A Reevaluation of the Cap-binding Protein, eIF4E, as a Rate-limiting Factor for Initiation of Translation in Reticulocyte Lysate*

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The cap-binding eukaryotic initiation factor, eIF4E, is a key target for the regulation of translation in mammalian cells and is widely thought to be present at very low molar concentrations. Here we present observations with the reticulocyte lysate that challenge this view. When reticulocyte ribosomes are harvested by centrifugation, most (~75%) of the eIF4E remains in the postribosomal supernatant (PRS). In a reconstituted translation system we find that the ribosome-associated eIF4E alone can sustain much of the overall activity, suggesting that much of the factor in the PRS is functionally redundant. Consistent with this, our estimates of eIF4E in the reticulocyte lysate reveal much higher concentrations than previously reported. The association of a small proportion of eIF4E with the ribosomal fraction appears to be functional and dependent on interaction with the factor eIF4G. This fraction of eIF4E is, as expected, more highly phosphorylated than that in the PRS; however, at least half the total phosphorylated eIF4E in reticulocyte lysate translation systems resides in the PRS fraction, suggesting that, while phosphorylation may enhance activity, it is not in itself sufficient to promote utilization of the factor. We also show that the eIF4E-binding factor, eIF4E-BP1 or PHAS-I, which regulates eIF4E activity in insulin-responsive cells, is present in the reticulocyte PRS at an approximately 1:1 molar ratio relative to eIF4E and demonstrate by coimmunoprecipitation studies that the binding of PHAS-I and eIF4G to eIF4E is mutually exclusive. These data are consistent with a potential regulatory role for PHAS-I in the reticulocyte lysate.

Translation of cellular mRNAs occurs by a cap-dependent mechanism whereby the 40S ribosomal subunit interacts with the 5'-terminal cap structure and subsequently migrates to the AUG start codon (1, 2). This process involves the activities of a number of protein factors of the eIF4G family (3-5). The formation of the complex termed eIF4F is thought to allow joining of the ribosomal subunit to mRNA through its interaction with the mRNA cap structure on the one hand and association with ribosomes on the other (6). eIF4F consists of three polypeptides (7-9): (i) eIF4E, which specifically binds the cap; (ii) eIF4A, which possesses RNA helicase activity; and (iii) eIF4G, a 220-kDa polypeptide on SDS gels. There is a strong requirement for eIF4E and eIF4G in cap-dependent initiation (1, 10-13). Recent work has shed light on the potential role of eIF4G in translation initiation (14, 15). These studies have identified an eIF4E-binding motif on eIF4G and have also identified likely sites of interaction with eIF4A and eIF3. Thus eIF4G seems to mediate joining of the mRNA and ribosomes by interaction with both the cap-binding protein, eIF4E, and with eIF3 already bound to ribosomes. eIF4A is believed to catalyze the unwinding of upstream mRNA secondary structures. It is recycled through the eIF4F complex during successive rounds of initiation (6, 16, 17), possibly explaining the presence of and requirement for, abundant quantities of uncomplexed eIF4A protein (6, 8, 18). The unwinding activity of eIF4A is stimulated in the presence of eIF4B which may interact directly with mRNA (19-22) and/or 18S rRNA (23) through RNA recognition motifs.

eIF4E has been the focus of intensive study in recent years. Current data suggest that this factor exists in particularly low abundance both in the reticulocyte lysate (24, 25) and in HeLa cells (26), indicating that it may quantitatively limit translation rates. The biological importance of eIF4E levels is illustrated by in vivo overexpression experiments, which result in a transformed phenotype (27) or aberrant growth (28). Several observations stress the importance of eIF4E phosphorylation in regulation of eIF4E activity during cell growth and development (see Rhoads (5) and references therein) (29-31). Growth stimulation correlates with increased de novo phosphorylation of eIF4E (reviewed in Rhoads (5) and Morley (31)) and eIF4F complex formation (30-34), while the reverse applies when translation rates are down-regulated during mitosis (35), the heat-shock response (26, 36) and viral infection (37, 38). Recent work has determined that the primary site of eIF4E phosphorylation in reticulocytes (39) and CHO cells (40) is serine 209. Interestingly, Saccharomyces cerevisiae and plant homologs of eIF4E lack a serine at the equivalent position (41, 42) (for comparison, see Hernández and Sierra (43)). The effect of phosphorylation on the affinity of eIF4E for cap structures and components of the translation machinery remains uncertain. While hyperphosphorylation of eIF4E does not affect its recovery from HepG2 cell extracts on m7GTP-Sepharose (32), other experiments do suggest an enhanced affinity for cap analogs and mRNA (44). eIF4E recovered with eIF4G from Ehrlich ascites tumor cells is highly phosphorylated relative to uncomplexed eIF4E (45); however, the phosphorylation of eIF4E alone may not alter its affinity for other elements of the translational apparatus (15, 32).

It is now becoming clear that eIF4E interacts specifically with both eIF4G and at least one other binding protein, PHAS-I.
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Materials—All reagents were reagent grade and purchased from Sigma unless indicated otherwise.

Buffers—Buffer A, 40 mM Tris/HCl, pH 7.4, 0.25 mM dithiothreitol, 0.1 mM EDTA, 50 mM sodium fluoride, 20 mM β-glycerophosphate, 2 mM benzamidine, 0.05% (v/v) Tween 20, 10% (v/v) glycerol; Buffer B, 40 mM Mops/KOH, pH 7.2, 125 mM NaCl, 2.5 mM EGTA, 40 mM β-glycerophosphate, 40 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 7 mM β-mercaptoethanol; Resuspension buffer (RB), 20 mM Mops/KOH, pH 7.2, 10 mM NaCl; 1.1 mM MgCl₂; 0.1 mM EDTA; Buffer A, 75 mM KCl; 0.5 mM dithiothreitol; 5% (v/v) glycerol; RIPA, 50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 0.5% (w/v) Triton X-100, 25 mM KCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol; IEF sample buffer, 9% urea, 7% (v/v) Ampholine, pH 3.5–10 (Pharmacia Biotech Inc.), 2% (w/v) β-mercaptoethanol, 5% (w/v) CHAPS, 1 μM m7GTP, 1 μM microcystin (Calbiochem), 2% benzamidine, 0.5% phenylmethylsulfonyl fluoride; Low salt buffer (LSB), 20 mM Mops/KOH, pH 7.2, 10 mM NaCl, 25 mM KCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol; IEF sample buffer, 9% urea, 7% Ampholine, pH 3.5–10 (Pharmacia Biotech Inc.), 2% (v/v) β-mercaptoethanol, 5% (v/v) CHAPS, 1 μM m7GTP, 1 μM microcystin (Calbiochem).

m7GTP-Sepharose Chromatography—Prior to use, the m7GTP-Sepharose affinity resin (Pharmacia) was equilibrated in RB, and the buffer was removed by aspiration using a needle attached to a vacuum line. Depletion of eIF4E was achieved in batch by addition of 4.5 volumes of undiluted reticulocyte lysate onto 1 volume of m7GTP-Sepharose. Following agitation on ice for 15 min, and recovery of the unbound fraction by a brief centrifugation in a microcentrifuge. In all cases, unbound material from a parallel treatment using Sepharose 4B was used as the nondepleted control.

In vitro Translation—Reticulocyte lysates were prepared as described (51). Translation reactions contained 50% (v/v) reticulocyte lysate with the following added components (final concentrations): 25 μM hemin, 25 μg/ml creatine phosphokinase, 75 mM KCl, 0.8 mM magnesium acetate, 50 μM amino acids without methionine and leucine, 200 μM leucine, 3 mM d-glucose, 5 mM MgCl₂ creatine phosphate. The translational activity of the system was measured by inclusion of 0.1 μCi/ml ([³⁵S]methyl) and measurement of trichloroacetic acid-precipitable radioactivity by scintillation counting.

Fractionation and Reconstitution of the Reticulocyte Lysate—Ribosomal components of the intact reticulocyte lysate were separated from the soluble fraction by centrifugation at 100,000 rpm (approximately 430,000 × g) for 25 min at 4 °C using the TL-100.2 rotor in a Beckman TL-100 ultracentrifuge, essentially as described elsewhere (52). When required for subsequent translation assays, the undiluted reticulocyte lysate was used as the starting material and the top two-thirds of the postribosomal supernatant removed for use, while the bottom third of the supernatant was discarded. The ribosomal pellet was resuspended in RB buffer to 0.1 of the starting volume, yielding a 10-fold concentrated (10×) stock of ribosomes. For reconstitution, 0.45 volume of postribosomal supernatant was combined with 0.05 volume of 10× ribosomes in a 50% translation reaction as above, with the additional inclusion of 15 mM 2-aminopurine. For measurements of the subcellular distribution of translation factors (see “Results and Discussion”) the reticulocyte lysate was prec incubated under full translation conditions (as above) prior to ultracentrifugation. In these cases the ribosomal pellet was resuspended in RB buffer to 0.2 of the volume of the original incubation.

Cap-binding purification of eIF4E by Fast Protein Liquid Chromatography—Chromatography on Mono Q (Pharmacia) to separate the reticulocyte lysate into fractions enriched in eIF4E or eIF4G was based on the method of Lamphere and Panniers (45), with some modifications. Briefly, 700 μl of reticulocyte lysate previously incubated for 15 min under full translation conditions was loaded onto a 1-ml Mono Q column equilibrated with 10 ml of Buffer A containing 50 mM KCl. The “free” and complexed forms of eIF4E were step-eluted with Buffer A containing 130 mM KCl and 300 mM KCl, respectively. Pooled eluates were dialyzed against 100 mM KCl by addition of KCl-DEAE Sepharose purified by affinity chromatography using a 0.3 ml m7GTP-Sepharose column, previously equilibrated in Buffer B. Bound eIF4E was eluted in 0.5 ml of Buffer B containing 150 μM m7GTP, concentrated by acetone precipitation and the sample dissolved directly into IEF sample buffer (see above).

Co-immunoprecipitation—Following preincubation under full translational conditions, 40 μl of translation mix was brought to 200 μl with RIPA prior to addition of affinity purified polyclonal antiserum as indicated in the figures. The mixture was incubated on ice for 90 min and 100 μl recovered by addition of 50 μl of a 50% (v/v) Protein A-Sepharose 4B suspension (Pharmacia) in RIPA. Following agitation at 4 °C for 30 min, the resin was recovered by centrifugation, washed three times in 1 ml of RIPA and bound proteins eluted directly into SDS or IEF sample buffer, as indicated in the figure legends.

Expression and Purification of Recombinant eIF4E, [His]₆-tagged PHAS-I, and L Protease—Recombinant proteins were expressed in, and purified from, Escherichia coli (BL21(DE3) strain containing the plasmid pLS5s (Novagen)) as described for eIF4E (53), PHAS-I (54), and L protease (Lb) (55). In the final step of purification, recombinant protein was dialyzed overnight at 4 °C against LSB and the protein concentration measured by the Bradford assay (Bio-Rad).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—Samples were analyzed on 12% polyacrylamide mini-gels (Protein II, Bio-Rad) as described elsewhere (33).

Western Blotting—After SDS-PAGE or VSIIF, proteins were transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore) under semidry conditions (Semi-Phor, Hoefer Scientific Instruments). Blots were incubated with primary antiserum; eIF4G, polyclonal rabbit anti-peptide, raised against the peptide WALWFFKNDKSKTWQNL; PHAS-I, polyclonal rabbit anti-peptide raised against the peptide CSSPDKRAGGEESOFE (46); eIF4B, polyclonal goat anti-eIF4B protein antiserum, kindly provided by Dr. J. Hershley; eIF4A, monoclonal antiserum, the kind gift of Dr. M. Altman. Bands were revealed by incubation with the appropriate alkaline phosphatase-conjugated secondary antibody. Anti-peptide antibodies were affinity purified on AFl-Gel 10 (Bio-Rad) prior to use, in accordance with the manufacturer’s instructions.

RESULTS AND DISCUSSION

The availability of eIF4E is widely believed to limit translation in eukaryotic cells (5, 49). This belief is based on measurements of initiation factor concentrations in HeLa cells (26) and reticulocytes (24, 25), which indicated a very low ratio of eIF4E to ribosomes. However, another common observation is that eIF4E is found in cells in both low and high molecular weight forms (33, 45, 56). The latter, which is usually isolated from ribosomal salt-wash fractions, is a complex of eIF4E with other initiation factors, notably eIF4A and eIF4G, which together make up the eIF4F complex (7, 9). This is believed to be the functional form of eIF4E, and good correlations between rates of protein synthesis and the proportion of eIF4E associated with these complexes have been observed in a number of phys-
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Fig. 1. Extensive depletion of eIF4E in the reticulocyte lysate causes only a moderate inhibition of protein synthesis. A, the PRS of a reticulocyte lysate was subjected to batchwise treatment with either Sepharose 4B (Control) or the affinity matrix m7GTP-Sepharose 4B (Depleted) for 15 min on ice as described under “Experimental Procedures.” Samples corresponding to 1 μl of the parent reticulocyte lysate were analyzed by SDS-PAGE and the depletion of eIF4E monitored by immunoblotting, as described (PRS, lanes 1 and 2). In addition, equivalent samples were analyzed in a similar manner following reconstitution of the reticulocyte lysate (see below) (Incubation, lanes 3 and 4). The immunoblots were quantified by scanning densitometry and the data are presented as a histogram of optical density expressed as arbitrary units (AU). B, control and depleted postribosomal supernatant (0.45 volume) were recombined with resuspended 10× ribosomes (0.05 volume) and translation mix (0.5 volume) prior to incubation at 30 °C. Aliquots (2 μl) were removed at the times shown and processed for incorporation of [35S]methionine into acid-precipitable protein. These data are representative of those obtained in three separate experiments.

Fig. 1A shows Western blots demonstrating the degree of depletion of the PRS as well as the quantities of eIF4E remaining after reconstitution of the translation system with ribosomes (“incubation”). In the control incubation, the PRS was mock-depleted using Sepharose 4B resin (see “Experimental Procedures”). Lanes 1 and 2 show that all detectable eIF4E was removed from the PRS following treatment with m7GTP-Sepharose. The small proportion of eIF4E that reappeared upon reconstitution (lane 4) is attributable to the added ribosomes. The influence of this treatment on translation of endogenous mRNA in the reconstituted lysate system is shown in Fig. 1B. Surprisingly, despite the extensive removal of eIF4E (80–90%), only a 30% reduction in [35S]methionine incorporation was observed after 60 min. Although the rate of translation is seen to fall relative to the undepleted control during the late part of the time course depicted in Fig. 1B, this was a characteristic of this experiment and was not a reproducible finding. Experiments in which the batch depletion was applied instead to the reconstituted system following recombination of the PRS with ribosomes consistently show an effect more in line with the extent of eIF4E depletion (data not shown). These results raise two interesting questions. First, the maintenance of approximately 70% of the protein synthesis activity in a system depleted of 80–90% of its content of eIF4E is not consistent with the concept that this factor is present in rate-limiting amounts (5, 25, 26, 49); rather it indicates that much of the factor in the PRS fraction is non-functional. This is consistent with the finding that eIF4E may be withheld from participation in initiation complexes through sequestration by PHAS-I in other cell systems (47, 48). The second point relates to the more severe effect of depleting the reconstituted lysate, rather than the PRS alone, prior to reconstitution (data not shown). This result may be explained by the concomitant removal of eIF4G by the m7GTP-Sepharose affinity matrix due to its interaction with eIF4E. Alternatively, the ribosomal fraction may contain a highly active (e.g. highly phosphorylated) form of eIF4E (59), which is removed by depleting the reconstituted lysate. In order to address these points we have undertaken a more rigorous study of the distribution of eIF4E between the ribosome-associated and PRS fractions of actively translating reticulocyte lysates, and, for each population of the factor, we have analyzed its extent of phosphorylation and its association in complexes with eIF4G and PHAS-I.

Subcellular Distribution of eIF4E and Associated Proteins in the Reticulocyte Lysate—Fig. 2A shows the results of an experiment where an intact RRL was incubated for 15 min at 30 °C under full translational conditions (see “Experimental Procedures”) and subsequently separated into soluble (PRS) and ribosomal fractions. The complete removal of all ribosomal components from the PRS was confirmed by sucrose density gradient analysis (data not shown). Equivalent samples of PRS and resuspended ribosomes were analyzed by gel electrophoresis followed by Western blotting, using antibodies recognizing eIF4E, eIF4G, eIF4B, eIF4A, and PHAS-I (see “Experimental Procedures”). The intensity of the resultant bands was quantified by densitometry and these data are summarized in Table I. In all cases the immunochromical signal of the blot was within the linear range of antibody response (data not shown). The distribution of the various proteins differed substantially; approximately 80% of the total eIF4G co-sedimented with ribo-
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**A**

![Diagram](image)

**B**

![Diagram](image)

**Fig. 2.** Subcellular distribution of eIF4E and associated proteins in the reticulocyte lysate; effects of cleavage of eIF4G by L protease. A, following a 15-min preincubation at 30°C under full translation conditions (see "Experimental Procedures"). B, reticulocyte lysates were fractionated by centrifugation at 100,000 rpm (approximately 430,000 × g) for 25 min in a Beckman TL-100 centrifuge. The S 100 was removed (PRS), and the pellet was resuspended in RB buffer on ice to 0.2 of the original incubation volume (see "Experimental Procedures"). Samples corresponding to 1 μl of unfractionated reticulocyte lysate were analyzed by SDS-PAGE and Western blotting using the specific antibodies shown. B, the reticulocyte lysate was further incubated for 10 min at 30°C following the addition of a partially purified form of recombinant FMDV L protease prior to fractionation and immunoblot analysis as for A. CpN = eIF4G cleavage product, N-terminal domain; CpC = eIF4G cleavage product, C-terminal domain. These data are representative of those obtained in three separate experiments.

| TABLE I |

Quantification of the distribution of initiation factors between the PRS and ribosomes in the RRL under translation conditions

Data presented in Fig. 2 were quantified by scanning densitometry and are represented as the percentage distribution of total immunoreactive protein between the PRS and resuspended ribosomes. Similar data were obtained in three separate experiments.

| Control (Fig. 2A) | FMDV protease (Fig. 2B) |
|-------------------|------------------------|
| eIF4G             | 20                     | Trace                   |
| eIF4A             | 60                     | 40                     |
| eIF4E             | 60                     | 40                     |
| PHAS-I            | 100                    | 100                    |

**Fig. 3.** Emetine does not prevent the loss of eIF4E from the ribosomal fraction following cleavage of eIF4G by L protease. Three parallel reactions of intact reticulocyte lysate under full translation conditions (see "Experimental Procedures") were incubated for 15 min at 30°C. Emetine (25 μM final concentration) was added for 2 min where indicated, prior to the addition of recombinant L protease (or an equivalent volume of LSB) and the incubation continued for a further 10 min at 30°C. Reactions were stopped by cooling on ice and lysates were fractionated by ultracentrifugation, as described in Fig. 2. The resultant ribosome pellets were resuspended as described and aliquots corresponding to 3 μl of undiluted reticulocyte lysate were analyzed by SDS-PAGE and the presence of eIF4E monitored by Western blotting. These data were reproduced on two separate occasions.

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elF4E into the postribosomal supernatant following L protease treatment. This intriguing result can be explained by at least three distinct mechanisms. First, if all “ribosomal” elF4E is normally cap-associated, the most probable explanation for these data is that L protease-induced cleavage of elF4G results in the dissociation of elF4E from the mRNA cap. A cleavage-induced conformational alteration in the elF4G domain containing the elF4E-binding site may be transmitted to elF4E itself, reducing its affinity for the cap structure. Although elF4E alone does have a high affinity for mRNA cap structures in vitro (44), this is apparently stimulated in the presence of elF4G (4, 6, 19); hence, cleavage of elF4G might conversely reduce its affinity for the cap. However, work in several laboratories, including our own, has shown that the C-terminal 2 elF4E complex retains its ability to bind to cap analogs (9, 14, 58). A second alternative is that the association between elF4E and the cap is very transient, with elF4E being released from the cap as the 40S subunit begins to scan toward the initiation codon. In this model the elF4E present in 48S complex (62, 64), where the 40S subunit is presumably located at the initiation codon, would be attached solely via its interaction with elF4G. Finally, one might argue that only a small proportion of ribosomal elF4E is cap-associated and that the majority is in fact involved in 43S preinitiation complexes formed in the absence of mRNA. elF4E in such complexes, presumably bound via the N-terminal domain of elF4G, would be released into the soluble fraction following cleavage of elF4G. Prevaling evidence is against the presence of elF4E in 43S preinitiation complexes (62, 64), but uncertainty on this point remains (65).

elF4E in the Ribosomal Fraction of the Reticulocyte Lysate Is Highly Phosphorylated—There are strong circumstantial links between elF4E phosphorylation and enhanced activity of this factor (5, 29, 30, 49). Fig. 4A shows typical measurements of elF4E phosphorylation obtained when samples of PRS and resuspended ribosomes are subjected to VSIEF followed by Western blotting (see “Experimental Procedures”). Clearly, the small proportion of elF4E that is ribosome-bound is enriched for the phosphorylated form. This effect is reproducible between lysates, although the actual degree of phosphorylation shows some variation. Nonetheless, it is noteworthy that first, we never observe 100% phosphorylation of ribosomal elF4E and, second, the soluble elF4E pool is itself approximately 30% phosphorylated. By combining data from Figs. 2A and 4A we can estimate the distribution of the phosphorylated form of elF4E between subcellular fractions in the reticulocyte lysate (Table II) and find that, while the ribosomal fraction is enriched for the phosphorylated form, the postribosomal supernatant contains more than half of the total phosphorylated elF4E protein in the reticulocyte lysate. However, the PRS contains only small amounts of elF4E (Fig. 2A). Hence, while phosphorylation of elF4E may affect its ability to participate in protein synthesis, it cannot alone be sufficient to promote an association with elF4G. Indeed, unpublished studies discussed by Mader et al. (15) have indicated that phosphorylation of elF4E does not alter its interaction with elF4G in a far Western blotting assay. Rather, concomitant phosphorylation of elF4E and elF4G may be required to stimulate their interaction (32).

elF4E Involved in Complexes Is More Phosphorylated than the “Free” Form When These Are Separated by Fast Protein Liquid Chromatography—As an alternative approach to differentiating between populations of elF4E in the reticulocyte lysate we used Mono Q chromatography to separate complexed from uncomplexed elF4E in samples of actively translating reticulocyte lysate (see “Experimental Procedures”), using a procedure similar to Lamphere and Panniers (45). pools corresponding to elF4E-elF4G complexes (“heavy”) and uncomplexed (“light”) fractions were analyzed by gel SDS-PAGE (data not shown) and VSIEF (Fig. 4B). In support of the data obtained with fractions separated by ultracentrifugation (Fig. 2A), approximately 80% of the elF4E was recovered at a KCl

Fig. 4. The phosphorylation status of elF4E in subcellular fractions of the reticulocyte lysate. A, the intact reticulocyte lysate was incubated for 15 min at 30 °C prior to centrifugal fractionation into PRS and ribosomes. The following samples were then removed for VSIEF, and the proportion of total elF4E in the phosphorylated state was visualized by immunoblotting (as described under “Experimental Procedures”). Precipitated proteins were resuspended directly into IEF sample buffer and subjected to VSIEF. A sample of the Mono Q load, corresponding to 2 μl of reticulocyte lysate, was also analyzed (Starting material). Quantification of scanning yielded values for the percentage of phosphorylation. The data are representative of two separate experiments. B, reticulocyte lysate was incubated under full translation conditions as above prior to chromatography on Mono Q as described under “Experimental Procedures.” Material corresponding to uncomplexed elF4E (Light) was eluted from the column at 130 mM KCl, while complexed elF4E (Heavy) was eluted at 330 mM KCl. elF4E was isolated as described under “Experimental Procedures.” Precipitated proteins were resuspended directly into IEF sample buffer and subjected to VSIEF. A sample of the Mono Q load, corresponding to 2 μl of reticulocyte lysate, was also analyzed (Starting material). Quantification by scanning yielded values for the percentage of phosphorylation. The data are representative of six independent experiments. C, reticulocyte lysate was incubated as in B; immunoprecipitation of elF4E was carried out using affinity-purified antibodies recognizing elF4G, elF4E, and PHAS-I, as described under “Experimental Procedures.” Immune complexes were isolated with Protein A-Sepharose in batch, as described, and the bound protein eluted directly into IEF sample buffer. Starting material shows VSIEF analysis of 4 μl of the original translation incubation. These data are representative of four separate experiments.
concentration previously found to elute the uncomplexed form of eIF4E protein (45), while 20% showed elution properties typical of the eIF4E-eIF4G complex (data not shown). This latter fraction was, however, more highly phosphorylated (60%) (Fig. 4B). These data thus support those in Fig. 4A, indicating that the functional pool of eIF4E is enriched for, but does not contain exclusively, the phosphorylated form of the protein.

Anti-eIF4G Antibody Co-immunoprecipitates Mostly Phosphorylated eIF4E, While Anti-PHAS-I Antibody Co-immunoprecipitates eIF4E That Is Less Phosphorylated—To confirm data on the phosphorylation status of eIF4E in complexed versus “free” form (Fig. 4, A and B), we examined the phosphorylation status of eIF4E in immunoprecipitates obtained with antibodies against eIF4E, eIF4G, and PHAS-I (see “Experimental Procedures”). Fig. 4C shows that, relative to the starting material (31% phosphorylated) or that recovered with anti-eIF4E antibody (29% phosphorylated), eIF4E co-immunoprecipitating with anti-eIF4G antibody is more highly phosphorylated (54%). However, the eIF4E co-immunoprecipitating with anti-PHAS-I antibody appears, if anything, to be less phosphorylated (20%) than the starting material.

Thus we find that eIF4E, selected by three distinct methods (Figs. 4, A–C), on the basis of its association with active translational complexes, is consistently enriched for the phosphorylated form. This observation supports the large body of evidence (5, 29, 30) which correlates increased eIF4E phosphorylation with enhanced eIF4F activity. While there is in vitro evidence for a greater affinity of phosphorylated eIF4E for mRNA cap structures (44), it is not clear at this stage which aspect(s) of eIF4E activity are affected by its phosphorylation in vivo. Numerous studies linking increased eIF4E phosphorylation and enhanced complex/eIF4F formation (30, 32–34) would indicate an improved ability to bind eIF4G; however, no evidence has been presented to refute the alternative possibility that eIF4E phosphorylation occurs only as a consequence of recruitment into complexes.

Binding of eIF4G or PHAS-I to eIF4E Is Mutually Exclusive—Recent work has provided evidence for a common eIF4E-binding motif within both eIF4G and PHAS-I (15). This suggests a competitive scenario where PHAS-I and eIF4G are unable to interact with eIF4E simultaneously. Recent studies with purified proteins by Haghighat et al. (50) have provided evidence in support of this hypothesis. Most work in favor of an important regulatory role for PHAS-I in controlling protein synthesis has involved insulin-responsive cells (48, 60). However, the potential role of PHAS-I in reticulocytes is not known at this time. As seen in Fig. 2A, we have detected significant quantities of PHAS-I in the reticulocyte lysate. Indeed the preferential retention of this protein in the post-ribosomal supernatant is consistent with current views for the role of PHAS-I which would preclude an association with any ribosomal component.

We have therefore extended the use of co-immunoprecipitation to investigate molecular interactions between eIF4G, eIF4E, and PHAS-I in the intact reticulocyte lysate under full translational conditions. Fig. 5 shows Western blots of co-immunoprecipitated material following SDS-PAGE. While this procedure is not quantitative, each employed antiserum predictably precipitated its specific antigen. Fig. 5 shows that, in addition, anti-eIF4G antibody co-immunoprecipitates eIF4E, while anti-eIF4E co-immunoprecipitates eIF4G and PHAS-I, and anti-PHAS-I antibody co-immunoprecipitates eIF4E. The doublet due to PHAS-I is likely to represent two differentially phosphorylated forms, as seen in 3T3-L1 cells (48, 60). These data are internally consistent in their support for interactions between eIF4E and eIF4G, as well as between eIF4E and PHAS-I. However, the failure of anti-eIF4G antibodies to co-immunoprecipitate PHAS-I and, conversely, the absence of eIF4G in anti-PHAS-I immunoprecipitates, in spite of the presence of eIF4E in both cases, provides evidence for a mutually exclusive interaction of PHAS-I and eIF4G with eIF4E in reticulocytes. It is likely, therefore, that PHAS-I and eIF4G compete for available eIF4E, whereas the affinity of PHAS-I (47, 48, 60), and possibly that of eIF4G, for eIF4E, is influenced by phosphorylation. These data are in agreement with recent work by Mader et al. (15) and Haghighat et al. (50). While we have established that eIF4E phosphorylation is insufficient to explain association with eIF4G (see Figs. 2A and 4A, summarized in Table 1), it is not known whether the phosphorylation status of eIF4E influences its association with PHAS-I. Fig. 4C shows that eIF4E co-immunoprecipitated with PHAS-I antibody is only 20% phosphorylated, compared with a phosphorylation status of approximately 30% in the starting material. The significance of this small difference is not clear; whereas dephosphorylation of eIF4E might favor binding of PHAS-I, it may equally occur as a consequence of association with PHAS-I.

The Molar Ratio of eIF4E to PHAS-I in the Postribosomal Supernatant of the Reticulocyte Lysate Is Approximately 1:1—To further assess the potential role of PHAS-I in the regulation of protein synthesis in reticulocyte lysates, we have estimated the molar concentrations of eIF4E and PHAS-I proteins in this system. To date, the only quoted estimate of the ratio of PHAS-I to eIF4E in the reticulocyte lysate is 1:20 (47), although no data were presented. We have used purified, recombinant eIF4E (Fig. 6) and PHAS-I (Fig. 7) proteins as
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Fig. 6. Quantification of the levels of eIF4E protein in the reticulocyte lysate. A, recombinant, bacterially expressed eIF4E protein (2 μg), prepared as described under "Experimental Procedures," was analyzed by SDS-PAGE and Coomassie staining; the resultant gel is presented. B, the immunological response to increasing amounts of recombinant eIF4E was tested by Western blotting (upper panel) and quantified by densitometric scanning (lower panel; AU = arbitrary units). C, the same experiment included a dose response of eIF4E antibodies to three different volumes of reticulocyte lysate. An estimation of eIF4E concentration in the reticulocyte lysate (see "Results") was made by taking the average of the calculated concentration, obtained from each of the three doses of reticulocyte lysate. D, to ensure reliability of the Western blotting method, 0.5 μl of reticulocyte lysate (lane 1) and 5.5 ng of recombinant eIF4E (lane 3) were tested for their ability to give an additive immunological response when combined (lane 2). These data are representative of those obtained in five experiments using three different lysate preparations.

standards in Western immunoblotting assays, using a procedure similar to that employed by Pause et al. (17). Fig. 6A shows >90% purity of the recombinant eIF4E protein. Fig. 6B shows a Western blot demonstrating a linear immunological response to increasing levels of recombinant eIF4E. In the same experiment, three different volumes of reticulocyte lysate were used to estimate the average endogenous concentration of eIF4E (Fig. 6C). From these data we calculate that 1 ml of reticulocyte lysate contains approximately 10.7 ± 0.8 μg (S.D., n = 3) of eIF4E. Fig. 6D confirms this result by showing that 0.5 μl of reticulocyte lysate (lane 1) yields an immunological response similar to that observed with 5.5 ng of recombinant eIF4E (lane 3). A similar value was obtained in assays on three separate lysates (data not shown). In molar terms this value is equivalent to a concentration for eIF4E of ~0.4 μM, as compared to previous estimates of 8 nM (24) and 33 nM (25) in the reticulocyte lysate and a calculated 0.35 nM in HeLa cells (26). Consistent with eIF4A being one of the most abundant initiation factors (18, 26), the recent estimation of 3.4 μM (calculated from 17) reveals eIF4A levels approximately 10-fold greater than those calculated above for eIF4E. Initiation factor concentrations are often best expressed relative to the translational apparatus itself. We have measured the molar concentration of ribosomes in different preparations of reticulocyte lysate and obtain a value of 0.2 μM ± 0.04 (S.D., n = 4). Therefore, we observe a eIF4E to ribosome ratio of 2:1 in reticulocytes, compared with previous data of 0.02 in reticulocytes (24) and 0.26 in HeLa cells (26). Our revised estimates of eIF4E abundance are therefore significant in that they are at least an order of magnitude greater than those published to date. Furthermore, these estimates are consistent with observations published herein that quantities of eIF4E per se are not limiting for translation in the reticulocyte lysate (see Fig. 1) and that the majority of this protein is not engaged in translation under optimal conditions (see Fig. 2A).

In Fig. 7, we show a similar method to quantify the concentration of PHAS-I in reticulocyte lysates. The recombinant PHAS-I protein used was electrophoretically distinct from endogenous PHAS-I, because of a histidine tag used to facilitate its purification (data not shown, see "Experimental Procedures"). Assuming that the histidine tag had no influence on the immunoreactive properties of the recombinant protein, we estimate the concentration of PHAS-I to be 3.2 ± 0.4 μg/ml of reticulocyte lysate (S.D., n = 3) (see Fig. 7B), representing a molar concentration of ~0.3 μM. From these data the ratio of eIF4E to PHAS-I in the reticulocyte lysate (and particularly in the PRS, containing ~75% of total eIF4E and all endogenous
PHAS-1 (see Fig. 2A) is close to 1:1. This close molar stoichiometry between eIF4E and PHAS-1 is strongly suggestive of a role for PHAS-1 in the regulation of translation in rabbit reticulocytes as well as in insulin responsive cells.

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