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RABBIT RETICULOCYTE DOUBLE-STRANDED RNA-ACTIVATED PROTEIN KINASE AND THE HEMIN-CONTROLLED TRANSLATIONAL REPRESSOR PHOSPHORYLATE THE SAME M_r 1500 PEPTIDE OF EUKARYOTIC INITIATION FACTOR 2α

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1. Introduction

The inhibition of polypeptide chain initiation that occurs when rabbit reticulocyte lysate is incubated in the absence of hemin [1–5] or in the presence of a low level (10–100 ng/ml) of dsRNA [1,6–8] appears to be mediated by the phosphorylation of the M_r 35 000 (α) subunit of eIF-2, the initiation factor that promotes binding of Met-tRNA$_f$ to 40 S ribosomal subunits [9–13], but involves the action of two distinct protein kinases [14]. The inhibitor formed in the absence of hemin (HCR) occurs in the post-ribosomal supernate, is rapidly activated by incubation with NEM [15], and is associated with the autophosphorylation of a M_r 90 000 100 000 protein [1,16 19], whereas the dsRNA-activated protein kinase (dsI) is found in the ribosomal fraction, is activated by dsRNA, and is associated with the phosphorylation of a M_r 67 000 protein(s) [1,7,8,20]. Peptide analyses of eIF-2α, phosphorylated either by HCR or dsI, have shown a similar pattern of phosphopeptides suggesting that these two protein kinases phosphorylate the same site or sites [20–23]. When eIF-2α, phosphorylated by HCR, is subjected to exhaustive trypsin digestion, almost all the phosphate can be localized to a single, M_r 1500 peptide [24]; we report here that this same single peptide is phosphorylated by dsI.

Abbreviations: dsRNA, double-stranded RNA; eIF, eukaryotic initiation factor; HCR, hemin-controlled translational repressor; NEM, *N*-ethylmaleimide; dsI, double-stranded RNA-activated protein kinase; SDS, sodium dodecyl sulfate, Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; poly(I · C), double strand polyinosinic acid · polycytidylic acid

2. Methods

Cell-free protein synthesizing samples contained 25 μl reticulocyte lysate in 55 μl final vol. and at 20 μM hemin final conc. Additional details have been described in [25]. HCR was highly purified as in [26]. It had 0.13 mg protein/ml and spec. act. 54 000 units/mg, where one unit represents the amount required to inhibit by 50% the difference in protein synthesis between no HCR and an amount that gives maximal inhibition when incubation is at 34°C for 90 min. The precursor of HCR (prorepressor) was partially purified as in [16]. It contained 2.5 mg/ml and had, following activation by NEM, spec. act. 25 000 units/mg. Soluble eIF-2 was purified from the post-ribosomal supernatant fraction as in [27]. It was 76% pure when analyzed on SDS–polyacrylamide gels.

The precursor of dsI (latent dsI) was partially purified from the 0.50 M KCl wash of rabbit reticulocyte ribosomes, prepared as in [28]. The protein fraction that did not adsorb to DEAE-cellulose at 0.05 M KCl [28] was precipitated with 65% ammonium sulfate. The precipitate was dissolved in a minimal volume of 0.10 M KCl in HDE buffer (6 mM Hepes (pH 7.15), 1 mM dithiothreitol, 0.1 mM EDTA) and dialyzed against 0.10 M KCl in HDE overnight. Protein (~130 mg) was applied to a 1.5 × 8 cm phosphocellulose column, equilibrated in 0.10 M KCl in HDE, followed by extensive washing with 0.10 M KCl in HDE. The column was then developed with a linear gradient of 110 ml 0.20 M to 110 ml 0.60 M KCl in HDE. The fractions eluting at ~0.20–0.25 M KCl contained the latent dsI and were pooled to 3.0 mg protein/ml final conc.

To prepare phosphorylated eIF-2α for peptide anal-

ysis, 10 μg eIF-2 were incubated in 6 mM Hepes (pH 7.15), 0.05 M KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.06 mM [γ-^{32}P]ATP (initial spec. act. 20 Ci/mmol), and either 0.25 μg HCR or 15 μg latent dsI plus 3 ng poly(I · C) in 30 μl final vol. for 25 min at 34°C. After incubation, each sample was subjected to electrophoresis on a 0.6 × 10 cm, 7% SDS–polyacrylamide gel for 7 h, the protein was eluted from 1 mm gel slices, and the ^{32}P-labeled, α-subunit was pooled [24]. Each sample was then digested with 100 μg trypsin/ml for 5 days at 37°C in 0.02 M Tris–HCl (pH 8.1), 0.02 M KCl, 1 mM $CaCl_2$, ~0.50 ml final vol. An additional 100 μg trypsin/ml was added after each 24 h interval. Following digestion, each sample was desalted on Sephadex G-15, equilibrated in 0.02 M NH_4HCO_3, lyophilized, and dissolved in 20 μl H_2O. Small aliquots were then analyzed by electrophoresis on 20% SDS–polyacrylamide gels and by two-dimensional fingerprint analysis on thin-layer cellulose sheets as in [24].

Materials were obtained as in [24], except that the cellulose sheets were from E. Merck, and poly(I · C) was from P. L. Biochemicals.

3. Results

To verify that the phosphorylation of eIF-2α by the partially purified dsI was indeed due to a protein kinase activity distinct from HCR, we tested the effect of poly(I · C), NEM, and no treatment on the activities of latent dsI and the prorepressor (fig.1). Incubation of the prorepressor with [γ-^{32}P]ATP (lane 5) showed some labeling of a band migrating at ~M_r 90 000, corresponding to the autophosphorylation of HCR [1,16–19]. Preincubation of the prorepressor with NEM (lane 6) enhanced the phosphorylation of this band, but incubation with poly(I · C) (lane 7) did not increase labeling of this band and showed no phosphorylation of a M_r 67 000 component. In contrast, preincubation of latent dsI with NEM and then incubation with [γ-^{32}P]ATP and eIF-2 (lane 4) showed no change from eIF-2 incubated with [γ-^{32}P]ATP alone (lane 1); in neither case did the M_r 35 000 (α) subunit of eIF-2 become phosphorylated. Incubation of latent dsI with poly(I · C), eIF-2, and [γ-^{32}P]ATP (lane 3), however, showed considerable phosphorylation of an M_r 67 000 component and the M_r 35 000 subunit of eIF-2. Little phosphorylation of these components occurred when poly(I · C) was omitted (lane 2). The samples in lanes 1–4 did show a faint labeled band in the M_r 90 000 region that does not represent HCR, since it was not increased with NEM and it did not promote phosphorylation of eIF-2α (lane 4).

The α subunit of eIF-2, phosphorylated with either dsI or HCR, was isolated by electrophoresis on and elution from 7% SDS–polyacrylamide gels. Analysis

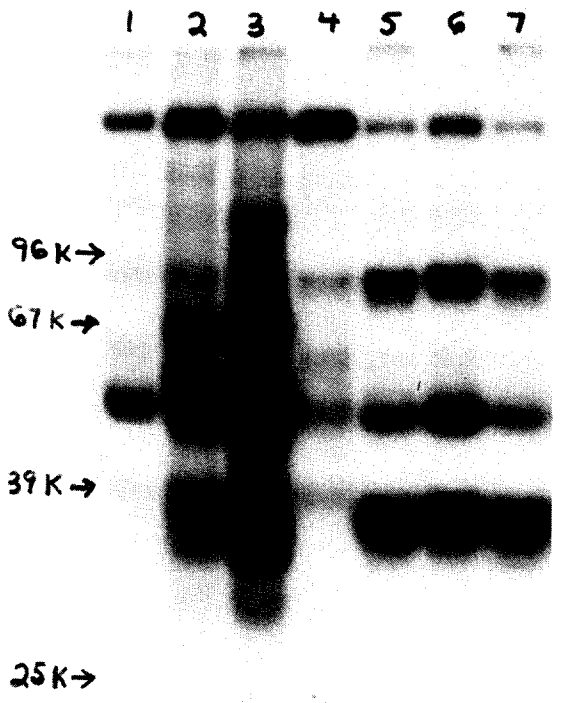

Fig.1. Effect of poly(I · C) and NEM on protein kinase activities of latent dsI and the prorepressor. Protein kinase samples, prepared and incubated as in section 2 (spec. act. [γ-^{32}P]ATP was 4 Ci/mmol), contained the following additions in 20 μl final vol.: (lanes 1–7) 1 μg eIF-2; (lane 2) 15 μg latent dsI; (lane 3) 15 μg latent dsI + 2 ng poly(I · C); (lane 4) 15 μg latent dsI, preincubated with 5 mM NEM for 5 min at 34°C and then with 5 mM dithiothreitol for 5 min more at 34°C; (lane 5) 2 μg prorepressor; (lane 6) 2 μg prorepressor, preincubated with 5 mM NEM for 5 min at 34°C and then with 5 mM dithiothreitol for 5 min more at 34°C; (lane 7) 2 μg prorepressor + 2 ng poly(I · C). Samples were electrophoresed on a 16 cm long × 0.15 cm thick, 7% polyacrylamide–SDS slab gel for 9 h at 90 V. The gel was stained with Coomassie blue, destained, then autoradiographed. M_r values were determined from molecular weight markers run in parallel lanes.

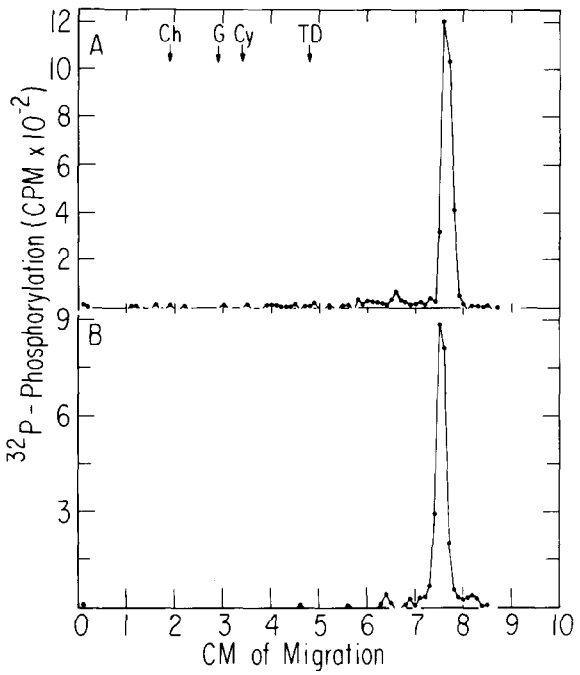

Fig.2. Analysis of tryptic digests on 20% SDS–polyacrylamide gels. Aliquots of tryptic digests of eIF-2α, phosphorylated by dsI (A) or HCR (B), were analyzed on 20% SDS–polyacrylamide disc gels [24]. The gels were immediately sliced at 1 mm intervals, and the Cerenkov radioactivity was determined as in [24,27]. The arrows at the top indicate the migration of standards run on parallel gels: Ch, chymotrypsinogen; G, globin, Cy, cytochrome C; TD, pyronin Y tracking dye.

of small aliquots of the protein kinase reactions on parallel 7% gels, that were fixed, stained, scanned, then counted as in [24,27] indicated that ~0.6 mol phosphate were incorporated/mol protein in each case. Each α-subunit preparation was then subjected to extensive trypsin digestion as in section 2. When aliquots of each were analyzed on 20% SDS–polyacrylamide gels (fig.2), they showed that ~90% of the total radioactivity migrated as a single low M_r band that appeared identical whether phosphorylation had been with dsI (fig.2A) or HCR (fig.2B). Chromatography of each trypsin digest on a calibrated Sephadex G-25 column showed an identical elution pattern with an estimated M_r of the phosphopeptide of 1500 ([24], not shown). Finally, when each digest was analyzed in two dimensions on thin-layer cellulose sheets (fig.3), the same, single phosphopeptide was identified whether derived from eIF-2α phosphorylated with dsI (fig.3A) or HCR (fig.3B).

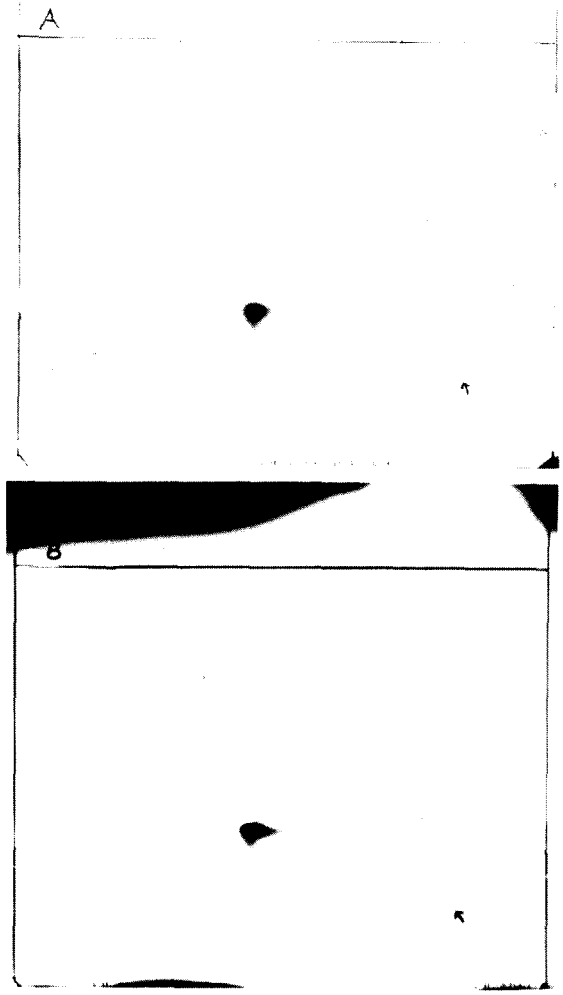

Fig.3. Fingerprint analysis of tryptic digests. Aliquots of tryptic digests of eIF-2α, phosphorylated by dsI (A) or HCR (B), were spotted onto thin layer cellulose sheets (arrow), electrophoresed in 0.69 N formic acid at 500 V for 70 min (to the left) in a Savant TLE-20, and then chromatographed for 3.5 h in *n*-butanol:pyridine:acetic acid:water (3:1:1:2, by vol.) (toward the top with the upper line representing the solvent front). The sheets were dried and then autoradiographed.

4. Discussion

Our findings indicate that the phosphorylation of the α subunit of eIF-2 by either dsI or HCR can be localized to the same M_r 1500 peptide, suggesting that these two protein kinases phosphorylate eIF-2α at the same single site. Work in progress is directed at determining whether this phosphorylation is into a single serine residue and whether the same serine(s) is (are)

phosphorylated by dsI and HCR. Our findings are consistent with analyses [20–23], which demonstrated that eIF-2α, phosphorylated by HCR or dsI and then digested with protease, gives the same phosphopeptide pattern. We have been able to demonstrate, however, that with more complete trypsin digestion, this phosphorylation can be resolved into a single M_r 1500 peptide [24]. These findings thus extend previous observations which indicated that the inhibition of protein synthesis in reticulocyte lysate by hemin deprivation or dsRNA, though due to the activation of different protein kinases, appears to be due to the same mechanims. Although there is some uncertainty as to how phosphorylation of eIF-2α by either HCR or dsI promotes inhibition of polypeptide chain initiation, inhibition does involve, at least in part, reduced binding of Met-tRNA$_f$ to 40 S ribosomal subunits [1,29–34]. In [35] we have shown that inhibition by HCR involves the accumulation of mRNA-containing, 48 S initiation complexes, suggesting that the rate of 60 S subunit addition and 80 S complex formation is also reduced. We have found that inhibition by dsRNA has a similar effect (M. G., D. A. Kaplansky, unpublished), strengthening the concept that HCR and dsI have the same mechanism of action.

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