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Regulation of double-stranded RNA-activated eukaryotic initiation factor 2α kinase by type 2 protein phosphatase in reticulocyte lysates

(inhibition of protein chain initiation/phosphorylation–dephosphorylation of eukaryotic initiation factor 2α/translational control)

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ABSTRACT **Protein synthesis initiation in reticulocyte lysates is inhibited by low concentrations (1–20 ng/ml) of double-stranded RNA (ds RNA) due to the activation of a ds RNA-dependent cAMP-independent protein kinase (ds I) that phosphorylates the α subunit of the eukaryotic initiation factor eIF-2. In lysates, ds I is present in the latent inactive form and is associated with the ribosome complement. Latent ds I is solubilized by extraction with high-salt buffers and can be purified in its latent form. Activation of purified latent ds I requires ds RNA and ATP and is accompanied by the ds RNA-dependent autophosphorylation of a polypeptide doublet of 70,000 and 72,000 daltons ("70k/72k"), which represent different phosphorylated states of the same polypeptide. These are phosphorylated in the sequence 70k→72k; increased phosphorylation of 72k is associated with increased ds I activation. Lysates (or Sepharose 6B ribosomes) treated with ds RNA display a similar ds I phosphoprotein profile, and this is accompanied by the phosphorylation of endogenous eIF-2α (38,000 daltons). Delayed ^{32}P pulses in ds RNA-inhibited lysates indicate that the phosphates on ds I and eIF-2α turn over. Under defined conditions, activated ds I in lysates is selectively dephosphorylated by endogenous protein phosphatase(s), and this is accompanied by the dephosphorylation of eIF-2α. Similarly, purified activated ds I is rapidly dephosphorylated by unfractionated lysate protein phosphatase(s) and by type 2 protein phosphatase but not by type 1 protein phosphatase. The dephosphorylation of ds I occurs in the sequence 72k→70k and is correlated with ds I inactivation. The heat-stable protein phosphatase inhibitor-2, which selectively blocks type 1 protein phosphatase, does not significantly affect the dephosphorylation of ds I by type 2 protein phosphatase or by unfractionated lysate phosphatases. The data support the conclusion that a ds I phosphatase activity with type 2 characteristics is involved in the regulation of ds I activity.**

The addition of low levels (1–20 ng/ml) of double-stranded RNA (ds RNA) to reticulocyte lysates results in rapid inhibition of protein synthesis (1–4). The inhibition is largely due to the ds RNA-dependent activation of a specific cAMP-independent protein kinase (ds I) that phosphorylates the α subunit (38,000 daltons) of the eukaryotic initiation factor eIF-2 (eIF-2α) (3–6). A similar inhibition of protein synthesis by heme-regulated eIF-2α kinase (HRI) is observed in heme-deficient lysates (4, 7–10). Although the two eIF-2α kinases are distinct molecular entities, both appear to phosphorylate the same site(s) on eIF-2α (4, 11), a phenomenon that probably accounts for their similar modes of inhibition. A putative model for the mechanism of inhibition is based on the diminished capacity of phosphorylated eIF-2α to interact with other initiation components, thereby impairing its ability to recycle.

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In previous studies (3, 5), we have described some of the parameters of the ribosome-bound ds I and have demonstrated that both latent and active ds I can be extracted from the ribosomes with high-salt buffers (3, 5, 6); we and others have described the purification and characterization of both the latent and activated forms of ds I (5, 6, 12–15). In the course of purification of latent ds I we observed that, at all stages, the activation of ds I requires ds RNA and ATP and is accompanied by the phosphorylation of a polypeptide doublet that migrates in $NaDodSO_4$/polyacrylamide gel electrophoresis as 70,000- and 72,000-dalton components ("70k/72k") (5, 6, 12). During activation, the two polypeptides are phosphorylated in an asymmetric manner. The 70,000 component is phosphorylated first and this is followed by rapid phosphorylation of the 72,000 polypeptide (12), which suggests that 70k is the precursor of 72k and that the two phosphoproteins therefore represent different phosphorylated states of the same polypeptide. In addition, we have noted that the increase in the phosphorylated 72,000 polypeptide is associated with increased ds I activity. The model suggested by these results is that (*i*) purified ds I is most active in a multiply phosphorylated state and (*ii*) activation of purified ds I proceeds through an autokinase mechanism. More significantly, we have found that the activation of endogenous ds I in lysates or Sepharose 6B ribosomes results in a similar phosphoprotein pattern.

The findings that (*i*) the two eIF-2α kinases (HRI and ds I) are activated by phosphorylation both in the purified state and in the lysate and (*ii*) the phosphorylation of eIF-2 in heme-deficient lysates is a reversible process (16–18) emphasize the regulatory roles of protein phosphatases in these inhibitions. It is clear from previous studies, for example, that, in inhibited lysates, there is a relatively rapid turnover of the phosphate on eIF-2α (16, 17), which indicates that the steady-state level of phosphorylated eIF-2α is dependent on the eIF-2α kinase–phosphatase equilibrium.

In the present study, we compare the effects of crude and partially purified rabbit protein phosphatases on the phosphoprotein profile and activity of ds RNA-activated eIF-2 kinase (ds I). We find that (*i*) ds I activated in the whole lysate can be rapidly dephosphorylated by an endogenous ds I phosphatase activity, (*ii*) this dephosphorylation of lysate ds I is accompanied by the rapid dephosphorylation of lysate eIF-2α, (*iii*) treatment of purified activated ds I with unfractionated lysate protein phosphatases results in rapid dephosphorylation of the 72,000

Abbreviations: ds RNA, double-stranded RNA; eIF-2, eukaryotic initiation factor 2; eIF-2α, α subunit of eIF-2; ds I, ds RNA-activated eIF-2α kinase; HRI, heme-regulated eIF-2α kinase; 70k/72k, polypeptide doublet of ds I (referred to as 67K/68.5K in ref. 12); PPase 1 and PPase 2, type 1 and type 2 protein phosphatase; I-2, protein phosphatase inhibitor 2.

polypeptide coupled with a transitory shift to the 70,000 component and a concomitant loss in inhibitory capacity, and (*iv*) the dephosphorylation and inactivation of purified ds I are primarily due to the action of a type 2 protein phosphatase (PPase 2) activity.

METHODS AND MATERIALS

Rabbit reticulocytes and reticulocyte lysates were prepared as described (19). Protein synthesis was assayed in 25-μl incubation volumes as described (20). In two experiments (see Figs. 1 and 2), the creatine~P/creatine phosphokinase energy-regenerating system normally used in protein-synthesizing lysates was replaced by the addition of 1 mM fructose 1,6-diphosphate/0.1 mM NAD^+, which enables lysate glycolysis to generate ATP at a rate sufficient to support linear protein synthesis (11, 21); the further addition of $^{32}P_i$ permits generation of constant-specific-activity [γ-^{32}P]ATP (ref. 11; see Fig. 1 legend). *Penicillium chrysogenum* mycophage ds RNA or reovirus ds RNA (20 ng/ml) was added where indicated. In experiments in which high concentrations of ds RNA were used, poly(I-C) (25 μg/ml) was added; high poly(I-C) does not activate ds I or significantly inhibit protein synthesis (2, 3). pH 5 fractions of protein-synthesis incubation aliquots were prepared as described (11, 18). Phosphoprotein analysis of pH 5 fractions (see Fig. 1 legend) was by $NaDodSO_4$/polyacrylamide gel electrophoresis (0.1% $NaDodSO_4$/10% acrylamide/0.26% bisacrylamide) and autoradiography was as described (7). Protein kinase assays were carried out in 20-μl volumes as described (3, 6) unless otherwise indicated. Phosphoprotein analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography was as above. Latent ds I was prepared as described (6); the step 6 glycerol-gradient latent ds I preparation was generally used. All latent ds I preparations were completely dependent on low ds RNA (20 ng/ml) and ATP (20–50 μM) for activation.

Preparation of PPase 2 from Lysate S100 and Lysate Ribosomes. Lysate S100 (10 ml) was chromatographed on DEAE-cellulose (0.5 × 20 cm) equilibrated with 10 mM Tris·HCl, pH 7.7/2 mM dithiothreitol/0.1 mM EDTA/5% glycerol (buffer A). Stepwise elution was carried out with buffer A containing 0.1 M, 0.2 M, or 0.3 M KCl. Peak fractions of PPase 2, which eluted in the 0.3 M KCl eluant, were concentrated to 1 ml (0.9 mg of protein) and applied to a Sephacryl S-200 column (1.7 × 50 cm) equilibrated with buffer A/50 mM KCl. PPase 2 activity eluted in a sharp peak at a molecular mass of 80,000–90,000 daltons and was concentrated to 30 μg of protein/ml. Generally, 1 to 2 μl (30–60 ng) of this preparation was used directly as the source of soluble PPase 2 (ds I phosphatase). A ribosomal PPase 2 activity from a lysate ribosomal salt wash was similarly purified. Both PPase 2 preparations were free of type 1 protein phosphatase (PPase 1) activity and were insensitive to protein phosphatase inhibitor 2 (22–25), which specifically blocks the action of PPase 1.

Materials. We thank J. Gordon Foulkes (The University of Dundee, Scotland) for preparations of skeletal muscle protein phosphatase inhibitor 2 (90,000 units/mg of protein) (22–24) and PPase 1 (45 units/ml) (26). We thank Joanne Petithory (Brandeis University, Waltham, MA) for preparing [^{32}P]phosphorylase *a* (26, 27) and for determining the phosphorylase phosphatase specific activities (24, 28) of the PPases used in the assays shown in Figs. 4 and 6. We thank Vivian Ernst (Brandeis University) for preparing reticulocyte lysates. *P. chrysogenum* mycophage ds RNA was a gift of Hugh Robertson (Rockefeller University, New York). Reticulocyte eIF-2 (≈70% purity) and reovirus ds RNA were prepared by one of us (D.H.L.). [^{14}C]-Leucine, $^{32}P_i$, and [^{32}P]ATP were purchased from New England Nuclear; poly(I-C) was obtained from Sigma.

RESULTS AND DISCUSSION

^{32}P-Pulse Labeling of ds I and eIF-2α in Reticulocyte Lysates Inhibited by ds RNA. We have previously described a convenient method for the analysis of phosphoprotein profiles directly in reticulocyte lysates inhibited by heme deficiency and ds RNA (11). The method is based on the finding that lysate glycolysis generates ATP at a rate that can support linear protein synthesis without the addition of an ATP generating system (21). Moreover, in the presence of fructose 1,6-diphosphate (1 mM), NAD^+ (0.1 mM), and $^{32}P_i$, lysates generate glycolytic [γ-^{32}P]ATP having a constant specific activity (11). As a consequence, the phosphoprotein profiles for any incubation interval in normal or inhibited lysates can be monitored directly by $NaDodSO_4$/polyacrylamide gel electrophoresis using conditions that clearly distinguish phosphorylated eIF-2α and the phosphoprotein doublet of ds I (the 70,000- to 72,000-dalton doublet). Phosphoprotein profiles at various time intervals of lysates inhibited by addition of ds RNA (20 ng/ml) at the start of incubation are shown in Fig. 1. Pulses of $^{32}P_i$ were added at the times shown; each track represents the profile generated during the indicated interval. A ds RNA-dependent phosphorylation of the 70k/72k doublet of ds I and of eIF-2α (38,000 daltons) is clearly observed after a 0- to 5-min pulse (tracks 1 and 2), a period that precedes the onset of inhibition (3). A 0- to 10-min pulse (tracks 3 and 4) results in maximal phosphate incorporation into both ds I and eIF-2α. The ds RNA-dependent phosphorylation of ds I in the whole lysate displays a 70k/72k doublet similar to that observed *in vitro* with highly purified ds I (6, 12). Moreover, as with purified ds I, the upper (72k) polypeptide of lysate ds I is significantly more highly labeled than the lower (70k) polypeptide, even in the earliest pulse (track 2). A series of delayed $^{32}P_i$ pulses are shown in tracks 5–11. These phosphoprotein profiles indicate that the phosphates in the ds I doublet are being turned over due to the action of endogenous protein phosphatase(s). This is most evident in the 10- to 20-min pulse (tracks 10 and 11). The less highly labeled phosphoproteins observed in all of the profiles of the delayed pulses (tracks 6–11) compared with those of the 0- to 10-min pulse (track 4) represent phosphate moieties that have slower turnover rates. In all of the delayed pulses, the rapid turnover of the phosphate of eIF-2α in inhibited lysates is clearly demonstrated because even the profiles of the most de-

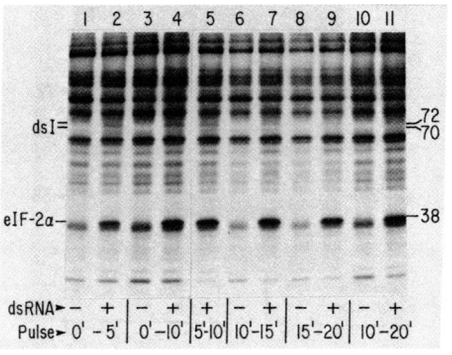

FIG. 1. pH 5 fractionation of phosphoprotein profiles of ds RNA-inhibited lysates pulsed with $^{32}P_i$: Generation of glycolytic ATP. Protein synthesis reaction mixtures (25 μl) were incubated at 30°C with 20 μM hemin, 1 mM fructose 1,6-diphosphate, 0.1 mM NAD^+, and *P. chrysogenum* ds RNA (20 ng/ml) where indicated. Each assay mixture was pulsed with $^{32}P_i$ (100 μCi; 1 Ci = 3.7 × 10^{10} becquerels) for 5- or 10-min intervals as indicated. Aliquots (10 μl) were added to 0.8 ml of 50 mM NaF/4 mM EDTA and immediately made pH 5 with acetic acid (18). The pH 5 precipitates were recovered by centrifugation, dissolved in $NaDodSO_4$ dissociation buffer, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The figure is an autoradiogram.

layed pulses (tracks 7, 9, and 11) display levels of phosphorylated eIF-2α comparable with that in the 0- to 10-min pulse (track 4). These data suggest that phosphates on both ds I and eIF-2α undergo rapid turnover in ds RNA-inhibited lysates.

Effect of High ds RNA Levels on Phosphoprotein Profiles in Reticulocyte Lysates. The data in Fig. 1 suggest that the phosphate moieties of activated ds I are subject to rapid turnover by endogenous protein phosphatase activity. This was further examined by the delayed addition of high levels of poly(I-C) (25 μg/ml), which does not significantly inhibit lysate protein synthesis and also prevents the activation (and phosphorylation) of ds I *in vitro* and in the lysate (2, 3). The turnover of phosphate in ds I and eIF-2α in response to delayed pulses of $^{32}P_i$ is shown in Fig. 2 (tracks 2, 4, and 8). However, in a 5- to 15-min pulse (tracks 3–6), high levels of poly(I-C) (high ds RNA) were added to one incubation mixture (track 5) at the midpoint (10 min) of the pulse. This blocked further ds I activation and permitted extensive dephosphorylation of both ds I (72,000 daltons) and eIF-2α (38,000 daltons) (compare track 5 with tracks 2 and 4). A control assay in which a high level of ds RNA was added at the start of incubation (track 6) displayed little or no phosphorylation of ds I or eIF-2α in the 5- to 15-min pulse. These results indicate that phosphate incorporated into ds I and eIF-2α during a 5- to 10-min pulse (see track 2) is rapidly lost once further activation of the kinase is prevented. The data show the presence of protein phosphatase activities that act on ds I.

Effect of Ribosome-Associated Protein Phosphatase on Ribosome-Bound ds I. In reticulocyte lysates, ds I is associated with ribosomes (3, 4). Filtration of lysates through Sepharose 6B yields a ribosome complement that contains all of the lysate ds I in its latent physiological state (3). Sepharose 6B ribosomes were monitored for endogenous protein phosphatase activity by a two-step procedure. Bound ds I was activated (step 1) by incubating the Sepharose 6B ribosomes with ds RNA and [γ-^{32}P]ATP. To detect endogenous protein phosphatase activity (step 2), 5 mM EDTA was added and the loss of label was monitored during further incubation (Fig. 3). The phosphoprotein pattern generated during ds I activation (step 1) shows marked phosphorylation of the 70k/72k doublet (ds I) and eIF-2α (track 4). These phosphorylations are not observed in the absence of ds RNA (track 1). In step 2 phosphatase assays, in which no ds RNA was initially added (in step 1), there was no significant loss of phosphate from any phosphoprotein in step 2 (tracks 2 and 3). When ds RNA was present in step 1, however, it was observed in step 2 assays that Sepharose 6B ribosomes contain an endogenous protein phosphatase activity (ds I phosphatase) that dephosphorylates the 72,000-dalton component of ds I. This can be seen after 5 min of step 2 incubation (track 5) and is most apparent after 20 min (track 6). The decrease of label in the 72,000-dalton component of ds I is concomitant with a transitory increase in label in the 70,000-dalton component due to the 72k→70k shift (track 6 and diagram at bottom). Addition of the heat-stable protein phosphatase inhibitor 2 (I-2), which specifically inhibits PPase 1 (22–25), has only a slight blocking effect on the dephosphorylation of ds I (track 7). Since this I-2 preparation completely blocks the activity of PPase 1 but not PPase 2 in reticulocytes (29) and other tissues (25), we tentatively concluded that the endogenous ds I phosphatase is a PPase 2. Of interest was the result that no significant dephosphorylation of eIF-2α was observed under the same conditions (tracks 5 and 6).

Comparison of Soluble and Ribosomal ds I Phosphatase: Effect of EDTA. We have previously found that the S100 fraction of reticulocyte lysates rapidly dephosphorylates ds I (12). To examine this further, lysate S100 was fractionated on DEAE-cellulose. A fraction enriched for PPase 2 activity, which eluted at 0.2–0.3 M KCl (see *Materials and Methods*), was further fractionated on Sephacryl-S200; a PPase 2 activity that dephosphorylated ds I eluted as a discrete peak at a molecular mass of 80,000–90,000 daltons. A similar protein phosphatase activity contained in the ribosomal salt wash and purified in the same way displayed similar specificity toward ds I. For convenience, these activities are referred to here as ds I phosphatases. Both fractions were free of PPase 1 and were insensitive to I-2, which blocks the activity of PPase 1 but not PPase 2.

For the two-step analysis of ds I phosphorylation and activation (step 1) and ds I dephosphorylation (step 2) in the lysate or with purified components, 5 mM EDTA was routinely added after step 1 activation to permit expression and measurement of the ds I phosphatase activity in step 2. To determine whether

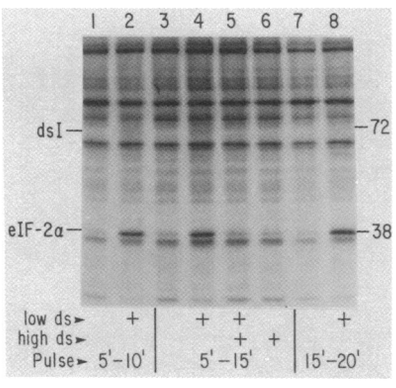

FIG. 2. Effect of high ds RNA levels on phosphoprotein profiles: Evidence for ds I phosphatase. Protein synthesis reaction mixtures (25 μl) and assay conditions were as described in Fig. 1. $^{32}P_i$ (100 μCi) was added to each assay mixture at the indicated interval. Where indicated, mixtures contained *P. chrysogenum* ds RNA (low ds) at 20 ng/ml. In one assay (track 6), poly(I-C) (25 μg/ml) (high ds) was added at the start of incubation. In another assay (track 5), a high level of poly(I-C) was added at the midpoint (10 min) of the $^{32}P_i$ pulse (5–15 min) to block further ds I activation. Aliquots (10 μl) were fractionated by pH 5 precipitation and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The figure is an autoradiogram.

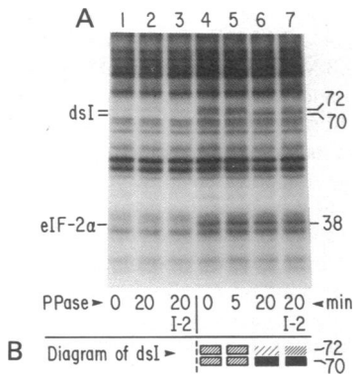

FIG. 3. Effect of ribosome-associated protein phosphatase on ribosome-bound ds I. (*A*) Ribosome-bound ^{32}P-labeled ds I was prepared in (total vol, 120 μl) 10 mM Hepes/KOH, pH 7.2/2 mM Mg(OAc)$_2$/50 mM KCl/1 mM dithiothreitol/25 μM [γ-^{32}P]ATP (33 Ci/mmol) containing *P. chrysogenum* ds RNA (20 ng/ml) and 30 μl of fresh lysate Sepharose 6B ribosomes (0.36 A_{260} unit) (3). A duplicate control mixture contained no ds RNA. After 10 min at 37°C, EDTA (5 mM) was added to both mixtures to block further kinase activity. Aliquots (20 μl) of each mixture were then further incubated at 37°C for 0, 5, or 20 min to monitor endogenous protein phosphatase (PPase) as indicated. Two mixtures (tracks 3 and 7) were supplemented with 50 units of I-2. All mixtures were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (see Fig. 1). The figure is an autoradiogram. (*B*) Schematic representation of the 70k/72k phosphorylated bands in corresponding assay mixtures (tracks 4–7). Tracks: 1–3, controls (no ds RNA); 4–7, mixtures contained ds RNA.

EDTA affected protein phosphatase activity, we examined the activity of the partially purified ds I phosphatases on phosphorylated ds I in the presence and absence of EDTA (Fig. 4). The phosphoprotein profile of the ds I kinase activity only (step 1) is shown in track 1. Further incubation in the presence (track 2) or absence (track 3) of 5 mM EDTA produced a low level dephosphorylation of ds I (track 2), due to contaminating phosphatase(s) activity. In the presence of 5 mM EDTA, the addition of ds I phosphatase isolated from the soluble (track 5) or ribosome-associated fractions (track 8) resulted in the extensive and rapid dephosphorylation of the 72,000-dalton component of ds I and the transitory shift of label to the 70,000-dalton component. When EDTA was omitted during the dephosphorylation step (tracks 6 and 9), ds I activation was not blocked; however, a reduced but still significant net dephosphorylation of the 72,000-dalton component occurred, as indicated by the shift of label to the 70,000-dalton component. These findings reflect an equilibrium between the kinase and phosphatase activities that is readily altered by increasing the level of either ds I or ds I phosphatase. As would be expected for PPase 2 (25), the addition of I-2 does not significantly affect the dephosphorylation of ds I by either ds I phosphatase activity (tracks 7 and 10). One observation of further interest was the finding that the added I-2 was partially phosphorylated as indicated by the labeled phosphoprotein migrating at 30,000 daltons (tracks 4, 7, and 10). This phosphorylation is due to a contaminating protein kinase in the ds I preparation and is not a ds RNA-dependent event (data not shown). Its significance is not known. In addition, it should be added that no significant dephosphorylation of eIF-2α was detected under these assay conditions (tracks 2–10).

The specificity of this ds I phosphatase was further examined by using phosphorylated HRI, eIF-2, and ds I as substrates. As shown in Fig. 5, the partial dephosphorylation of the 72,000-dalton polypeptide of ds I by ds I phosphatase was similar in the presence or absence of phosphorylated eIF-2α (tracks 1–4). Significantly, under the same conditions, no dephosphorylation of eIF-2α or HRI was observed (tracks 3–8). Under all of the various incubation combinations, including assay mixtures containing both ds I and HRI (tracks 7 and 8), ds I was selectively dephosphorylated by low levels of ds I phosphatase activity whereas little or no dephosphorylation of HRI, eIF-2α, or eIF-2β could be detected (tracks 5–8).

Comparison of PPase 1 and PPase 2 and Effect of I-2 on Dephosphorylation of ds I. The dephosphorylation of activated ^{32}P-labeled ds I and eIF-2α by PPase 1 and PPase 2 (ds I phosphatase) are compared in Fig. 6. In this experiment, the activation of ds I and the phosphorylation of eIF-2α were carried out in step 1 assays. All of the step 2 phosphatase incubations were carried out for 20 min in the presence of 5 mM EDTA. In the absence of added protein phosphatase, there was little or no dephosphorylation of phosphorylated ds I or eIF-2α in step 2 assays (track 2). The addition of crude unfractionated ly-

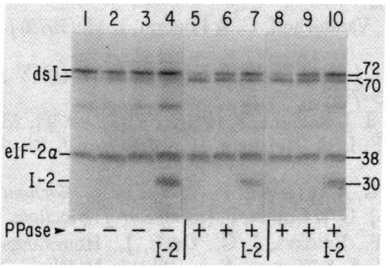

FIG. 4. Comparison of soluble and ribosomal ds I phosphatases: Effect of EDTA. Step 1 ds I activation assay mixtures (20 μl) contained 10 mM Tris·HCl (pH 7.6), 60 mM KCl, 2 mM Mg(OAc)$_2$, 50 μM [γ-^{32}P]ATP (30 Ci/mmol), reovirus ds RNA (20 ng/ml), 1 μg of eIF-2, and 1.5 μl of glycerol-gradient latent ds I (step 6, ref. 6). Incubation was for 10 min at 30°C. In step 2 protein phosphatase assay mixtures (26 μl), the following additions were used: tracks 2, 5, and 8, 5 mM EDTA; tracks 2–4, no protein phosphatase; tracks 5–7, 0.01 unit of soluble PPase 2; tracks 8–10, 0.01 unit of ribosomal PPase 2; tracks 4, 7, and 10, 45 units of I-2. Incubation was for 10 min at 30°C. Track 1 represents step 1 activation only. The figure is an autoradiogram.

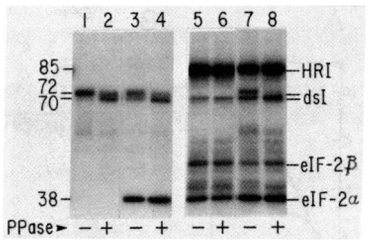

FIG. 5. Specificity of ds I phosphatase. Step 1 ds I activation assay mixtures (20 μl) contained 10 mM Tris·HCl (pH 7.6), 60 mM KCl, 2 mM Mg(OAc)$_2$, 50 μM [γ-^{32}P]ATP (30 Ci/mmol), 0.25 μg of eIF-2, and reovirus ds RNA (20 ng/ml) (tracks 1–4, 7, and 8), 1.5 μl of glycerol-gradient latent ds I (step 6, ref. 6) (tracks 1–4, 7, and 8), and 50 ng of purified HRI (step 7, ref. 30) (tracks 5–8). After 10 min at 30°C, 5 mM EDTA was added to all mixtures to block further activation. Step 2 protein phosphatase assay mixtures (27 μl) also contained 0.01 unit of soluble PPase 2 (see Fig. 4) (tracks 2, 4, 6, and 8). All assay mixtures were further incubated for 6 min at 30°C and then analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The figure is an autoradiogram.

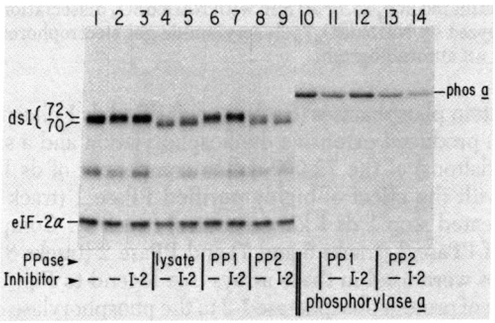

FIG. 6. Dephosphorylation of ^{32}P-labeled ds I by PPase 1 and PPase 2: Effect of inhibitor 2. Tracks 1–9: step 1 ds I activation assay mixtures (20 μl) contained 25 mM Tris·HCl (pH 7.5), 2 mM Mg(OAc)$_2$, 60 mM KCl, 25 μM [γ-^{32}P]ATP (30 Ci/mmol), 0.5 μg of eIF-2, reovirus ds RNA (20 ng/ml), and 1.5 μl of glycerol-gradient latent ds I (step 6, ref. 6). Incubation was at 30°C for 10 min, and the reaction was terminated by the addition of 5 mM EDTA. Protein phosphatase assays (step 2) were carried out by further incubation of the mixtures for 20 min at 30°C with 0.5 μl of lysate (tracks 4 and 5), 0.09 unit of PPase 1 (tracks 6 and 7), or 0.01 unit of PPase 2 (soluble ds I phosphatase; see Fig. 4) (tracks 8 and 9); the amounts of PPase 1 and PPase 2 used displayed equivalent activity against phosphorylase *a* (see below). Where indicated, 45 units of I-2 was added during step 2 incubations. Track 1 is a step 1 ds I activation assay only. Tracks 2 and 3 are controls for endogenous phosphatase activity in the ds I preparation. Tracks 10–14: phosphorylase *a* phosphatase activity was assayed in 20-μl mixtures containing 20 mM Tris·HCl (pH 7.6), 1 mM EDTA, 5 mM dithiothreitol, and 4 μg of ^{32}P-labeled phosphorylase *a* (≈7,500 cpm). One mixture (track 10) contained no phosphatase, two mixtures (tracks 11 and 12) contained 0.09 unit of highly purified rabbit skeletal muscle PPase 1, and two mixtures (tracks 13 and 14) contained 0.01 unit of partially purified soluble ds I phosphatase (PPase 2) (see Fig. 4). Two mixtures (tracks 12 and 14) also contained 45 units each of I-2. Incubation was for 10 min at 37°C. Under these assay conditions, the amounts of the PPase 1 and PPase 2 preparations added were determined empirically and were based on the amount of each phosphatase required to dephosphorylate ^{32}P-labeled phosphorylase *a* (tracks 10–14) by approximately 50%. Only PPase 1 activity was inhibited by I-2 (track 12); PPase 2 activity was slightly stimulated by inhibitor 2 (track 14). The figure is an autoradiogram.

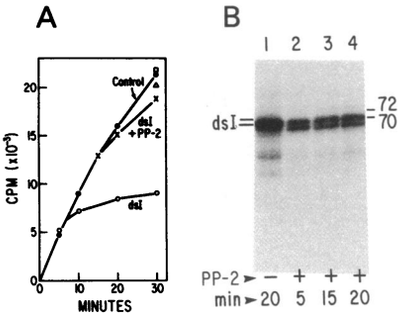

FIG. 7. Effect of PPase 2 on the inhibitory activity of activated ds I. Glycerol-gradient latent ds I (5 μl) (step 6, ref. 6) was activated (step 1) for 10 min at 30°C in a 10-μl mixture containing 20 mM Tris·HCl (pH 7.5), 2 mM $Mg(OAc)_2$, 1 mM unlabeled ATP, and reovirus ds RNA (20 ng/ml). Control assay mixtures without ds RNA or latent ds I were similarly incubated. In step 2 phosphatase assays, 2.5 μl of step 1 reaction mixtures were incubated for 15 min at 30°C in a mixture (5 μl) containing 0.01 unit of soluble ds I phosphatase (PPase 2, see Fig. 4). The entire volume was then assayed in a protein-synthesis reaction mixture (25 μl) in the presence of poly(I-C) (25 μg/ml). (*A*) ●, Control (no additions); □, mixture containing ds I and lacking ds RNA; ○, mixture containing ds I and ds RNA and lacking PPase 2; ×, mixture containing ds I, ds RNA, and PPase 2; △, mixture containing ds RNA and lacking ds I. (*B*) Extent of ds I phosphorylation and dephosphorylation. Step 1 ds I activation assay mixtures (12 μl) were as in *A* except that unlabeled ATP was replaced by 63 μM [γ-^{32}P]ATP (30 Ci/mmol). After 10 min at 30°C, 2.7-μl aliquots were added to step 2 phosphatase assays (5 μl) containing PPase 2 where indicated (tracks 2–4) as above. No EDTA was present. Mixtures were incubated at 30°C, and reactions were terminated at 5, 15, or 20 min with $NaDodSO_4$ dissociation buffer and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The figure is an autoradiogram.

sate protein phosphatases (track 4) or purified ds I phosphatase (track 8) produced extensive dephosphorylation and a shift (to 70,000 daltons) of the 72,000-dalton component of ds I, compared with the effect of highly purified PPase 1 (track 6) and an untreated step 1 ds I kinase control (track 1). Comparable levels of PPase 1 (tracks 6 and 7) and PPase 2 (tracks 8 and 9) activities were used in these assays (see legend to Fig. 6). The addition of protein phosphatase I-2 to the phosphorylase *a* phosphatase assays completely blocked the action of PPase 1 (track 12) but not of PPase 2 (track 14); in fact, some stimulation of the PPase 2 activity was observed (track 14). Similarly, I-2 did not block the action of ds I phosphatase (track 9) but in fact produced a slight stimulation. On the other hand, the action of crude lysate protein phosphatases on phosphorylated ds I was partially blocked by I-2 (track 5). The reason for this is not known, but these data clearly demonstrate the relative efficiency and specificity of a PPase 2 for ds I. As noted above (see Figs. 4 and 5), the isolated protein phosphatases display little activity toward phosphorylated eIF-2α under the conditions of the assay. Only the crude lysate protein phosphatases are effective, producing about a 50% dephosphorylation of eIF-2α, an effect that is partially blocked by I-2 (Fig. 6, tracks 4 and 5).

Treatment of Activated ds I with ds I Phosphatase Decreases its Inhibitory Effect in Lysates. The activation of partially purified latent ds I yields an eIF-2α kinase activity that is a potent inhibitor of protein synthesis when added to lysates in nanogram amounts (Fig. 7*A*). In the protein synthesis assays, the lysates were supplemented with high concentrations of ds RNA (20 μg/ml) to block further ds I activation. However, brief *in vitro* treatment of the activated ds I with low levels of partially purified ds I phosphatase in the absence of EDTA (see Fig. 4) essentially inactivates the inhibitory effects of the same amount of ds I (Fig. 7*A*). In control experiments, direct addition to lysates of ds I phosphatase had no effect on the rate of protein synthesis. The phosphoprotein profiles of the inhibitory ds I and of the partially inactivated ds I are shown in Fig. 7*B*. The removal of approximately 70% of the phosphate from the 72,000-dalton component of ds I after 5 min of incubation is sufficient to achieve partial inactivation. Under the assay conditions, a steady-state level of partially dephosphorylated ds I is achieved in 5 min as a result of the ds I kinase–phosphatase equilibrium (Fig. 7*B*). The results affirm our previous conclusion that the 72,000-dalton component of ds I must be in a highly phosphorylated state to express its maximal eIF-2α kinase and inhibitory properties (12). These data also provide further evidence for the significance of protein phosphatases in the regulation of protein synthesis by ds RNA-activated eIF-2α kinase.

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