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The Phosphorylation State of the Reticulocyte 90-kDa Heat Shock Protein Affects Its Ability To Increase Phosphorylation of Peptide Initiation Factor 2 α Subunit by the Heme-Sensitive Kinase†

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ABSTRACT: The rabbit reticulocyte M_r 90000 protein associated with the heme-sensitive eIF-2α[1] kinase has been identified previously as the mammalian heat shock protein of this size class (hsp 90). Purified reticulocyte hsp 90 when added exogenously to the kinase increases its activity. This stimulatory effect is abolished after incubation of hsp 90 with a highly purified type 1 phosphoprotein phosphatase isolated from reticulocytes. Phosphorylation of dephosphorylated hsp 90 by casein kinase II but not by cAMP-dependent protein kinase restores the biological activity of hsp 90 to stimulate eIF-2α phosphorylation.

Exposure of cells to elevated temperatures or a variety of stressful environmental conditions results in a rapidly expressed set of metabolic changes referred to collectively as the heat shock or stress response [reviewed in Lindquist (1986)]. The changes include elevated synthesis of a small number of peptides, the heat shock or stress proteins. Prominent among these are peptide species of about 90 kDa in a wide variety of cell types. Concomitant with synthesis of the heat shock proteins is a reduction in translation of most preexisting mRNA species. The mechanisms involved in the redirection of the translational machinery are not well understood. However, in heat-treated mammalian cells translational control appears to be exerted at the level of peptide chain initiation, hallmarked by a decrease in 40S ribosomal initiation complexes and polysome disaggregation (Duncan & Hershey, 1987). Several contributing events have been implicated including the phosphorylation of the α subunit of eIF-2 (Duncan & Hershey, 1984), which has been shown also to occur in Ehrlich ascites tumor cells following heat treatment (Scorsone et al., 1987). A kinase similar to the heme-controlled eIF-2α kinase of reticulocytes is activated during heat shock of HeLa cells (DeBenedetti & Baglioni, 1986).

Heme deficiency in reticulocytes or their lysates causes inhibition of protein synthesis due to phosphorylation of the α subunit of peptide initiation factor 2 (eIF-2α) by a heme-sensitive enzyme system originally named the heme-controlled repressor, HCR (Gross & Rabinovitz, 1972), before it was recognized as a protein kinase. In this paper this enzyme will be called the eIF-2α kinase. It is distinct from another eIF-2α kinase that is activated in the presence of double-stranded RNA (Farrell et al., 1977). Highly purified preparations of the eIF-2α kinase contain a 100-kDa peptide that appears to be associated with the enzyme and to undergo phosphorylation in active kinase preparations (Trachsel et al., 1978; Gross & Mendelewski, 1978), a 95-kDa peptide that is the catalytic subunit of the kinase (Kudlicki et al., 1987), and a relatively abundant 90-kDa peptide (Wallis et al., 1980; Kudlicki et al., 1987) that when added exogenously in purified form increases eIF-2α phosphorylation by the kinase (Kudlicki et al., 1987; Rose 1988). Addition of small amounts of the 90-kDa peptide to reticulocyte lysates causes inhibition of protein synthesis (Rose, 1988). Recently, this reticulocyte 90-kDa protein has been shown to be structurally and functionally related to or identical with the hsp 90 family of proteins from other mammalian cells (Rose et al., 1987; Rose, 1988) and therefore is called reticulocyte hsp 90 below. The evolutionarily conserved hsp 90 proteins are highly elongated and have no known en-

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[1] Abbreviations: eIF-2α, the α subunit of the eukaryotic initiation factor 2; hsp, heat shock protein; SDS, sodium dodecyl sulfate.

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zymatic activity (Iannotti et al., 1988). However, species of hsp 90 form transient complexes with steroid hormone receptors (Catelli et al., 1985; Sanchez et al., 1985), pp60^{src} tyrosine kinase (Brugge et al., 1981; Oppermann et al., 1981), and the transforming proteins of the Y73 and FSV viruses (Yonemoto et al., 1982).

Mammalian hsp 90 has been shown to undergo several posttranslational covalent modifications including methylation (Wang et al., 1981), ADP-ribosylation (Carlsson & Lazarides, 1983), and phosphorylation (Kelley & Schlesinger, 1982). As isolated from chicken liver hsp 90 was found to have 5.8 mol of phosphate per dimer (Iannotti et al., 1988). Reticulocyte hsp 90 can be phosphorylated by the catalytic subunit of the cAMP-dependent kinase and by casein kinase II (Kudlicki et al., 1985). Tryptic digestion of the reticulocyte protein phosphorylated by the latter kinase gave three phosphopeptides, one of which was determined to be a 14 amino acid sequence containing both phosphoserine and phosphothreonine (Rose et al., 1987).

The results presented here demonstrate that incubation with a Mn^{2+}-dependent type 1 [cf. Ingebritsen and Cohen (1983)] phosphoprotein phosphatase separated at a late stage of purification from the eIF-2α kinase abolishes the ability of hsp 90 to increase eIF-2α phosphorylation by the eIF-2α kinase. Inactivation of hsp 90 by the phosphatase is dependent on Mn^{2+} and appears to involve dephosphorylation. The activity of hsp 90 is restored by rephosphorylation in vitro by casein kinase II. The results provide the first demonstration that a biochemical activity of hsp 90 is sensitive to its phosphorylation state, implicate the protein in translational regulation by the eIF-2α kinase, and prompt the suggestion that casein kinase II may play a role in translational control mediated by the eIF-2α phosphorylation.

Experimental Procedures

Materials. Chromatography media and chemicals were used as previously indicated (Kudlicki et al., 1987).

Isolation of Proteins from Rabbit Reticulocytes. Fractionation of the rabbit reticulocyte postribosomal supernatant to give the PC_{100} fraction was carried out as described in Wollny et al. (1984). The eIF-2α kinase was further purified, and the type 1 phosphoprotein phosphatase and 90-kDa protein were separated as detailed in Kudlicki et al. (1987). Casein kinase II was isolated as given in Rose et al. (1987), and the catalytic subunit of the cAMP-dependent protein kinase was purified as described previously (Grankowski et al., 1979) but omitting the histone–Sepharose chromatography.

Purification of eIF-2 from the 0.5 M salt-wash fraction of reticulocyte polysomes was carried out as reported previously (Odom et al., 1978) with minor modifications described in Szyszka et al. (1989).

Enzyme Assays. (a) eIF-2α phosphorylation was carried out as reported previously (Kudlicki et al., 1987). Specific details are provided in the figure and table legends. (b) Phosphorylation of the 90-kDa protein was done under conditions reported in Rose et al. (1987) and as described under Results with the protein kinase specified in the legends. (c) For the phosphoprotein phosphatase assay, details of the Mn^{2+}-dependent assay and determination of released phosphate are given in Wollny et al. (1984). Preincubation of the 90-kDa protein with the type 1 phosphatase was carried out under conditions described under Results; usually about 14 μg of the M_r 90 000 protein was dephosphorylated with 1.1 μg of the type 1 phosphatase in the presence of 2 mM Mn^{2+} in a total volume of 40 μL during a 10-min incubation at 35 °C.

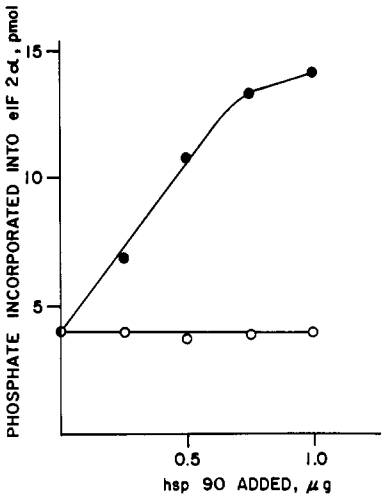

FIGURE 1: Effect of a type 1 phosphatase on the activity of hsp 90 for eIF-2α phosphorylation. Phosphorylation of eIF-2α by the eIF-2α kinase was carried out for 5 min at 35 °C. Each incubation mixture contained about 5 μg of eIF-2, 50 ng of eIF-2α kinase, and 0.1 mM [γ-^{32}P]ATP (about 500 Ci/mol). hsp 90 was added to the reaction mixtures in the amounts given on the abscissa. The 90-kDa protein was preincubated with the phosphatase for 10 min at 35 °C in the presence of the phosphatase either without Mn^{2+} (closed circles) or with 2 mM Mn^{2+} (open circles).

Protein determination and peptide composition of protein fractions were analyzed as described in Kudlicki et al. (1987).

Results and Discussion

Phosphatase Treatment Abolishes the Ability of the hsp 90 To Increase eIF-2α Kinase Activity. Reticulocyte hsp 90 was isolated by gel electrophoresis under nondenaturing conditions from preparations of the eIF-2α kinase by the procedure described previously (Kudlicki et al., 1987). The protein is homogeneous as judged by SDS one-dimensional gel electrophoresis and is free of eIF-2α kinase or phosphoprotein phosphatase activities. As shown in Figure 1 and reported previously (Kudlicki et al., 1987; Rose, 1988), this hsp 90 causes a concentration-dependent increase in eIF-2α phosphorylation by the eIF-2α kinase when it is added to the in vitro phosphorylation reaction mixture. The assay was carried out under conditions in which the initial rate of eIF-2α phosphorylation was measured. Phosphorylation of eIF-2α was quantitated after the peptide band had been excised from a polyacrylamide gel on which the components of the phosphorylation reaction mixture had been separated by electrophoresis in SDS. Radioactivity in the excised peptide was determined by Cerenkov counting. The ability of hsp 90 to increase eIF-2α phosphorylation by the kinase is lost by preincubation of hsp 90 with a Mn^{2+}-dependent type 1 phosphatase in the presence of Mn^{2+} (Figure 1, open circles) but not if Mn^{2+} is omitted from the preincubation reaction mixture (closed circles). Inactivation of hsp 90 was dependent on the amount of phosphatase used and the time of preincubation (data not shown). The phosphatase used in these experiments was isolated from preparations of the eIF-2α kinase as described previously (Kudlicki et al., 1987). The phosphatase copurified with the eIF-2α kinase until the last step of the isolation procedure, preparative gel electrophoresis under nondenaturing conditions. The results suggest that the phosphatase may form a loose complex with hsp 90 in crude fractions and perhaps intact cells. The phosphatase has been characterized (Szyszka et al., 1989) as a Mn^{2+}-dependent type 1 enzyme according to the classification suggested by Ingebritsen and Cohen (1983). A 2 mM Mn^{2+} optimum was

Table I: Casein Kinase II Restores Biological Activity of hsp 90[a]

type of hsp 90 added	^{32}P in eIF-2α pmol	% of control
(1) none (control)	2.5	100
(2) untreated[b]	6.0	240
(3) dephosphorylated	2.5	100
(4) dephosphorylated, then rephosphorylated by CK II	5.5	220
(5) dephosphorylated, then rephosphorylated by cApK	2.1	84

[a] Phosphorylation of eIF-2α was carried out in the absence (1) or presence (2–5) of hsp 90 treated as indicated. Dephosphorylation of hsp 90 was as described in the text; then EGTA was added and the sample (about 0.6 μg of hsp 90) incubated with unlabeled ATP and either casein kinase II (CK II), about 70 ng, or the catalytic subunit of cAMP-dependent protein kinase (cApK), 60 ng, in 30-μL reaction mixtures for 20 min at 35 °C. All samples were heated for 5 min at 70 °C; then eIF-2α phosphorylation was carried out for 5 min at 35 °C after the addition of 5 μg of eIF-2, about 40 ng of eIF-2α kinase, and [γ-^{32}P]ATP (0.1 mM, about 2 Ci/mmol). Incorporation of [^{32}P]-phosphate into eIF-2α was quantitated as described in the text. [b] hsp 90 was not incubated with the type 1 phosphatase. Identical values were obtained for hsp 90 that was incubated with the phosphatase in the absence of Mn^{2+}.

determined for in vitro dephosphorylation of hsp 90.

For the experiments reported here, hsp 90 was incubated with the phosphatase in the absence or presence of 2 mM Mn^{2+}; then a 1.2-fold molar excess of EGTA to Mn^{2+} was added to the reaction mixture. Subsequently, the hsp 90 fraction was tested for its ability to increase eIF-2α phosphorylation by the eIF-2α kinase. Control experiments indicated that the activity of the phosphatase was reduced to a very low level in the presence of Mn^{2+} and EGTA (data not presented). Also, EGTA prevents inhibition of the eIF-2α kinase by Mn^{2+}. Other experiments have shown directly that neither phosphorylated eIF-2α nor the phosphorylated M_r 100 000 peptide of the eIF-2α kinase is dephosphorylated significantly by this type 1 phosphatase.

Rephosphorylation by Casein Kinase II Restores the Stimulatory Effect of hsp 90 on eIF-2α Phosphorylation. The effects of various forms of hsp 90 on eIF-2α phosphorylation by the eIF-2α kinase are shown in Table I. The experimental system was similar to that used in the experiments of Figure 1 except that where indicated the dephosphorylated hsp 90 was incubated with nonradioactive ATP and either casein kinase II or the catalytic subunit of the cAMP-dependent kinase before its activity for increasing eIF-2α phosphorylation by the eIF-2α kinase was measured. The activity of hsp 90 was restored by incubation of the dephosphorylated protein with the former but not by the latter kinase under conditions in which hsp 90 was phosphorylated. Both kinases were shown previously to efficiently phosphorylate hsp 90 (Kudlicki et al., 1985). To avoid possible direct effects by casein kinase II or the cAMP-dependent kinase on the subsequent eIF-2α phosphorylation, after rephosphorylation the reaction mixture containing hsp 90 was heated at 70 °C for 5 min. This treatment inactivates both casein kinase II and the catalytic subunit of the cAMP-dependent kinase but was shown previously not to affect the ability of phosphorylated hsp 90 to stimulate eIF-2α phosphorylation (Rose, 1988). Figure 2 shows a stained gel and autoradiogram of reaction mixtures similar to those used for the experiment of Table I.

Rephosphorylation of hsp 90 by casein kinase II was analyzed in more detail with the results presented in Figure 3. The amount of phosphate incorporated into hsp 90 is plotted against the increase in eIF-2α phosphorylation caused by the

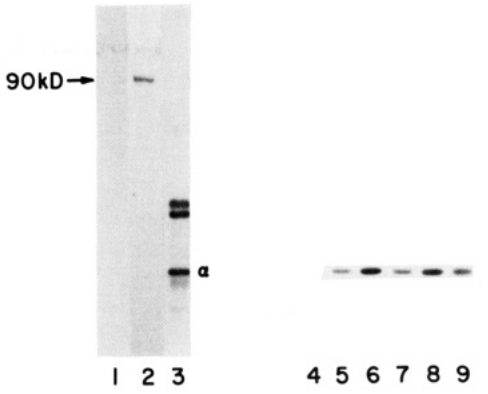

FIGURE 2: Analysis of eIF-2α phosphorylation by SDS–polyacrylamide gel electrophoresis. Tracks 1–3 are from a Coomassie-stained gel and tracks 4–9 from an autoradiogram. Track 1, eIF-2α kinase only, 50 ng of protein; track 2, about 1 μg of hsp 90; track 3, about 5 μg of eIF-2. Track 4 represents an autoradiogram from a reaction mixture without eIF-2α kinase; tracks 5–9 are reaction mixtures as described in Table I (1–5). Track 5, no hsp 90; track 6, plus native hsp 90; track 7, plus dephosphorylated hsp 90; track 8, dephosphorylated hsp 90 rephosphorylated by casein kinase II; track 9, dephosphorylated hsp 90 rephosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

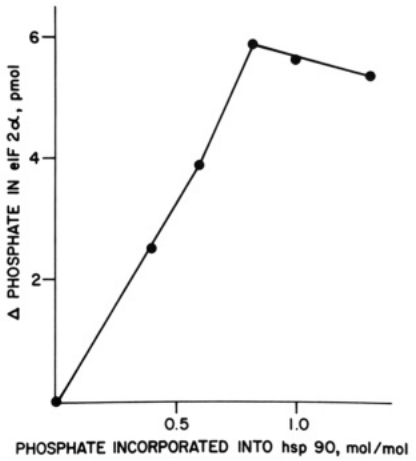

FIGURE 3: Relation between rephosphorylation of hsp 90 and recovery of its biological activity. For each point about 1 μg of hsp 90 (11 pmol) was dephosphorylated for 10 min at 35 °C with 0.15 μg of phosphatase in the presence of Mn^{2+} and then rephosphorylated for different times with [γ-^{32}P]ATP and 0.25 μg of casein kinase II. To stop the phosphorylation reaction, samples were placed in ice and immediately 10 ng of heparin [cf. Hathaway and Traugh (1982)] was added; then the samples were heated for 5 min at 70 °C. The pretreated hsp 90 samples were added to eIF-2α phosphorylation assays containing 5 μg of eIF-2, 50 ng of eIF-2α kinase, and 0.1 mM [γ-^{32}P]ATP (500 Ci/mol). This phosphorylation reaction was carried out for 5 min at 35 °C. Reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis. Radioactivity in eIF-2α and in the 90-kDa peptide was quantitated. eIF-2α phosphorylation was 3.5 pmol without hsp 90 or with hsp 90 that had been incubated with the phosphatase but not rephosphorylated by casein kinase II.

phosphorylated hsp 90. The activity of hsp 90 reaches a maximum at about 0.7 mol of phosphate/mol of hsp 90 protein. This value may be subject to considerable error in that it was calculated without correction from the molar amounts of radioactive phosphate in bands of hsp 90 excised from SDS electrophoresis gels assuming the molar amount of hsp 90 protein to be that added originally to the reaction mixture in which phosphorylation was carried out. Although the exact numbers may be subject to considerable error, the data show that extensive phosphorylation of hsp 90 causes a decline in activity, suggesting that not all of the sites that are phosphorylated by casein kinase II contribute to its activity in the

eIF-2α phosphorylation reaction. The data presented in Figure 3 were derived from a time course of hsp 90 rephosphorylation by casein kinase II. Similar results were obtained by determining hsp 90 phosphorylation with increasing amounts of casein kinase II.

Considered together, the results indicate that reticulocyte hsp 90 is isolated in the phosphorylated form and that its ability to increase eIF-2α phosphorylation by the eIF-2α kinase can be eliminated by dephosphorylation with the type 1 phosphatase. The activity of hsp 90 can be restored by rephosphorylation with casein kinase II but not by the catalytic subunit of the cAMP-dependent kinase. The results suggest that phosphorylation of hsp 90 by one or more specific kinases may be an important step in regulating the activity of the eIF-2α kinase and in turn protein synthesis. Gross and Kaplansky (1985) reported that activation of the heme-sensitive eIF-2α kinase is inhibited by Mn^{2+}. It appears that the effects they observed may have involved a Mn^{2+}-dependent phosphatase and hsp 90 dephosphorylation. Whether or not casein kinase II is responsible for hsp 90 phosphorylation in vivo and for the role these components play in translational regulation during the stress response remains to be established. However, it should be noted that the reticulocytes used as a primary source for the proteins in this study were induced during severe anemia caused by nearly lethal treatment of the rabbits with phenylhydrazine. Whether this treatment induces biological effects related to the stress response observed in other cells is uncertain; however, this appears to be a possibility.

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Registry No. eIF-2α kinase, 82249-72-7.

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