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IDENTIFICATION OF A PROTEIN KINASE ACTIVITY IN RABBIT RETICULOCYTES THAT PHOSPHORYLATES THE mRNA CAP BINDING PROTEIN

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SUMMARY: The 25 kDa mRNA cap binding protein can be purified in a partially phosphorylated state and the extent of its phosphorylation appears to be regulated during heat shock and mitosis in mammalian cells. We demonstrated that a nonabundant serine protein kinase activity exists in rabbit reticulocytes that phosphorylates the 25 kDa cap binding protein in both the free (eIF-4E) and complexed (eIF-4F) state. This kinase was not inhibited by the cAMP-dependent protein kinase inhibitory peptide IAAGRTGRRNAIHDILVAA, did not phosphorylate S6 ribosomal protein, did not phosphorylate p220 of eIF-4F as protein kinase C does and no other substrates for this kinase were apparent in reticulocyte ribosomal salt wash. The molecular identity of this kinase, the specific site(s) of eIF-4E that it phosphorylates and its *in vivo* regulatory role remain to be studied.

Eukaryotic initiation factor-4F (eIF-4F), composed of p25, p48, and p220 subunits, plays an incompletely understood role in the denaturation of mRNA structure and attachment of capped mRNA to ribosomes (1-7). This complex may be loosely associated or its assembly physiologically regulated because it is generally easier to purify isolated p25 (eIF-4E) than the eIF-4F complex. Initial studies suggest that eIF-4F is a limiting component of the translational apparatus that might play a role in mRNA discrimination at the level of translational initiation (8-11). Because of its apparent ability to denature native mRNA structure and its relative nonabundance within cells, eIF-4F appears to be an ideal target for some post-transcriptional regulatory events.

Approximately 10-20% of eIF-4E purified from reticulocytes or HeLa cells is phosphorylated (12-14). In addition, eIF-4E appears to be dephosphorylated following heat shock of HeLa cells (13). Purified eIF-4F can at least partially reverse the inhibition of translation of mRNAs in lysates prepared from heat shocked Ehrlich cells (15). These observations suggest that phosphorylation and dephosphorylation of eIF-4E and/or p25 of eIF-4F may

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Abbreviations used are: eIF-4F, eukaryotic initiation factor-4F; p25, 25 kDa subunit of eIF-4F; p48, 46-48 kDa subunit of eIF-4F which is probably the same polypeptide as eIF-4A; p220, 220 kDa subunit of eIF-4F; eIF-4E, eukaryotic initiation factor 4E (isolated p25); RSW, ribosomal salt wash; IEF, isoelectric focusing; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NP-40, octylphenoxypolyethoxyethanol; DTT, DL-dithiothreitol; PMSF, phenylmethylsulfonylfluoride.

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have a regulatory role. In this report we describe the identification of a nonabundant protein kinase activity in rabbit reticulocytes that phosphorylates both eIF-4E and p25 of the eIF-4F complex. A partial purification of this kinase is described and evidence presented that indicates it is not one of the protein kinases already identified in reticulocytes.

MATERIALS AND METHODS

Reagents. All reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. [γ-^{32}P]-ATP (3,000-6,000 Ci/mmol) was synthesized and its purity determined as previously described (16). [^{14}C]-Methylated eIF-4F was prepared by adapting a method using [^{14}C]HCHO (51 Ci/mmol, New England Nuclear) (17).

Purification of eIF-4F and eIF-4E. Rabbit reticulocyte eIF-4F was purified as described elsewhere (4). eIF-4E was purified from rabbit reticulocyte lysate by m^{7}GTP sepharose affinity chromatography (18).

Isoelectric focusing of ^{14}C-labeled p25 of eIF-4F. Slices of dried polyacrylamide gels containing the protein of interest were rehydrated in at least two exchanges (1-2 ml) of 9 M urea, 2% NP-40 and 5% 2-mercaptoethanol for 1 h prior to isoelectric focusing. Verticle slab isoelectric focusing gels (0.75 mm thick) were 4% polyacylamide (30 parts acrylamide per 1.6 parts bisacrylamide), 9.2 M urea, 2% NP-40, 0.1% TEMED, 0.02% ammonium persulfate and contained 2% ampholytes (LKB, 60% pH 3.5-10 and 40% pH 5-7) (19). The cathode solution was degassed 20 mM NaOH and the anode solution was 10 mM phosphoric acid. After pre-focusing, proteins were applied to the cathode end of gels and focused as described in legends to figures. Isoelectric focusing protein standards (FMC Corporation, Rockland, ME) were used to determine optimal conditions.

Dye Ligand Chromatography. Rabbit reticulocyte lysate (11.3 g protein) was diluted to a final volume of 100 ml with Buffer A (20 mM Tris-pH 7.5 [4^{o}C], 2 mM EDTA, 10 mM $MgCl_2$, 0.25 mM PMSF and 1 mM DTT) and applied to a 2.5 X 6 cm column of Blue Sepharose CL-6B (Pharmacia) at 4^{o}C. The column was washed with 160 ml of Buffer A and bound proteins eluted stepwise with 10 mM ATP in Buffer A, then 15 mM ATP plus 0.3 M KCl in Buffer A and lastly 15 mM ATP plus 1 M KCl in buffer A as depicted in Figure 2A. Approximately 1.2 ml fractions were collected, pooled as indicated and made 60% ammonium sulfate at 4^{o}C. Protein precipitates were pelleted by centrifugation and dissolved in Buffer A containing 30% glycerol, aliquoted and stored at -70^{o}C. Protein concentrations were determined by the method of Bradford (20).

High Performance Liquid Chromatography. A Pharmacia FPLC system using a weak anion exchange column, Mono P HR 5/20 (5 X 200 mm) (Pharmacia) was used for this procedure. Starting material was dye ligand partially purified kinase (see Table I). The buffer was 20 mM Tris-pH 7.5 (4^{o}C), 2 mM $MgCl_2$, 1 mM DTT and 20% glycerol. Proteins were eluted with linear 0 to 0.5 M KCl gradients. Fractions were immediately concentrated to approximately 100 µl using microconcentrators (Centricon 30, Amicon), diluted 1X with buffer containing 60% glycerol and 2 mM DTT and stored at -70^{o}C.

Protein Kinase Assays. Reaction mixtures for determining kinase activity were 25 µl in volume and contained: 20 mM Hepes-pH 7.4 (30^{o}C), 5 mM $MgCl_2$, 0.1 mM ATP and 5 µCi of [γ-^{32}P]ATP (specific activity 3-6 mCi/mmol) unless specified otherwise. Incubations contained 3.6 µg of enzyme preparation plus 1.4 µg of eIF-4F, were for 10 min at 30^{o}C and were terminated by adding 25 µl of 2X electrophoresis sample buffer. Samples were analyzed by 10% SDS-PAGE by the method of Laemmli and autoradiograms prepared as previously described (21,22).

Phosphoamino Acid Analysis. eIF-4E was phosphorylated as described above using HPLC prepared kinase, analyzed by SDS-PAGE and the [^{32}P]eIF-4E passively eluted in 10 mM NH_4HCO_3 and 0.05% SDS. The eluted protein was hydrolyzed under nitrogen in 6N HCl for 2 h at 110^{o}C. Samples were analyzed by cellulose thin-layer (Eastman) electrophoresis at pH 3.5 after a described method (23). The standards were localized by ninhydrin staining and radiolabeled amino acids detected by autoradiography.

RESULTS AND DISCUSSION

To study the enzymatic regulation of eIF-4E phosphorylation rabbit reticulocytes were chosen because they are enriched with initiation factors and approximately 10-20% of eIF-4E purified from this source is phosphorylated (Fig. 1) (12,13). Using a dye ligand chromatographic procedure, a protein kinase activity that phosphorylated p25 of eIF-4F was purified approximately 100-fold (Fig. 2 and Table I). This kinase activity increased progressively from eluate Pools 1 through 3 while other apparent protein kinase activities decreased beyond Pool 2 (Fig. 2B). Although pool 3 also contained an activity that phosphorylated a 67 kDa protein found in eIF-4F preparations this activity had a different elution profile than the kinase phosphorylating p25 of eIF-4F (Fig. 2B). Similarly, there was a minor

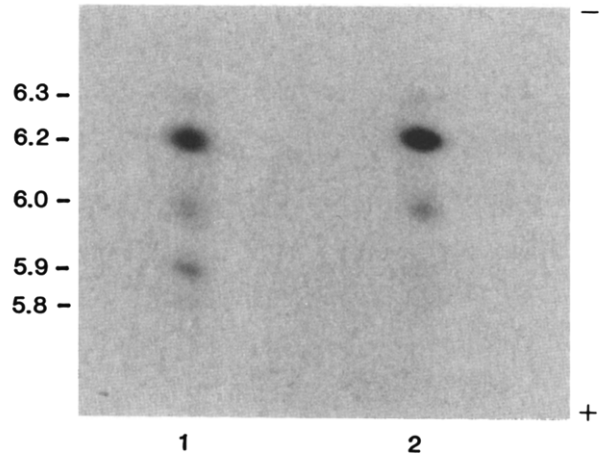

Figure 1. Isoelectric focusing analysis of the p25 subunit of [^{14}C]methylated eIF-4F. Purified ^{14}C-labeled eIF-4F (10.5 µg) was incubated at 30°C for 30 min in 20 mM Hepes-pH 8.0, 100 mM KCl, 1 mM DTT and 0.1 mM EDTA (30 µl final volume) in the presence or absence of calf intestinal alkaline phosphatase (5 units, Boehringer Mannheim). The entire content of each incubation was analyzed by SDS-PAGE and the 25 kDa component in each lane removed and analyzed by IEF as described in Methods. The autoradiogram here shows the ^{14}C-labeled proteins after 28 days of exposure. Lane 1 is from a control and lane 2 from a phosphatase treated [^{14}C]eIF-4F sample. The same pattern is seen when m^{7}GTP affinity purified eIF-4E is analyzed by IEF and silver stained (not shown).

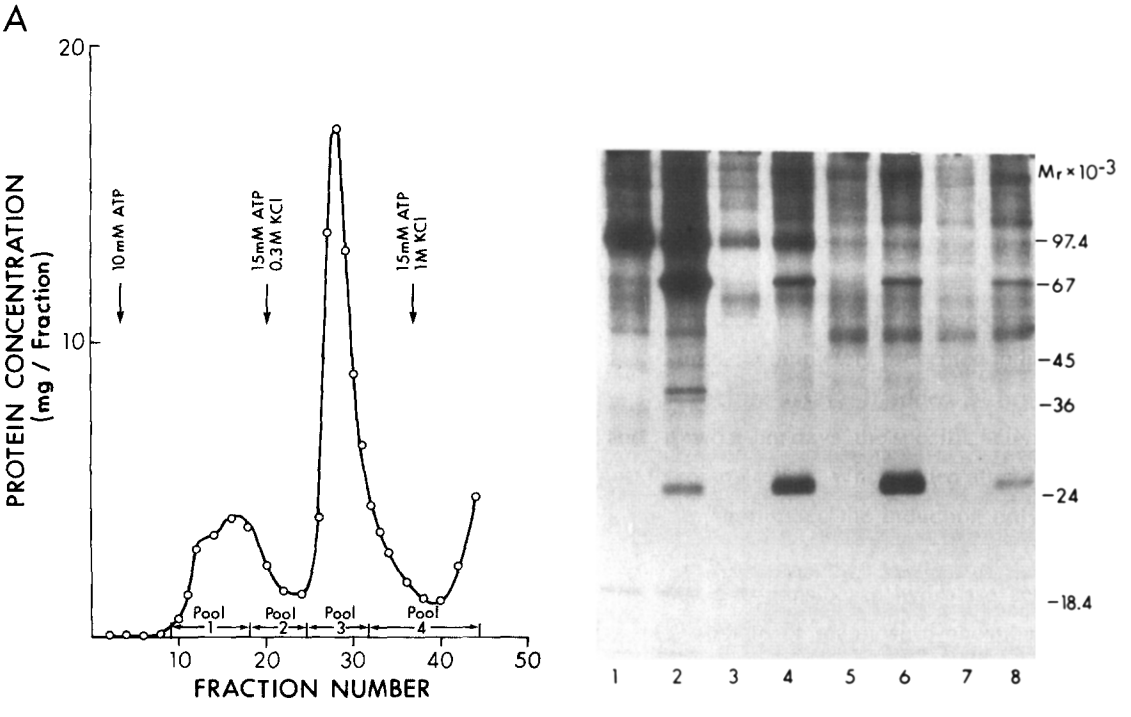

Figure 2. Partial purification of a protein kinase activity that phosphorylates p25 of eIF-4F and eIF-4E using dye ligand chromatography. The details of this step using Blue Sepharose CL-6B and reticulocyte lysate are described in Methods. Panel A. The stepwise elution of protein bound to the column with 10 mM ATP (eluate Pool 1), 15 mM ATP plus 0.3 M KCl (Pools 2 and 3) and 15 mM ATP plus 1 M KCl (Pool 4) is shown. Each fraction was approximately 1.2 ml. The amount of protein in each fraction is shown on the ordinate. Panel B. The results of phosphorylation studies using eIF-4F as substrate are shown. The autoradiogram in Panel B was obtained after a 10 h exposure. Proteins phosphorylated in the absence (odd lanes) or presence (even lanes) of eIF-4F are shown consecutively: lanes 1 and 2, Pool 1; lanes 3 and 4, Pool 2; lanes 5 and 6, Pool 3; and lanes 7 and 8, Pool 4.

TABLE I. PURIFICATION TABLE

PURIFICATION STEP	TOTAL PROTEIN mg	TOTAL ACTIVITY units[1]	SPECIFIC ACTIVITY units/mg	FOLD PURIFICATION
RETICULOCYTE LYSATE	11,300	*	*	*
POOL 3 FROM DYE LIGAND COLUMN	8	8.5	1.1	100**
POOL 7 FROM MONO P COLUMN	0.13	0.6	4.6	400

unit = pmol ^{32}P incorporated into eIF-4E per min at 30°C
* activity too low to quantitate accurately by beta counter
** estimated by densitometric scanning of autoradiograms

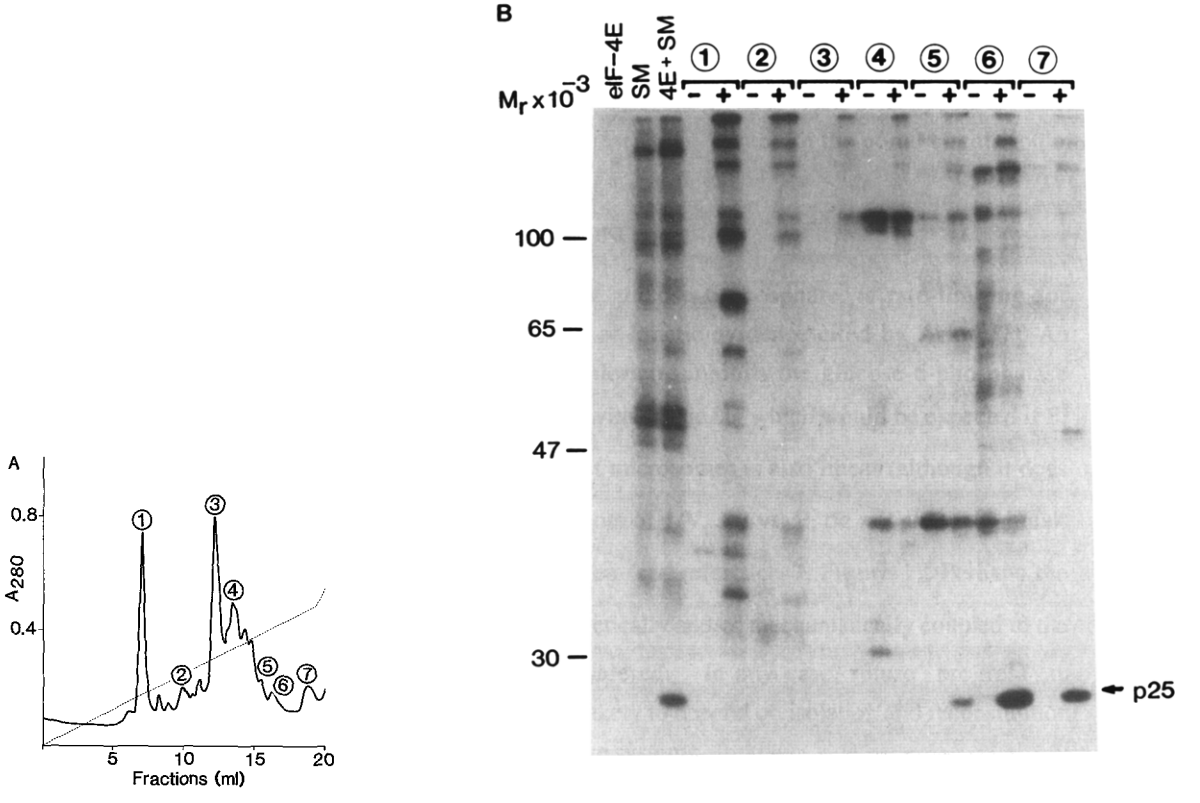

Figure 3. Further purification of a kinase that phosphorylates both eIF-4E and p25 of eIF-4F using anion exchange HPLC. Fractions obtained by dye ligand chromatography were used as starting material (see Table I). A total of 8 mg of material was applied to a Pharmacia Mono P column in 3 ml of 20 mM Tris-pH 7.5 (4°C), 2 mM $MgCl_2$, 1 mM DDT and 20% glycerol and eluted with a 20 ml linear (0-0.5 mM KCl) gradient. Peak fractions of protein were collected and immediately concentrated as described in Methods. Fractions were assayed using 4 µg of eluted protein and 0.6 µg of crude rabbit eIF-4E as described in Methods. Panel A. The elution profile of proteins (A_{280}) is shown with the peak fractions assayed identified by numbers. The KCl gradient is depicted by the broken line. Panel B. The autoradiogram of SDS-PAGE analyzed incubation samples following a 20 h exposure is shown. The first 3 lanes represent crude eIF-4E alone, starting material alone, and starting material plus eIF-4E, respectively. Numbered pairs of incubations without (-) and with (+) crude eIF-4E represent assays of eluted protein: peak 1 from panel A (lane 1), peak 2 (lane 2), peak 3 (lane 3), peak 4 (lane 4), peak 5 (lane 5), peak 6 (lane 6), and peak 7 (lane 7). The location of phosphorylated eIF-4E is labeled p25.

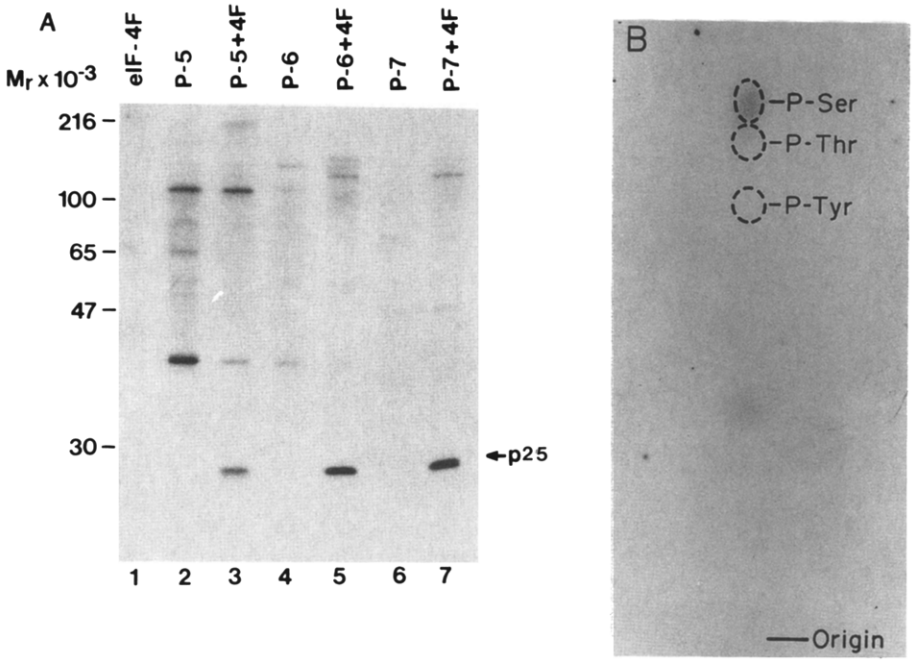

Figure 4. The kinase activity identified phosphorylates p25 of eIF-4F but not p220 of eIF-4F. Panel A. Rabbit reticulocyte eIF-4F (1.6 µg) was incubated with kinase as described in Methods. The autoradiogram shown was obtained after a 7 h exposure. Each lane represents: eIF-4F alone (lane 1), peak 5 of Fig. 3 panel A alone (lane 2) and plus eIF-4F (lane 3), peak 6 alone (lane 4) and plus eIF-4F (lane 5), peak 7 alone (lane 6) and plus eIF-4F (lane 7). Panel B. eIF-4E kinased under conditions similar to those is Panel A was eluted from gels, hydrolyzed and analyzed by thin layer electrophoresis as described in Methods. An autoradiogram of phosphoamino acids and the location of standards is shown here.

contaminant that appeared to phosphorylate p220 of eIF-4F (Fig. 2B, lanes 5-8). Protein eluted from the column with ATP and KCl (63 mg) represented <1% of the starting material (11.3 g). Purification of the kinase activity was approximately 100-fold after the first step as estimated by densitometric quantitation of autoradiograms (Table I).

An additional HPLC step resulted in preparations that phosphorylated p25 of eIF-4F but none of its other subunits. This is an important point because protein kinase C phosphorylates both p220 and p25 of eIF-4F (24). A weak anion exchange column (Mono P HR 5/20) produced a 4-fold increase in enzyme specific activity (Fig. 3 and Table I). To conserve highly purified eIF-4F, crude rabbit reticulocyte eIF-4E was used as a substrate in these assays and accounted for many of the high molecular weight phosphoproteins observed in the plus eIF-4E lanes. While a trace amount of kinase activity was present in peak 1 (Fig. 3B, lane 4), the majority was identified in the high salt fractions. Incubation of these fractions with highly purified eIF-4F demonstrated the presence of a kinase activity that phosphorylated p25 but not p220 or p48 of eIF-4F (Fig. 4). This distinguishes this kinase, tentatively called 4E kinase, from protein kinase C which phosphorylates p220 at a more rapid rate than p25 of eIF-4F (24).

Initial results indicate that the nonabundant kinase acitivity described here is not one of the well characterized serine protein kinases that have already been purified from rabbit reticulocytes. The 4E kinase did not use [γ-^{32}P]GTP as a phosphate donor (not shown) nor did the cAMP dependent protein kinase inhibitory peptide IAAGRTGRRNAIHDILVAA influence its ability to phosphorylate eIF-4E (not shown) (25-27). Additional studies using highly purified protein kinases prepared in other laboratories did not identify one that phosphorylated eIF-4E *in vitro* at a similar rate (24). Protein kinase C and cAMP dependent protein kinase phosphorylate p220 of eIF-4F at a more rapid rate than p25 of eIF-4F (24). The kinase activity described here phosphorylates serine residue(s) of eIF-4E and could be separated from kinases that phosphorylated p220 of eIF-4F

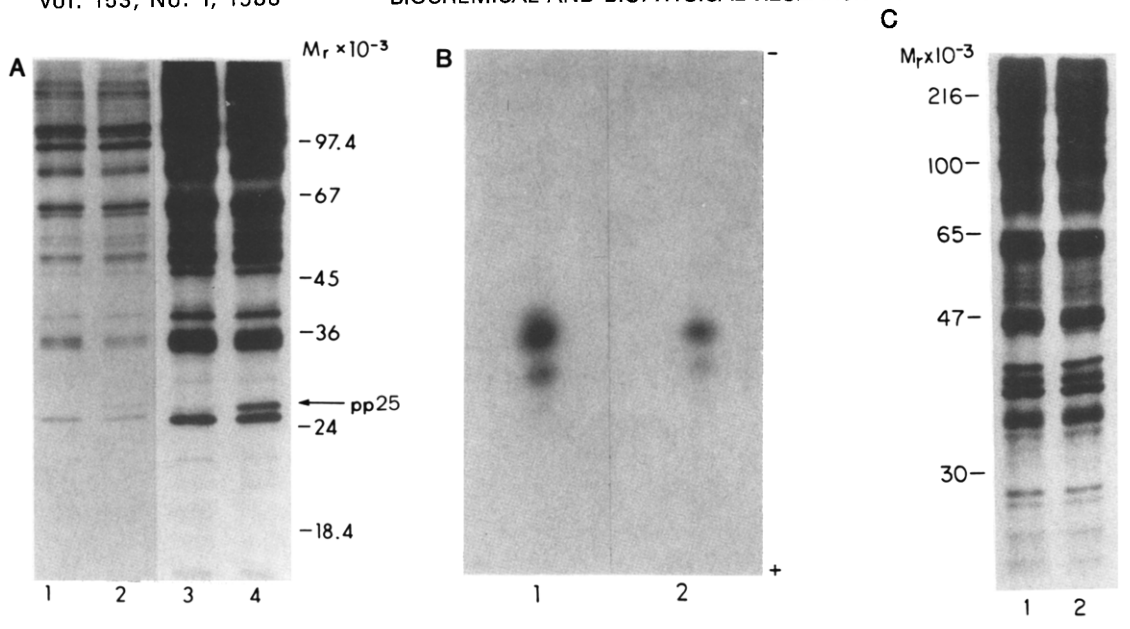

Figure 5. The only apparent substrate in RSW detected was eIF-4E. Panel A. Incubations were as described in Methods and the autoradiogram shown obtained following 7 h (lanes 1 & 2) and 24 h (lanes 3 & 4) exposures. Incubations contained 370 μg of RSW alone (lane 1 & 3) and RSW plus 2 μg of HPLC prepared kinase (lanes 2 & 4). The location of purified eIF-4E in an adjacent lane identified by Coomassie Blue staining is labeled p25. Panel B. The relative pI of phosphorylated p25 of eIF-4F and the 25-28 kDa phosphoprotein induced in kinase treated RSW is shown. Phosphorylated p25 from 3.5 μg of kinase treated eIF-4F (lane 1) and phosphorylated 25-28 kDa protein from 12 μg of kinase treated RSW (lane 2) were separated by SDS-PAGE, identified by autoradiography, cut from the gel and analyzed by IEF and autoradiography as described in Methods. Panel C. Incubations contained 100 μg of reticulocyte ribosomes alone (lane 1) and ribosomes with 2 μg of kinase (lane 2). Incubations were for 10 min as described in Methods and the autoradiogram was obtained following a 24 h exposure.

again indicating that it is unlikely to be either protein kinase C or a cAMP dependent protein kinase (Fig. 4). When reticulocyte RSW, a source of all initiation factors purified to date, was incubated with preparations of 4E kinase the only apparent substrate phosphorylated was 25-28 kDa. Analysis of this 25-28 kDa phosphoprotein by isoelectric focusing demonstrated that it had the same pI as ^{32}P-labeled p25 of eIF-4F prepared using 4E kinase (Fig. 5). In addition, Cleveland peptide mapping of the ^{32}P-labeled substrate in RSW produced the same pattern of phosphopeptides obtained with p25 of eIF-4F phosphorylated *in vitro* and digested with V8 protease (not shown). These results indicate that eIF-4E was the only apparent substrate for 4E kinase in RSW. Incubation of 4E kinase preparations with ribosomes failed to demonstrate phosphorylation of a 32 kDa protein indicating that this kinase is unlikely to be one of the recently purified S6 kinases (Fig. 5C) (28-32).

In summary, we have identified a protein kinase activity in rabbit reticulocytes that phosphorylates eIF-4E and p25 of eIF-4F and have tentatively named it 4E kinase. It does not appear to be a protein kinase previously characterized in rabbit reticulocytes (33). Although this activity appears to be nonabundant, we have not excluded the possibility of an inhibitory molecule masking some of this activity. Further purification will enable the molecular identity of the 4E kinase to be established and its regulation understood. This will also provide a tool for studying the effect(s) of eIF-4E phosphorylation on the assembly of the eIF-4F complex, possible protein-protein interactions with other eIFs and eIF-4F catalyzed denaturation of mRNA structure. Two physiologic conditions where the regulation of this protein kinase may be of biologic interest are the heat shock response and mitosis in mammalian cells (13,34).

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