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Induction of eIF-4E phosphorylation by the addition of L-pyrroline-5-carboxylic acid to rabbit reticulocyte lysate

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Addition of L-pyrroline-5-carboxylic acid to reticulocyte lysates inhibits protein synthesis and induced phosphoproteins of 25 and 14 kDa. The 25 kDa phosphoprotein had the same M_r and p*I* as phosphorylated eIF-4E. Incubation of lysates with L-pyrroline-5-carboxylic acid did not alter the crosslinking of eIF-4E to reovirus mRNA caps. These results suggest that modifications of the translational apparatus other than eIF-4E phosphorylation may mediate the inhibitory effect seen with L-pyrroline-5-carboxylic acid and/or that phosphorylation of eIF-4E may effect functions subsequent to its interaction with the mRNA cap such as protein-protein interactions with other cap-specific translation factors.

eIF-4E; eIF-4F; mRNA cap; L-pyrroline-5-carboxylic acid; Translation; Glutathione

1. INTRODUCTION

Studies on rabbit reticulocyte lysates indicate that the nonprotein amino acid P5C, or a compound generated during its presence in lysates, decreases protein synthesis in an apparent mRNA selective manner [1]. P5C is a naturally occurring nonprotein amino acid that is present in human plasma and undergoes physiologic changes in concentrations with diet [2–4]. In addition, it has been indirectly shown to alter cellular $NADP^+$/NADPH ratios in intact cells, to affect purine synthesis in some cells by increasing the carbon atom flux through the oxidative limb of the pentose pathway and increasing PP-ribose-P synthesis, to be an intermediate in a proposed NADPH linked hydride ion shuttle that can be reconstituted from liver, and to possibly play a role in the symbiotic metabolic relatonship between bacteroids and plant cells in N_2-fixing soybean root nodules [5–11].

The mechanism(s) of the effect on protein synthesis observed in lysates containing P5C remain unknown [1]. Changes in the $NADP^+$/NADPH couple in these lysates may alter the activity of a protein kinase regulated by thio/disulfide status and affect the phosphorylation state of a key initiation factor [12–14]. GSSG has already been shown to induce the phosphorylation of eIF-2α presumably by activating the hemin regulated eIF-2α kinase [13,14]. Alternatively, changes in the thiol/disulfide status of a factor(s) or the oxidation reduction state of NADP(H) bound to an initiation factor might account for part of the observed effects [15–17]. Although several studies provide evidence that the thiol/disulfide status of some

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Abbreviations: eIF-4F, eukaryotic initiation factor-4F (p25, p48 and p220 subunits); eIF-4E, eukaryotic initiation factor-4E (isolated p25); eIF-2, eukaryotic initiation factor-2; eIF-4A, eukaryotic initiation factor-4A; eIF-4B, eukaryotic initiation factor-4B; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; DTE, dithioerythritol; P5C, L-pyrroline-5-carboxylic acid; PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate

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component(s) of the translational machinery alters its catalytic activity this has proven to be a difficult problem to solve [15,18–20]. The studies reported here were done to determine if phosphorylation of an initiation factor might explain the inhibitory effects on protein synthesis observed in lysates containing P5C.

2. MATERIALS AND METHODS

2.1. *Materials*

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. eIF-4F and eIF-2 were purified from rabbit reticulocytes as described previously [21,22]. Untreated rabbit reticulocyte lysates and P5C were prepared as described elsewhere [1,23,24].

2.2. *Phosphorylation studies*

Incubations were 25 μl in volume, had the quantity of reticulocyte lysate specified in legends and contained: 0.5 μmol Hepes, pH 7.4, 2.5 μmol KCl, 12.5 nmol $MgCl_2$, 1.5 units creatine phosphokinase, 0.375 μmol creatine phosphate, 12.5 μmol (40 μCi) [γ-^{32}P]ATP, 0.35 nmol hemin and 0.625 nmol each of 19 amino acids (minus methionine). Other additions are specified in legends. Lysate was added to start reactions which were incubated at 30°C for 1 min before [γ-^{32}P]ATP was added. Samples (5 μl) were taken at the specified times, analyzed by 10% SDS-PAGE, stained with Coomassie blue and used to prepare autoradiograms as described elsewhere [1].

2.3. *Isoelectric focusing*

Slices of 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. Vertical slab isoelectric focusing gels (0.75 mm thick) were 4% polyacrylamide (30/1.6 BIS), 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) [25]. The cathode solution (20 mM NaOH) was degassed and the anode solution was 10 mM phosphoric acid. After prefocusing, proteins were applied to the cathode end of gels and focused as described in legends to figures. Optimal IEF conditions were established using protein standards (FMC Corporation, Rockland, ME).

2.4. *Crosslinking of reticulocyte proteins to ^{32}P-labeled mRNA caps*

Reticulocyte lysate (11 μl) was incubated in a final volume of 25 μl with 30 mM Hepes, pH 7.6, 140 mM KCl, 3 mM DTE, 5 mM Mg $[C_2H_3O_2]_2$, hydrolyzed poly[U] and [^{32}P]cap-labeled reovirus mRNA as described previously [26,27]. [^{32}P]cap-labeled reovirus mRNA was prepared using [α-^{32}P]GTP as described elsewhere [27]. Incubations had P5C, m^7GDP (Pharmacia), or purified rabbit eIF-4F added as described in the legends, were performed at 30°C for the specified times and then treated with $NaBH_3CN$ followed by RNase A digestion [27]. Crosslinked proteins were identified by SDS-PAGE and autoradiography.

3. RESULTS AND DISCUSSION

To determine if P5C might induce the phosphorylation of eIF-2α the same lysates initially used to characterize the P5C effect were studied [1]. Incubations were essentially the same as those used for cell-free translations except that [γ-^{32}P]ATP was present in all incubations and GSSG or P5C in those specified. As expected, the addition of GSSG to such incubations induced the phosphorylation of a 36 kDa protein that co-migrated with purified eIF-2α (fig.1) [13,14]. However, the unexpected result was the induction of 25 and 14 kDa phosphoproteins in lysates containing P5C. Induction of both phosphoproteins was seen at 2 and 5 min of incubation in lysates containing P5C but not in control or GSSG-treated lysates (fig.1). With shorter exposures no differences were seen in the high molecular mass regions (fig.1B). The induction of 25 and 14 kDa phosphoproteins by the addition of P5C was observed 5 times in the lysate preparation used for these studies but was not observed in two other lysates.

The following studies were done to determine if the 25 kDa phosphoprotein was the mRNA cap binding protein (eIF-4E). Addition of purified eIF-4F to lysates containing P5C increased the quantity of the 25 kDa phosphoprotein (fig.2). On close inspection a less abundant phosphoprotein with the same M_r was also seen in control incubations (fig.2, lane 1). To investigate further the identity of the 25 kDa phosphoprotein induced by P5C, purified rabbit reticulocyte eIF-4F was radiolabeled with [^{14}C]HCHO and used as a standard. The p25 subunit of eIF-4F (both unlabeled and ^{14}C-labeled) had the same M_r as the phosphoprotein designated pp25 (fig.2). IEF analysis of both [^{14}C]p25 (from eIF-4F) and pp25 induced in lysates containing P5C showed that pp25 had the same p*I* as phosphorylated p25 from eIF-4F (fig.3). We conclude that pp25 induced in the reticulocyte lysates containing P5C was phosphorylated 25 kDa mRNA cap binding protein. eIF-4E has been shown to be phosphorylated in both rabbit reticulocytes and HeLa cells [28–30].

To determine if the function of eIF-4E was modified in lysates incubated with P5C we determined the ability of endogenous eIF-4E to crosslink to oxidized reovirus mRNA caps. No difference in the quantity of eIF-4E crosslinked to

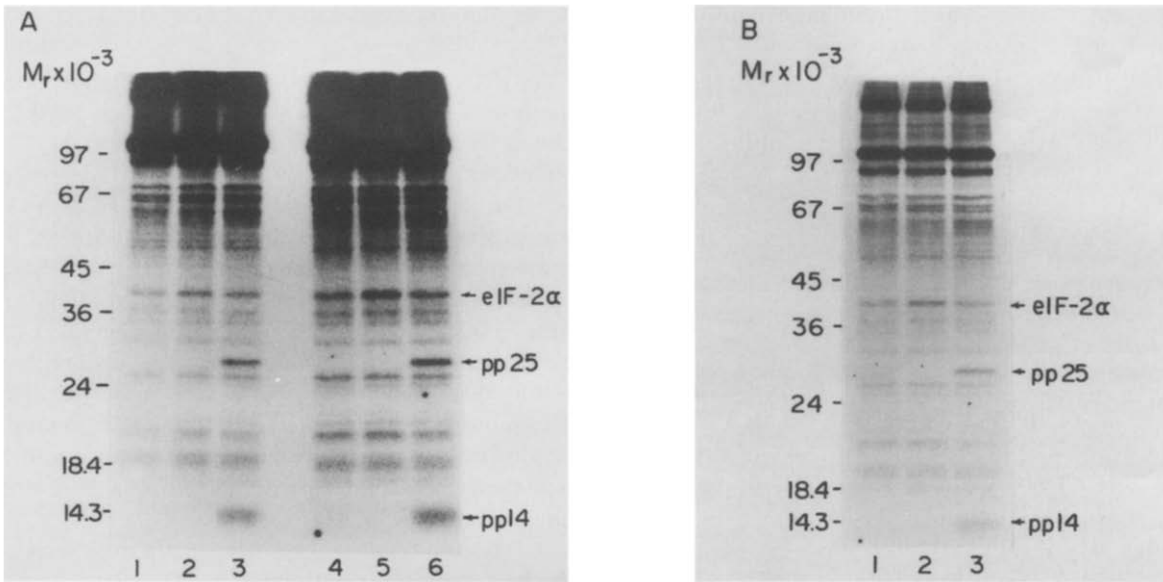

Fig.1. Induction of pp25 and pp14 in rabbit reticulocyte lysate containing L-P5C. In vitro phosphorylation studies using [γ-^{32}P]ATP were done in a total volume of 25 μl and contained 14 μl of reticulocyte lysate as described in section 2. Incubations contained no additions (control) or a final concentration of 1 mM GSSG or 1 mM P5C. Samples were taken after 2 min (lanes 1–3), and 5 min (lanes 4–6) of incubation at 30°C and analyzed by SDS-PAGE. (A) The autoradiogram following a 26 h exposure with the location of molecular mass markers is shown. Lanes 1, 2 and 3 contained samples taken at 2 min from control, GSSG- and P5C-treated incubations, respectively. Lanes 4, 5 and 6 contained 5 min samples from control GSSG- and P5C-treated incubations, respectively. The relative M_r of the α-subunit of eIF-2 was determined by Coomassie blue staining of purified eIF-2 in a similar gel with reticulocyte lysate in an adjacent lane. The relative position of eIF-2α is indicated and the phosphoproteins induced are labeled pp25 and pp14. Molecular mass markers used were phosphorylase *b* (97.4 kDa), bovine albumin (67 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa), β-lactoglobin (18.4 kDa) and lysozyme (14.3 kDa). (B) An exposure of lanes 4, 5 and 6 from panel A without an intensifying screen is shown to demonstrate the higher molecular mass phosphoproteins obscured in A. Lanes 1, 2 and 3 represent lanes 4, 5 and 6 from A, respectively.

^{32}P-caps of L class reovirus mRNA was detected in the presence or absence of P5C (fig.4). Preincubation of lysates with P5C for up to 40 min with or without exogenous ATP prior to the addition of cap-labeled mRNA (reovirus class S_3, S_4 and M_3) also failed to show any effect of P5C on crosslink-

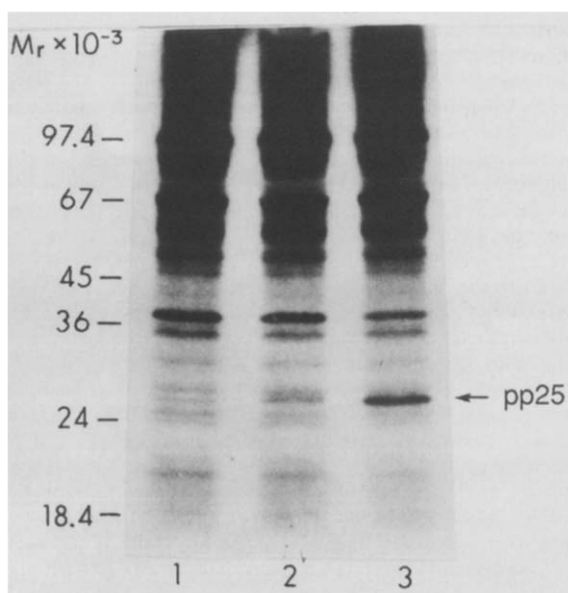

Fig.2. Addition of purified eIF-4F increased the quantity of pp25 in P5C-treated lysates. Incubations with [γ-^{32}P]ATP were identical to those in fig.1 except that they contained only 8 μl of lysate, had a final concentration of 8 μM hemin and where indicated less than 100 nmol P5C and/or 3 μg of eIF-4F. Incubations were performed as described in section 2 and 5 μl samples taken at 5 min were analyzed by SDS-PAGE. The autoradiogram shown was obtained after 48 h. Lanes: 1, control (no additions); 2, 1 mM P5C; 3, 1 mM P5C plus eIF-4F. Purified eIF-4F analyzed in an adjacent lane and stained with Coomassie blue demonstrated that the phosphoprotein designated pp25 co-migrated with the p25 subunit of eIF-4F.

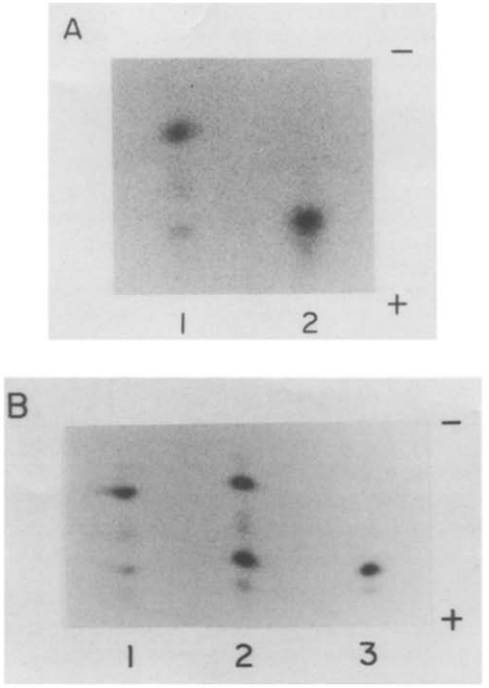

Fig.3. Isoelectric focusing analysis of [^{14}C]p25 of eIF-4F, endogenous pp25 from P5C-treated lysates and ^{32}P-labeled p25 of eIF-4F. (A) The ^{32}P-labeled pp25 from an incubation containing P5C and the ^{14}C-labeled p25 from a sample similar to that shown in lane 1 of fig.3 were cut from the dried gels. Each sample was rehydrated and analyzed by equilibrium isoelectric focusing as described in section 2. After focusing the gel was fixed, dried and exposed to film at −70°C without an intensifying screen. The autoradiogram shown was obtained following a 10 day exposure. The radiolabeled protein distribution following isoelectric focusing of the ^{14}C-labeled p25 of eIF-4F standard and the endogenous ^{32}P-labeled pp25 from lysate containing P5C are shown respectively in lanes 1 and 2. The anode and cathode regions of the gel are indicated by (+) and (−), respectively. The p*I* of the anodic component of the ^{14}C-labeled reference was approximately 5.9. (B) Isoelectric focusing of ^{32}P-labeled p25 in lysates containing P5C and exogenous ^{14}C-labeled and unlabeled eIF-4F. The p25 proteins from incubations equivalent to those shown in lanes 1, 2 and 3 of fig.3 were analyzed by IEF as described for panel A. The autoradiogram shown was obtained after a 20 day exposure without an intensifying screen where a ^{14}C-labeled p25 of eIF-4F reference (lane 1), p25 from a lysate with P5C, [^{14}C]eIF-4F and [γ-^{32}P]ATP (lane 2) and p25 from a lysate with P5C and unlabeled eIF-4F (lane 3) are shown.

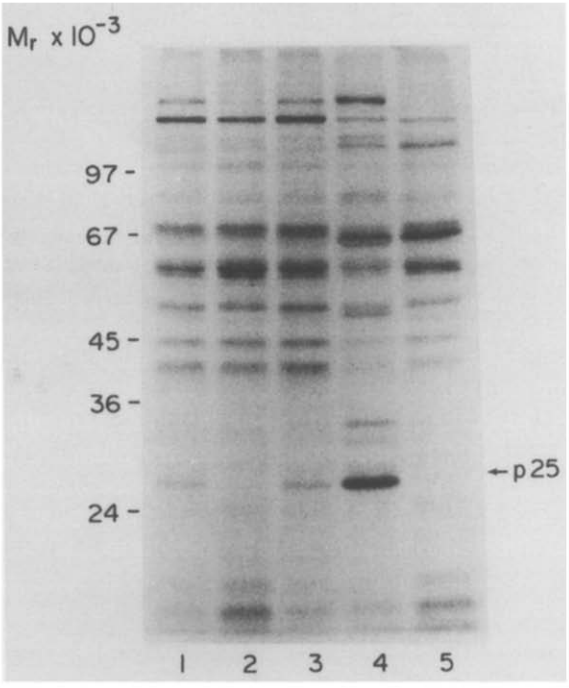

Fig.4. eIF-4E crosslinking to ^{32}P-labeled caps of reovirus mRNA in reticulocyte lysates containing P5C. Reticulocyte lysate and cap-labeled mRNA (L class) were preincubated at 30°C for 10 min with or without 1.5 mM P5C, then treated with $NaBH_3CN$ followed by RNase A digestion and analysis by SDS-PAGE and autoradiography (see section 2). The autoradiogram shown was obtained after a 4 day exposure and represents proteins crosslinked to labeled mRNA caps. Incubations were: control (lane 1), plus 0.9 mM m^{7}GDP (lane 2), plus 1.5 mM P5C (lane 3), plus 1.4 μg eIF-4F (lane 4) and plus eIF-4F and m^{7}GDP (lane 5).

ing of eIF-4E to mRNA caps (not shown). In addition to eIF-4E, we consistently observed an approx. 160 kDa protein present in lysates and eIF-4F preparations whose crosslinking to mRNA caps was inhibited by m^{7}GDP (fig.4) [26,31]. The system used for these studies was not saturated with regard to eIF-4E because addition of purified eIF-4F increased the signal of the radiolabeled 25 kDa protein (fig.4, lane 4). These results indicate that the phosphorylation of eIF-4E induced by P5C does not alter the ability of eIF-4E to interact with the mRNA cap structure. The mechanism by which eIF-4E phosphorylation was induced in lysates containing P5C remains unclear, but seems likely to involve a recently identified protein kinase that phosphorylates eIF-4E [32–34]. In addition, the identity of pp14 is not certain at this time. If pp14 is a proteolytic product of pp25 then it lacks the ability to interact with the reovirus cap structure (fig.4).

In view of the current suggestion that dephosphorylation of eIF-4E following heat shock or during mitosis in some way inhibits protein synthesis it is difficult to reconcile the induction of eIF-4E phosphorylation and the apparent mRNA selective inhibitory effect seen in lysates containing P5C [1,29,35]. This leads us to suggest that there might be additional undetected modifications of either eIF-4E, eIF-4F or other key translational components that are participating in this inhibitory phenomenon. Moreover, phosphorylation or possibly other undetected modifications may not alter cap recognition by eIF-4E but may instead affect subsequent steps such as protein-protein interactions with the p220 subunit of eIF-4F. Alternatively, interactions between eIF-4E and eIF-4A or eIF-4B might be altered.

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