. Immunoblots were visualized with goat anti-rabbit alkaline phosphatase-coupled second antibody (Sigma).

Vertical slab isoelectric focusing (VSIEF) of eIF-4 α

The isoelectric focusing method used was essentially as described [15], with the following modifications: the sample buffer contained 1 μ M Microcystin (Calbiochem) in addition to the components listed; the anode buffer (upper tank) was 0.01 M glutamic acid and the cathode buffer (lower tank) was 0.05 M histidine. Following transfer of proteins to PVDF, eIF-4 α was visualized by immunoblotting.

m 7 GTP–Sephacrose chromatography and partial purification of eIF-4F

For the isolation of eIF-4 α , cell extracts of equal protein concentration were diluted to 300 μ l with Buffer C (50 mM Mops/KOH, pH 7.2, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 80 mM β -glycerophosphate, 125 mM NaCl, 1 mM benzamidine, 7 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.1 mM GTP) prior to the addition of 25 μ l of a 50% (v/v) slurry of m 7 GTP–Sephacrose equilibrated in Buffer C. Samples were shaken for 10 min at 4 °C and the resin was isolated by centrifugation in a Microfuge. The beads were washed three times in Buffer C and bound protein was eluted with either SDS/PAGE or VSIEF sample buffer, as indicated. For the isolation of the eIF-4F complex, m 7 GTP–Sephacrose columns (0.5 ml) equilibrated in Buffer C were employed as described previously [4]. To deplete *Xenopus* oocyte and egg extracts of eIF-4 α [28], extracts (60 μ l) were incubated with 7.5 μ l of dry m 7 GTP–Sephacrose resin for 10 min on ice, the resin isolated by centrifugation in a Microfuge and the supernatant retained. The resulting supernatant depleted of endogenous PHAS-I/eIF-4 α was used in the experiments described below.

Immunoprecipitation of eIF-4 α , eIF-4 γ , the 70 kDa form of ribosomal S6 kinase (p70s6k), p90rsk, MAP kinase and PHAS-I

Cell extracts of equal protein concentration were diluted to a final volume of 200 μ l with Buffer D [50 mM Tris/HCl, pH 8, 120 mM NaCl, 40 mM NaF, 30 mM *p*NPP, 1% (v/v) Nonidet P-40 and 1% (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM PMSF] and incubated with antiserum specific for eIF-4 α , eIF-4 γ , p70s6k, p90rsk, MAP kinase or PHAS-I, as indicated, for 1.5 h on ice. Immunoglobulins were precipitated and recovered with Protein A–Sephacrose (Pharmacia) and precipitates were washed three times with Buffer D. For PHAS-I, bound proteins were eluted with SDS/PAGE sample buffer. To assay for ribosomal protein S6 kinase, immunoprecipitates were washed once in assay dilution buffer (50 mM Mops/KOH, pH 7.2, 1 mM dithiothreitol, 10 mM MgCl $_2$, 0.2% (v/v) Triton X-100, 5 mM *p*NPP, 2 mM benzamidine) and resuspended in 5 μ l of assay dilution buffer, and samples were assayed for their ability to phosphorylate 40 S ribosomal subunits as described [16].

Calmodulin–Sephacrose chromatography

Cell extracts of equal protein concentration were adjusted to 2 mM CaCl $_2$ prior to passage through a 1 ml calmodulin–Sephacrose column (Pharmacia) equilibrated in Buffer E (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 14 mM 2-mercaptoethanol, 0.2 mM EDTA, 2 mM benzamidine, 0.5 mM PMSF, 2 mM

CaCl₂). Following an extensive wash, bound protein was eluted with Buffer E supplemented with 2 mM EDTA and 4 mM EGTA. Protein was precipitated and analysed for the presence of eIF-4 proteins by immunoblotting as described.

RESULTS AND DISCUSSION

Early stimulation of protein synthesis rates in PBLs does not require p70s6k activity

Previously we have shown that activation of quiescent PBLs with phorbol ester [phorbol 12-myristate 13-acetate (PMA)] or Con A leads to a 2–4-fold increase in the rate of protein synthesis within 1 and 4 h respectively. This increase is under translational control and occurs in parallel with enhanced phosphate-labelling of eIF-4 α [14]. The role of phosphorylation in modulating the activity of eIF-4 α or its assembly into the eIF-4F cap binding complex is unclear. In an attempt to resolve this and to determine whether increased phosphorylation of eIF-4 α reflects events occurring at the level of kinase(s) or phosphatase(s), we have initiated studies of upstream signalling events in PBLs and the human T cell line JURKAT.

A conserved response of many cell types to mitogenic signals is the activation of the ribosomal protein S6 kinases p70s6k and p90rsk via distinct signalling pathways. This results in the enhanced phosphorylation of ribosomal protein S6, activation of downstream kinases and phosphorylation of transcription factors [1,2,17,18]. Activation of the p70s6k family of S6 kinases can be

specifically inhibited by the macrolide immunosuppressant rapamycin [19]. Rapamycin and its structural analogue, FK-506, can interact with both a common family of intracellular receptors (FKBPs) and specific binding proteins [17], with a distinct physiological response to each in T lymphocytes; the FK-506–FKBP complex inhibits the activity of calcineurin, resulting in decreased lymphokine gene transcription, while the rapamycin–FKBP complex inhibits proliferation via inhibition of p70s6k [19,20].

To examine the potential role of the p70s6k family of ribosomal protein S6 kinases in the stimulation of translation, PBLs were incubated with 20 nM rapamycin for 10 min prior to the addition of either PMA or Con A, and the incorporation of [³⁵S]methionine into protein was estimated as described. Figure 1(a) shows that during the first 2 h of incubation, rapamycin had little effect on the PMA- or Con A-enhanced translation rates. Following incubation for 3 h or more, a reproducible inhibition of translation was observed in response to rapamycin in Con A-stimulated cells which was not observed in PMA-stimulated cells. Similar results have been obtained using FK506; treatment of cells for longer times indicated that both of these compounds appear to prevent the increase in the rate of translation usually observed in response to Con A (results not shown). To determine whether rapamycin was functioning as predicted, the activity of p70s6k and p90rsk was assayed directly. Following incubation of cell cultures for 30 min with PMA, in the absence or presence of rapamycin, extracts were prepared and p70s6k and p90rsk were isolated by immunoprecipitation and their activity monitored

Figure 1 PMA- or Con A-induced activation of translation is unaffected by the presence of rapamycin

(a) Cell cultures (5 ml; 1×10^7 cells in Eagle's basal medium) were incubated with 18 μ Ci/ml [³⁵S]methionine, in the absence (Δ , $\blacktriangle$) or presence of 10 nM PMA ($\circ$, $\bullet$) or 10 μ g/ml Con A ($\square$, $\blacksquare$), with (open symbols; broken lines) or without (closed symbols) 20 nM rapamycin. Aliquots ($3 \times 500 \mu$ l) were removed at the times indicated, cells were isolated by centrifugation, and incorporation of radioactive methionine into protein was determined as described [14]. These data are derived from three separate experiments (error bars are S.D.). (b) and (c) Cell cultures (10 ml; 2×10^7 cells) were incubated for 30 min in the absence (lane 1) or presence (lanes 2 and 3) of 10 nM PMA, with (lane 3) or without (lanes 1 and 2) 20 nM rapamycin, prior to the preparation of extracts as described. Aliquots of extract containing equal amounts of protein (10 μ l; 25 μ g of protein) were then incubated with antiserum specific to p70s6k (b) or p90rsk (c). The antibody was precipitated with Protein A–Sepharose and the activity of S6 kinase was determined with 40 S ribosomal subunits as substrate, as described in the Experimental section. The migration of S6 is indicated. (d) PBLs (30 ml; 6×10^7 cells) were labelled with phosphate as described, prior to incubation in the absence or presence of PMA for 1.5 h, with or without rapamycin (RAPA), as indicated. Cells were then harvested, extracts prepared, eIF-4 α protein isolated by m⁷GTP–Sepharose chromatography and equal amounts of protein visualized by SDS/PAGE. The migration of eIF-4 α (α) is shown on the resulting autoradiogram. (e) PBLs (20 ml; 4×10^7 cells) were labelled with phosphate as in (d), prior to incubation in the absence or presence of PMA or Con A, with or without rapamycin (RAPA), for the times indicated. Cells were then harvested, extracts prepared, eIF-4F complex isolated as described in the Experimental section and equal amounts of protein visualized by SDS/PAGE. The migration of eIF-4 α and eIF-4 γ are shown on the resulting autoradiogram.

Figure 2 Stimulation of PBLs results in enhanced phosphate-labelling of eIF-4 γ and increased eIF-4F complex formation

(a) PBLs (70 ml; 1.4×10^8 cells) were labelled with 350 μ Ci/ml [32 P]P $_i$, washed and resuspended as described in Figure 1. Aliquots (10 ml) of cells were unincubated, or incubated for 2 h with 10 nM PMA or 10 μ g/ml Con A, in the absence or presence of 20 nM rapamycin (RAPA) or FK-506, as indicated. Cell extracts were prepared and eIF-4F was isolated by m⁷GTP–Sephacryl chromatography. Proteins were transferred to PVDF and decorated with anti-eIF-4 γ antiserum (upper panel) prior to exposure to film; the autoradiograph is presented in the lower panel. (b) PBLs (70 ml; 1.4×10^8 cells) were labelled with 500 μ Ci/ml [32 P]P $_i$ and cell extracts prepared as described above. Samples containing equal amounts of protein (300 μ g) prepared from control cells (lane 1), or cells stimulated with 10 nM PMA (lane 2) or 10 μ g/ml Con A (lane 3) for 2 h, were diluted to 200 μ l with Buffer D and incubated with rabbit anti-eIF-4 α polyclonal antiserum. Following the addition of Protein A–Sepharose, precipitates were recovered as described in the Experimental section and eluted protein was transferred to PVDF, prior to decoration with anti-eIF-4 γ or anti-eIF-4 α antiserum. The top panel shows the resulting immunoblot with anti-eIF-4 γ antiserum and the middle panel the corresponding autoradiograph. The bottom panel shows decoration of the PVDF-bound protein with anti-eIF-4 α antiserum. Quantification of data from three experiments is presented in Table 1. (c) Cell extracts prepared as described in (b) were precipitated with antiserum to eIF-4 γ and recovered proteins were visualized by SDS/PAGE and immunoblotting. The top panel shows the resulting immunoblot with anti-eIF-4 γ antiserum and the middle panel the corresponding autoradiograph. The bottom panel shows decoration of the PVDF-bound protein with anti-eIF-4 α antiserum. Quantification of data from three experiments is presented in Table 1. (d) Cell extracts prepared as in (c) were adjusted to 2 mM CaCl $_2$, prior to chromatography on calmodulin–Sepharose as described in the Experimental section. Eluted proteins were transferred to PVDF, and eIF-4 α and eIF-4 γ were visualized by immunoblotting. Quantification of data from three experiments is presented in Table 1.

using 40 S ribosomal subunits as substrate. Figure 1 shows that, in PBLs, PMA leads to the activation of both p70s6k (Figure 1b) and p90rsk (Figure 1c) within 30 min, with only p70s6k being sensitive to the presence of rapamycin (compare lanes 1 and 3). These data indicate that the activation of neither p70s6k nor any protein kinase directly downstream of it in the signalling pathway is required for the early increase in translation rates in primary blood lymphocytes stimulated with phorbol ester.

We have also determined the effect of rapamycin on the mitogen-induced enhancement of eIF-4 α phosphorylation in response to PMA and Con A. Cells were labelled with [32 P]P $_i$ prior to incubation with PMA for 1.5 h (Figure 1d) or for 0.5 and 1.5 h (Figure 1e) in the absence or presence of rapamycin. Cell extracts were then prepared, eIF-4 α purified by m⁷GTP–Sephacryl chromatography and equal amounts of protein visualized by SDS/PAGE. Figure 1(d) indicates that enhanced phosphate-labelling of eIF-4 α in PBLs in response to PMA for 1.5 h is largely unaffected by the presence of rapamycin. Figure 1(e) indicates that, in a separate cell preparation, rapamycin had little, if any, effect on the PMA-induced phosphorylation of eIF-4 α at 0.5 or 1.5 h. Similarly, following Con A treatment for 4 h, rapamycin had little effect on eIF-4 α phosphorylation in these cells. Likewise, neither FK-506 nor wortmannin (a specific inhibitor of phosphatidylinositol 3-kinase, believed to function upstream of p70s6k and MAP kinase [17] in some cell types) prevented the early activation of translation or the enhanced phosphate-labelling of eIF-4 α in response to PMA or Con A (results not shown). These data, therefore, show that at times less than 2 h post-stimulation, the early increase in translation rates and the increased phosphate-labelling of eIF-4 α in primary PBLs are largely independent of the activation of calcineurin or p70s6k, or of any immediate downstream effector of these signalling pathways.

Enhanced rates of translation are associated with increased levels of eIF-4 γ phosphate-labelling and eIF-4F complex formation

In numerous cell types, mitogenic stimulation results in the enhanced phosphorylation both of eIF-4 α and of the largest

subunit of the eIF-4F complex, eIF-4 γ (p220) [1,2]. To examine whether increased levels of eIF-4 γ phosphorylation are associated with activation of PBLs, we have isolated eIF-4 γ by a number of methods: (a) by virtue of its affinity for eIF-4 α on m⁷GTP–Sephacryl, (b) by co-immunoprecipitation with eIF-4 α , (c) directly by immunoprecipitation, and (d) by retention on calmodulin–Sepharose. For these studies, cells were labelled with [32 P]P $_i$ as described and extracts were prepared following treatment with either PMA or Con A for 2 h, in the absence or presence of rapamycin or FK-506. For Figure 2(a), the eIF-4F complex was then isolated by m⁷GTP–Sephacryl chromatography and equal amounts of protein were visualized by SDS/PAGE. Immunoblot analysis (upper panel) showed that, while the recovery of eIF-4 γ protein was similar in each case, phosphate-labelling of eIF-4 γ was enhanced in response to PMA (lower panel). These data are consistent with those presented in Figure 1(e), which also indicate enhanced phosphorylation of eIF-4 γ at 0.5 and 1.5 h following PMA addition to cells. Similar findings have been reported for reticulocytes and 3T3-L1 preadipocytes in response to PMA and insulin respectively [2,4], and with JURKAT cells either in response to PMA or following activation of the T cell receptor signalling pathway [2]. Figure 2(a) also indicates that, relative to the unstimulated cells, Con A treatment led to enhanced phosphate-labelling of eIF-4 γ ; however, in all cases examined, the effect of rapamycin or FK-506 upon the phosphorylation of eIF-4 γ was inconclusive, due to the partial effects which were consistently observed.

To complement these studies, eIF-4 α was isolated from [32 P]phosphate-labelled cell extracts by immunoprecipitation and the amount of associated eIF-4 γ protein was visualized by both immunoblotting and autoradiography. Figure 2(b) shows that, while similar levels of eIF-4 α were recovered in each case, stimulation of cells with either PMA (lane 2) or Con A (lane 3) resulted in enhanced recovery of total eIF-4 γ protein in the immunoprecipitate (quantification of these data is summarized in Table 1). In agreement with the data presented in Figures 1(e) and 2(a), PMA increased the phosphate-labelling of eIF-4 γ to a greater extent than did Con A. In a similar experiment utilizing precipitation with anti-eIF-4 γ antiserum, the data presented in Figure 2(c) indicate that treatment of cells with PMA or Con A

Table 1 Treatment of PBLs with PMA or Con A enhances eIF-4F complex formation

For details, see the text and Figure 2. Results are means $\pm$ S.D. ($n = 3$).

Treatment	eIF-4F complex formation (arbitrary units)		
	Anti-eIF-4 α *	Anti-eIF-4 γ †	Calmodulin–Sepharose†
Control	1.75 $\pm$ 0.19	0.23 $\pm$ 0.01	0.085 $\pm$ 0.030
10 nM PMA	2.76 $\pm$ 0.31	0.31 $\pm$ 0.02	0.26 $\pm$ 0.02
10 μ g/ml Con A	2.53 $\pm$ 0.30	0.30 $\pm$ 0.02	0.20 $\pm$ 0.02

* To express the data presented in Figure 2 in terms of increased eIF-4F complex formation, immunoblots from three separate experiments were quantified by densitometric scanning and the amount of eIF-4 γ protein recovered was divided by the amount of eIF-4 α recovered.

† To express the data presented in Figure 2 in terms of increased eIF-4F complex formation, immunoblots from three separate experiments were quantified by densitometric scanning and the amount of eIF-4 α protein recovered was divided by the amount of eIF-4 γ protein recovered.

resulted in increased recovery of eIF-4 α with eIF-4 γ (see Table 1). An increase in the association between eIF-4 α and eIF-4 γ was confirmed directly following the isolation of eIF-4 γ on calmodulin–Sepharose; in the presence of Ca²⁺, eIF-4 γ is retained on the resin while free eIF-4 α is recovered in the flow-through fraction. Figure 2(d) and Table 1 show that when cell extracts prepared from either PMA- or Con A-stimulated cells were fractionated on calmodulin–Sepharose, there was enhanced association of eIF-4 α with eIF-4 γ , with an increase in the specific activity (labelling) of eIF-4 γ in this complex. eIF-4 γ was found to migrate as a doublet (Figure 2d), possibly reflecting its partial degradation during the extensive washing of the resin, in spite of the presence of general proteinase inhibitors in the buffers. In all cases examined, this enhanced association between eIF-4 α and eIF-4 γ was unaffected by the presence of rapamycin in the culture medium (results not shown). It is possible, therefore, that the low rates of translation typically observed in unstimulated cells reflect, in part, limiting levels of this initiation factor complex. It is not inconceivable that activation of cell growth in response to PMA or Con A leads to the enhanced phosphorylation of both eIF-4 α and eIF-4 γ , thereby facilitating the formation or stability of the eIF-4F complex [2,4]. Indeed, assays performed with purified proteins have demonstrated that phosphorylation of eIF-4 γ increases the interaction of eIF-4F with the mRNA cap structure [2]; in addition, the ability of eIF-4 α to interact with the mRNA cap structure is greatly enhanced when it is present in the eIF-4F complex rather than in the uncomplexed form [1]. In response to a mitogenic signal, we propose that there is a transient activation of eIF-4F complex formation; this would increase the ability of poorly translated mRNAs to compete for this factor, and increase the helicase activity required to unwind convoluted 5' secondary structures typically associated with growth-associated mRNAs [1–3,5–8]. At this time, however, the role of protein phosphorylation in modulating the interactions between the subunits of eIF-4F is not understood.

Enhanced phosphate-labelling of eIF-4 α in PBLs and JURKAT cells reflects increased steady-state phosphorylation

To determine whether increased phosphate-labelling of eIF-4 α in PBLs represents enhanced steady-state phosphorylation or phosphate turnover, cell extracts were prepared and eIF-4 α partially purified by batch absorption on m⁷GTP–Sepharose. Eluates were then subjected to one-dimensional VSIEF and immunoblotting with affinity-purified anti-peptide antiserum. Figure 3 shows that eIF-4 α protein can be resolved into two main species; the lower intense band represents the less phosphorylated form

Figure 3 Enhanced phosphate-labelling of eIF-4 α following PBL or JURKAT cell activation reflects a modest stimulation of steady-state phosphorylation

PBLs (30 ml; 6×10^7 cells) were unincubated (lane 1), or incubated for 2 h in the absence (lane 4) or presence (lanes 2, 3 and 5) of 10 nM PMA, in the presence of 20 nM rapamycin (lane 3) or 1 μ M Microcystin (lanes 4 and 5). Cell extracts were prepared and eIF-4 α was isolated by batch absorption on to m⁷GTP–Sepharose as described in the Experimental section. Samples were then analysed by VSIEF and immunoblotting; the migration of eIF-4 α is shown, with the more phosphorylated (upper) form (P) indicated. JURKAT cells (3×10^6 cells in 10 ml), maintained in exponential phase (lanes 6–8), were incubated in the absence (lane 6) or presence of OKT-3 (lane 7) or 10 nM PMA (lane 8) for 30 min, prior to the preparation of extracts and analysis of the state of phosphorylation of eIF-4 α as described above. JURKAT cells (3×10^6 cells) were also grown to high density to slow cell growth, and extracts were prepared following incubation at 37 °C for 3 h either without (lane 9) or following dilution of cells in complete medium to 3×10^5 cells/ml, in the absence (lane 10) or presence (lane 11) of 20 nM rapamycin. eIF-4 α was isolated by batch absorption on to m⁷GTP–Sepharose and subjected to VSIEF as described above. The upper band in lanes 9–11 was only seen in this experiment and is due to cross-reaction of the antiserum with other cellular proteins in JURKAT cells.

and the upper intense band the more phosphorylated form. Occasionally (lanes 1, 2, 6 and 7), minor degradation products were observed, but this was not a reproducible finding. Quantification of individual bands from three separate experiments by high resolution densitometric scanning showed that there were small, but reproducible, changes in the steady-state phosphorylation of eIF-4 α concomitant with activation of protein synthesis. In quiescent PBLs (lane 1), $10 \pm 3\%$ (mean $\pm$ S.D., $n = 3$) of the total eIF-4 α protein was found in the more phosphorylated form; this increased to $19 \pm 3\%$ following stimulation with PMA, in either the absence (lane 2) or the presence (lane 3) of rapamycin. The general phosphatase inhibitor Microcystin induced a more modest change in the steady-state phosphorylation of eIF-4 α (lane 4), while only causing a slight increase in eIF-4 α [³²P]phosphate-labelling, independent of the activation of translation (S. J. Morley, unpublished work). This response was variable between preparations of cells, with the percentage of phosphorylated eIF-4 α determined at $14 \pm 6\%$ ($n = 3$). The reason for this variation is unclear. In combination with PMA, surprisingly, Microcystin was not able to effect any substantial additional increase in the steady-state

Figure 4 Treatment of PBLs with PMA results in the activation of p90rsk, MAP kinase and eIF-4 α kinase(s)

PBLs (12 ml; 2.4×10^7 cells) were incubated for the times indicated in the absence or presence of 10 nM PMA, with or without 20 nM rapamycin (RAPA), in the absence or presence of 1 μ M Microcystin (MC) as shown. Cells were harvested and extracts prepared as described in the legend to Figure 3, prior to analysis by SDS/PAGE. The top and middle panels show the migration of p90rsk and MAP kinase (MAPK) respectively, decorated with specific rabbit polyclonal antisera. The bottom panel shows the phosphorylation of bacterially expressed eIF-4 α protein by these extracts. For this assay, equal amounts of extract protein (prepared in the presence of phosphatase inhibitors) were incubated with 2 μ g of eIF-4 α protein in the presence of 200 μ M (3350 c.p.m./pmol) ATP, 10 mM MgCl₂ and 500 nM peptide inhibitor of protein kinase A for 20 min at 30 °C; eIF-4 α was isolated as in Figure 3 and its phosphorylation status monitored by SDS/PAGE, with the resulting autoradiogram presented.

phosphorylation of eIF-4 α ($19 \pm 2\%$ phosphorylated; lane 5) over that seen in response to PMA alone. Although speculative, it is possible that Microcystin is not only inhibiting phosphatase activity in the cell, but also preventing the activation of eIF-4 α kinase(s) at some upstream point [21].

The other cell type studied in this work was the human T leukaemic cell line JURKAT. Previously we have demonstrated enhanced phosphate-labelling of eIF-4 α in response to treatment of cells with PMA or on stimulation of the T cell receptor with a monoclonal antibody (OKT-3) [14]. Relative to control cells, VSIEF analysis of cell extracts prepared from PMA- or OKT-3-stimulated cells (Figure 3, lanes 6–8) indicated a modest, reproducible, increase in total immunoreactive eIF-4 α recovered in the more phosphorylated form [from $35 \pm 4\%$ (mean $\pm$ S.D., $n = 3$) in the control to $45 \pm 3\%$ in the stimulated cells]. This probably reflects the finding that, under optimal growth conditions [$(3\text{--}5) \times 10^5$ cells/ml], the rate of protein synthesis was stimulated by only 1.2–1.5-fold by either treatment [14]. We have now expanded these studies to examine JURKAT cells which have been grown for 5 days to a high density [$(2\text{--}3) \times 10^6$ cells/ml] to slow their growth. Under these conditions the rate of translation was greatly depressed. Within 3 h of dilution of these cells to optimal growth conditions, the rate of protein synthesis increased by 4-fold and the proportion of total immunoreactive eIF-4 α protein in the phosphorylated form rose from $40 \pm 3\%$ ($n = 3$) to $82 \pm 5\%$ (Figure 3, lanes 9 and 10). Although the addition of rapamycin slightly reduced the stimulation of protein synthesis brought about by dilution (15–20% decrease), the shift of the eIF-4 α population to the more phosphorylated variant was, if anything, potentiated ($95 \pm 4\%$ of total eIF-4 α in the phosphorylated form; compare lanes 9–11). These data suggest that, in PBLs and JURKAT cells, at early times following stimulation of cell growth, the increase in the steady-state level of

eIF-4 α phosphorylation can be correlated with enhanced translation rates, which does not require the activation of p70s6k.

Although activation of cell growth has been correlated with enhanced eIF-4 α phosphorylation in numerous systems (reviewed in [1–3,5]), little is known about the signal transduction mechanisms responsible for this event and no specific eIF-4 α kinase(s) has yet been identified. In some cell types, activation of protein kinase C may lead to the phosphorylation of eIF-4 α [1,2,4]. However, in PBLs, while treatment with PMA or stimulation of the T cell receptor leads to the activation of protein kinase C, it also results in the activation of the MAP kinase signalling pathway [2,21]. To date none of the individual components of the latter pathway have been found to phosphorylate eIF-4 α directly *in vitro* [1]. Another possible connection between the T cell receptor and eIF-4 α is ras. This latter family of proteins has a central role in regulating proliferation and differentiation [1,22,23], and there is strong evidence for a modulation of eIF-4 α phosphorylation mediated via a ras-dependent pathway in fibroblasts and PC12 cells [1,2,5,24,25]. However, as ras itself does not possess intrinsic kinase activity, at least one serine/threonine kinase must link ras to eIF-4 α . In an attempt to identify such kinase(s), we have examined the temporal correlation between the activity of eIF-4 α kinase and the activation of other known signalling molecules during PBL stimulation. Figure 4 shows that, relative to unstimulated cells, incubation of PBLs with PMA resulted in the rapamycin-insensitive activation of p90rsk and MAP kinases within 15 min, as judged by their retarded mobility on SDS/PAGE. Although Microcystin alone did not induce the activation of these kinases, it prolonged the effects of PMA, presumably by preventing the activity of phosphatases responsible for the inactivation of this kinase [17]. Extracts from cells treated with these agents were also assayed for their ability to phosphorylate bacterially expressed eIF-4 α protein; Figure 4 shows that, as with p90rsk and MAP kinases, the activity of eIF-4 α kinase was enhanced within 15 min of treatment with PMA, with a maximal effect seen at 30 min. High resolution densitometric scanning of data from three separate experiments showed that eIF-4 α kinase activity is increased by $30 \pm 5\%$ (mean $\pm$ S.D., $n = 3$) at 15 min and by $120 \pm 10\%$ at 30 min after treatment of cells with PMA. This increase in eIF-4 α kinase in response to PMA was largely insensitive to the presence of rapamycin. Microcystin alone was able to partially activate eIF-4 α kinase over levels found in untreated cells, but in conjunction with PMA it led to a lower level of kinase activity than with either alone. Although this was a reproducible finding, the reasons for it are unclear. One possible explanation consistent with the data presented in Figures 2–4 is that, while eIF-4 α kinase may be maintained in a less active form in resting lymphocytes by the action of a phosphatase, inactivation of the phosphatase alone is not sufficient to enhance phosphorylation of eIF-4 α , substantially alter the steady-state phosphorylation of eIF-4 α or activate translation. The identity of the putative eIF-4 α kinase remains elusive. One possible candidate is a protamine kinase purified from bovine kidney which will phosphorylate eIF-4 α *in vitro* [26]. However, in PBLs, protamine kinase activity was not increased in response to either PMA or Con A (results not shown).

To determine whether phosphatase activity directed against the eIF-4 α protein itself was altered following cell activation, extracts prepared in the absence of phosphatase inhibitors were incubated with eIF-4F complex labelled *in vivo* with [³²P]phosphate [4]. In three separate experiments, the rate of dephosphorylation of eIF-4 α and eIF-4 γ was measured by SDS/PAGE and autoradiography; the results from one such experiment are presented in Figure 5(a), with quantification of

Figure 5 Cell extracts prepared from PMA-stimulated cells exhibit a reduced ability to dephosphorylate exogenously added eIF-4 α

(a) PBLs (12 ml; 2.4×10^7 cells) were incubated in the absence or presence of 10 nM PMA for 1.5 h. Cell extracts were prepared as described in the Experimental section, except that buffers did not contain phosphatase inhibitors at this stage. Reticulocyte eIF-4F complex, labelled with [32 P]P_i *in vivo* (3500 c.p.m./ μ g), was purified by m⁷GTP–Sephacrose chromatography and ammonium sulphate precipitation, prior to dialysis [29]. Extracts containing equal amounts of protein were incubated with 32 P-labelled eIF-4F (1 μ l; 6560 c.p.m.), in the absence or presence of 2 μ M Microcystin (M) as indicated, and 2 μ l aliquots were removed into SDS/PAGE sample buffer at the times indicated. The resulting autoradiogram is presented; lane m shows the [32 P]eIF-4F preparation (1 μ g), with the migration of eIF-4 α and eIF-4 γ indicated. (b) Quantification of the data presented in (a) by densitometric scanning, expressed as the percentage of phosphate-labelled protein remaining as a function of time. These data are representative of those obtained in three separate experiments. Control extract: eIF-4 α ($\blacktriangle$), eIF-4 γ ($\bullet$); extract from PMA-stimulated cells: eIF-4 α ($\blacksquare$), eIF-4 γ ($\circ$); extract from PMA-stimulated cells incubated in the presence of Microcystin: eIF-4 α (∇), eIF-4 γ ($\blacktriangledown$).

these data presented in Figure 5(b). These experiments show that, while the phosphate on eIF-4 α has a half-life of less than 15 min in the unstimulated cell extract, this is increased to at least 30 min following treatment of cells with PMA. Control experiments showed that this dephosphorylation was prevented by the inclusion of Microcystin in the incubation (see Figure 5) and was not due to degradation of the added eIF-4F protein (results not shown). Therefore the data presented in Figure 3 suggest that the enhanced steady-state phosphorylation of eIF-4 α following PBL activation results in part from the activation of eIF-4 α kinase(s) and in part from the stabilization of phosphate incorporated into eIF-4 α during this process. Interestingly, the data presented in Figure 5 indicate that dephosphorylation of eIF-4 γ is stimulated following activation of PBLs, suggesting that phosphate turnover may play a significant role in the enhanced phosphorylation of this protein. At this time it is not possible to reproducibly analyse the steady-state phosphorylation of eIF-4 γ by VSIEF, as the protein fails to focus clearly (results not shown).

Activation of PBLs leads to a decreased interaction between eIF-4 α and PHAS-I

Recently it has been suggested that, in addition to phosphorylation, the interaction of eIF-4 α with other proteins, such as PHAS-I (4E-BP1) [10,11,18], may play a role in translational control by inhibiting the ability of eIF-4 α to participate in the initiation process. One current model is that, following treatment of adipocytes with insulin, PHAS-I is phosphorylated via the MAP kinase signalling pathway; this in turn leads to the dissociation of the eIF-4 α -PHAS-I complex and increased availability of eIF-4 α to participate in translation of mRNA. The finding that PHAS-I is phosphorylated by MAP kinase both *in vivo* and *in vitro* provides a possible direct link between activation of growth factor receptors and protein synthesis initiation factors

Figure 6 Activation of PBLs is associated with decreased interaction between eIF-4 α and PHAS-I

PBLs (20 ml; 4×10^7 cells) were unincubated (lanes 1, 4 and 7), or incubated for 30 min with 10 nM PMA (lanes 2, 5 and 8) or for 16 h with 10 μ g/ml Con A (lanes 3 and 6), prior to preparation of extracts. Equal amounts of extract protein were analysed by SDS/PAGE directly (lanes 1–3), following batch isolation of eIF-4 α on m⁷GTP–Sepharose (lanes 4–6), or following immunoprecipitation with antiserum to PHAS-I (lanes 7 and 8). Proteins were transferred to PVDF and decorated with anti-eIF-4 α (upper panel) or anti-PHAS-I (lower panel) antiserum as described in the Experimental section. These data are representative of those obtained in three separate experiments.

[10,11,18]. To see whether PHAS-I was present and if such a mechanism could be involved in activation of translation in PBLs, cell extracts were resolved by SDS/PAGE and blotted for the presence of PHAS-I and eIF-4 α . Figure 6 shows that PHAS-I is present in PBL cell extracts and that, following stimulation of cells with PMA or Con A, PHAS-I is phosphorylated, as judged by the retardation of a proportion of the protein on SDS/PAGE (Figure 6, compare lanes 1–3). Following acute stimulation of PBLs with PMA for 30 min, MAP kinase is activated (Figure 4; S. J. Morley and V. M. Pain, unpublished work), which appears to be temporally coincident with enhanced phosphorylation of PHAS-I (Figure 6, lane 2).

To examine the association between PHAS-I and eIF-4 α , eIF-4 α was isolated from cell extracts by m⁷GTP-Sepharose

Figure 7 Incubation of PHAS-I immunoprecipitates with *Xenopus* extracts containing activated MAP kinase or immunoprecipitated MAP kinase results in partial phosphorylation of PHAS-I and the release of a small percentage of associated eIF-4 α

(a) Cell extracts for immunoprecipitation were prepared from unstimulated PBLs as described in the Experimental section and 50 μ l aliquots were diluted to 200 μ l with Buffer D prior to incubation with 7 μ g of anti-PHAS-I antiserum for 1.5 h at 4 $^{\circ}$ C. Immunoglobulins were precipitated and recovered with Protein A–Sepharose, washed three times with Buffer D, once with kinase buffer [10 mM Mops/KOH, pH 7.2, 2 mM benzamide, 5 mM α NPP, 2 mM dithiothreitol, 10 mM MgCl_2 , 0.1% (v/v) Triton X-100, 200 μ M ATP] and resuspended in 5 μ l of kinase buffer. Resuspended pellets were then incubated for 15 min at 30 $^{\circ}$ C in the presence of 5 μ l of buffer (lane 1) or 5 μ l of *Xenopus* egg (lane 2) or oocyte (lane 3) extract depleted of eIF-4 α as described in the Experimental section. Reactions were then transferred on to ice and precipitates re-isolated and washed three times with 200 μ l of Buffer D, prior to elution of bound proteins with SDS/PAGE sample buffer. Proteins were transferred to PVDF and decorated with anti-eIF-4 α (upper panel) or anti-PHAS-I (lower panel) antiserum as described. The MAP kinase activity of the oocyte and egg extracts towards the epidermal growth factor receptor peptide (residues 662–682 [31]) was 0.1 and 0.9 pmol of phosphate/min per μ g of protein respectively. (b) Reticulocyte lysate (30 μ l) was adjusted to 200 μ l with Buffer D and PHAS-I immunoprecipitated as in (a). The resuspended precipitate was incubated in the presence of *Xenopus* oocyte (lane 1) or egg (lane 2) extract, and pellets were washed and bound proteins analysed as described in (a). The upper and lower panels show decoration of the blot with anti-eIF-4 α and anti-PHAS-I antisera respectively. (c) Reticulocyte lysate (60 μ l) was adjusted to 300 μ l with Buffer D, and PHAS-I was immunoprecipitated and resuspended as in (b). In parallel incubations, MAP kinase was immunoprecipitated from extracts prepared from *Xenopus* oocytes and eggs [28], as described in the Experimental section, and resuspended in kinase buffer. Resuspended PHAS-I immunoprecipitates were then incubated for 15 min at 30 $^{\circ}$ C in the presence of isolated MAP kinase derived from oocyte (lane 1) or egg (lane 2) extract, and pellets were recovered and washed as above, prior to SDS/PAGE and Western blot analysis of recovered protein. The upper and lower panels show decoration of the blot with anti-eIF-4 α and anti-PHAS-I antisera respectively.

chromatography and the bound proteins were analysed by SDS/PAGE and immunoblotting with antisera to eIF-4 α and PHAS-I (Figure 6, lanes 4–6). Stimulation of PBLs with PMA for 30 min resulted in a $40 \pm 10\%$ (mean $\pm$ S.D., $n = 3$) reduction in the amount of PHAS-I protein recovered with eIF-4 α on this matrix, with this decrease evident within 15 min (results not shown). In addition, as reported for adipocytes [10,11], the PHAS-I protein recovered by this method was predominantly in the less phosphorylated form. In response to 16 h of treatment with Con A, when protein synthesis rates are 5–10-fold higher than in the unstimulated cells [14], there was no apparent change in the association between PHAS-I and eIF-4 α (compare lanes 1 and 3); however, this possibly reflects the finding that the levels of both total eIF-4 α [27] and PHAS-I protein [11] are increased during these later times of T cell activation. In addition to selecting eIF-4 α using m⁷GTP–Sepharose, we have used immunoprecipitation with anti-PHAS-I antiserum to investigate the interaction between eIF-4 α and PHAS-I (Figure 6, lanes 7 and 8). When proteins recovered in the immunoprecipitates were analysed by SDS/PAGE, equal levels of PHAS-I were recovered in each case; consistent with the data presented above, a higher proportion of the phosphorylated form was present in extracts prepared from the PMA-stimulated cells. Quantification of data obtained from three separate experiments indicated that treatment of cells with PMA led to a $40 \pm 5\%$ ($n = 3$) decrease in the level of eIF-4 α recovered with anti-PHAS-I antiserum.

The data presented in Figure 6 indicate that PMA treatment of PBLs markedly decreased the amount of PHAS-I associated with eIF-4 α , consistent with the dissociation of PHAS-I from eIF-4 α [10,11]. To further examine the role of MAP kinase in this process in PBLs, the ability of an extract containing activated MAP kinase and of immunoprecipitated MAP kinase to dissociate the PHAS-I–eIF-4 α complex was analysed *in vitro*. For these experiments, PHAS-I and the associated eIF-4 α were immunoprecipitated from unstimulated PBL cell extracts (Figure 7a) or reticulocyte lysate (Figure 7b) and the protein recovered was incubated with extracts prepared from either *Xenopus* oocytes or eggs [28]. The latter extracts had been depleted of endogenous eIF-4 α and of a large proportion of the PHAS-I by passage

through m⁷GTP–Sepharose (results not shown), with egg extracts containing predominantly the activated form of MAP kinase and oocyte extracts the less active form ([28] and legend to Figure 7). Relative to results with buffer or oocyte extract, Figure 7(a) shows that incubation of the PBL PHAS-I immunoprecipitate with egg extract resulted in a small enhancement of phosphorylation of PHAS-I and a reduced association of eIF-4 α with PHAS-I, concomitant with the release of eIF-4 α into the supernatant (results not shown). When PHAS-I–eIF-4 α was isolated from the reticulocyte lysate, incubation of immunoprecipitates with egg extract again resulted in the release of eIF-4 α from the complex, suggesting that phosphorylation of PHAS-I may be associated with disruption of the eIF-4 α –PHAS-I complex (Figure 7b). Although many kinases are activated in the egg extracts [28], this interpretation is further strengthened by the finding that dissociation of eIF-4 α from PHAS-I can be partially prevented by the inclusion of the specific MAP kinase phosphatase, MPK-1, in the assay (results not shown).

To look more directly at the role of MAP kinase, it was immunoprecipitated directly from extracts prepared from *Xenopus* oocytes or eggs and incubated with anti-PHAS-I immunoprecipitates isolated from the reticulocyte lysate. As shown in Figure 7(c), incubation of the anti-PHAS-I immunoprecipitate with MAP kinase from *Xenopus* eggs resulted in a small increase in the phosphorylation of PHAS-I, as visualized by its enhanced retardation on SDS/PAGE, and the release of a small percentage (15%) of the associated eIF-4 α . The magnitude of this dissociation was less than expected considering that the anti-(MAP kinase) immunoprecipitate was able to phosphorylate myelin basic protein to a very high level (results not shown), suggesting that other kinases may have a role in the physiological phosphorylation of PHAS-I. Therefore the data presented in Figures 6 and 7 for PBLs are consistent with the proposed role for PHAS-I in modulating the availability of eIF-4 α to participate in the initiation phase of translation [10,11]. In agreement with this model, further studies with the reticulocyte lysate have shown that no detectable eIF-4 γ was recovered in anti-PHAS-I immunoprecipitates and no detectable PHAS-I was recovered in anti-eIF-4 γ immunoprecipitates (results not shown).

It is widely believed that the binding of mRNA to ribosomes is a rate-limiting step in protein synthesis initiation, which is possibly regulated in part by the phosphorylation status and level of eIF-4F complex in the cell [1,5,14,29,30]. Previous studies with PBL cell extracts have shown that they exhibit lesions in the initiation process primarily affecting the interaction of mRNA with the 40 S initiation complex [12,13], and that activation is associated with enhanced phosphorylation of eIF-4 α [14]. The data presented above now show that phosphorylation of eIF-4 α and eIF-4 γ increases co-incident with enhanced translation rates [14], at a time when there is a decrease in the association between eIF-4 α and PHAS-I. While the disruption of the eIF-4 α -PHAS-I interaction is incomplete and appears to precede the enhancement of eIF-4F complex formation, the role of eIF-4 α and eIF-4 γ phosphorylation in this process is not known. It is quite possible that interaction of the eIF-4 α -PHAS-I complex with the m⁷GTP cap structure of a selected population of mRNA renders it untranslatable; the dissociation of PHAS-I and the phosphorylation of eIF-4 α and eIF-4 γ may then play a role in mobilizing specific classes of mRNA for translation. Further work is needed in order to understand the role of PHAS-I in regulating the rate of translation and the level of eIF-4F complex formation in these cells.

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