
Hsp90 regulates protein synthesis by activating the heme-regulated eukaryotic initiation factor 2 α (eIF-2 α) kinase in rabbit reticulocyte lysates

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Heat shock protein 90 (Hsp90), an abundant and ubiquitous cytoplasmic protein has recently been indicated to participate in the regulation of protein synthesis by interacting with the heme-regulated eukaryotic initiation factor 2 α (eIF-2 α) kinase, also known as the heme-regulated inhibitor (HRI). However, there exists an ambiguity on the exact nature of its action. In this investigation, the interaction of Hsp90 and HRI has been examined both *in vitro* using purified proteins, and *in situ* in rabbit reticulocyte lysates subjected to heat shock and treatment with N-ethylmaleimide (NEM), a sulfhydryl reagent known to induce stress response. During heat shock or NEM-treatment of reticulocyte lysates, Hsp90 co-immunoprecipitated with activated HRI by anti-HRI monoclonal antibodies. Furthermore, the amount of Hsp90 being associated with HRI was a function of duration of heat shock and was correlated with the extent of HRI activation. Interestingly, simultaneous heat shock and NEM-treatment of reticulocyte lysates led to maximal association of HRI and Hsp90, leaving nearly no free HRI in the lysates. *In vitro*, with the purified proteins, the autokinase and the eIF-2 α kinase activities of HRI were enhanced when HRI was pre-incubated with Hsp90, both in the presence and absence of hemin. These data, therefore, clearly demonstrate that Hsp90 interacts with HRI during stress, and that this association leads to activation of HRI and thereby inhibition of protein synthesis at the level of initiation. Considering the ubiquitous nature of Hsp90 and the presence of HRI or HRI-like eIF-2 α kinase activity in a number of organisms, it is highly possible that Hsp90 may universally mediate down regulation of global protein synthesis during stress response.

1. Introduction

During heat shock and several other kinds of stress, a negative regulation of global protein synthesis has been universally documented. Although the regulation of translation during stress is exerted predominantly at the level of initiation, not much is known about the mechanism except the non-functional nature of the eukaryotic initiation factor 4 (eIF-4) group of polypeptides which are required for interaction of ribosomes with mRNAs to form 48S pre-initiation complexes (reviewed in Pal *et al* 1996). However, in specific cell types, namely, rabbit reticulocytes and HeLa cells, heat shock induced inhibition of protein synthesis was shown to be due to

increased phosphorylation of eIF-2 (Ernst *et al* 1982) caused by activation of the heme-regulated eIF-2 α kinase. Subsequently, it was shown that in rabbit reticulocyte lysates, addition of the heat shock protein 90 (Hsp90) inhibited endogenous protein synthesis, perhaps as a consequence of eIF-2 α phosphorylation (Rose *et al* 1989). Since then, a few laboratories have been trying to determine the relationship between Hsp90 and eIF-2 α kinase in order to unravel the mechanism of Hsp90-mediated regulation of protein synthesis (reviewed in Pal *et al* 1996). The role of eIF-2 α kinases in regulation of protein synthesis is briefly described below.

In eukaryotes, the role of eIF-2 α kinases in the regulation of peptide chain initiation is of great importance

Keywords. Hsp90; HRI; heat shock; NEM-activation; co-immunoprecipitation; protein synthesis

(reviewed in London *et al* 1987; Chen and London 1995; Pal *et al* 1996). During the process of initiation, eIF-2 is essential for the formation of eIF-2·Met-tRNAⁱ·GTP·40S pre-initiation complex. Phosphorylation of eIF-2 α by the eIF-2 α kinases results in an impairment of eIF-2 recycling leading to inhibition of protein synthesis (Levin *et al* 1976; Kramer *et al* 1976; Farrell *et al* 1977). The detailed mechanism of this inhibition is described elsewhere (Pal *et al* 1996).

Two distinct eIF-2 α kinases were initially characterized from rabbit reticulocytes as potent inhibitors of protein synthesis. Although regulated differently, both kinases phosphorylate the same site (ser 51) in eIF-2 α (reviewed in London *et al* 1987). The heme-regulated eIF-2 α kinase, also known as the heme-regulated inhibitor (HRI), is a dimer of a 92 kDa cytosolic polypeptide and is abundant in reticulocytes, while the double-stranded RNA (dsRNA)-activated eIF-2 α kinase, also known as the dsRNA-dependent inhibitor (dsI or PKR) is a 67 kDa ribosome-associated polypeptide and is ubiquitous (reviewed in Chen and London 1995). However, more recently, a third eIF-2 α kinase, GCN2, which gets activated under conditions of amino acid starvation, has been purified from yeast and is homologous to the mammalian eIF-2 α kinases (reviewed in Chen and London 1995). Thus HRI, dsI and GCN2, which regulate the process of initiation of protein synthesis in various systems are of immense importance (reviewed in London *et al* 1987; Hershey 1991; Chen and London 1995).

HRI has been purified from rabbit reticulocyte lysates and has been well characterized (Ranu and London 1976; Kramer *et al* 1976; Farrell *et al* 1977). Purified HRI specifically phosphorylates the α -subunit of eIF-2 and inhibits initiation of protein synthesis. Further characteristics of HRI have been reviewed in London *et al* (1987), Chen and London (1995) and Pal *et al* (1996). HRI-like inhibitors have also been purified from murine erythroleukemia (MEL) cells (Mellor *et al* 1993) and from Ehrlich ascites cells. However, they seem to be dissimilar among themselves and are distinct from both HRI and PKR (Olmsted *et al* 1993). In addition to these purified inhibitors, HRI-like activity has been reported in a number of non-erythroid cell types (reviewed in Pal *et al* 1996; Anand and Pal 1997). HRI cDNAs from rabbit reticulocytes and rat brain have been cloned and they share 82% homology (Chen *et al* 1991a,b; Mellor *et al* 1994). HRI was initially thought to be erythroid-specific (Pal *et al* 1991; Crosby *et al* 1994). However, Mellor *et al* (1994), using rat brain HRI cDNA, detected HRI expression in a variety of rat tissues although to a lesser extent (10 times) as compared to that in reticulocytes.

Activation of HRI in reticulocyte lysates, as measured by eIF-2 α phosphorylation and inhibition of protein synthesis, is caused by various conditions and reagents

such as heme-deficiency, treatment with oxidized glutathione, N-ethylmaleimide (NEM) and other sulfhydryl-reactive reagents, ethanol, heavy metal toxicity, heat shock, addition of oxidants and denatured proteins, nutritional starvation and viral infection (reviewed in Pal *et al* 1996). The precise mechanism of HRI activation in any of the above-mentioned cases, is however, largely unknown.

Among the conditions of HRI activation described above, heat shock induced activation and the role of Hsp90 is of interest here. Details of the recent analysis on Hsp90-HRI interaction in rabbit reticulocyte lysates have been described previously (Pal *et al* 1996). A few groups have independently worked on the Hsp90-HRI interaction, and two different and somewhat contradictory results have been reported. Initially it was reported that Hsp90 activates HRI *in vitro* (Szyszka *et al* 1989; Pal 1994) and it inhibits protein synthesis in hemin-supplemented reticulocyte lysates (Rose *et al* 1989). On the other hand, association of Hsp90 with latent HRI in hemin-supplemented lysates has also been reported (Matts and Hurst 1989). Interestingly, in the Hsp90-HRI complex, a number of other proteins were also detected (Matts and Hurst 1989; Xu *et al* 1997). Similarly, Mendez and de Haro (1994) reported an inactive HRI in the Hsp90-HRI complex in rabbit reticulocyte lysates. More recently, Hsp90 has been shown to interact with nascent HRI co-translationally (Uma *et al* 1997). Furthermore, the authors have suggested that Hsp90 helps HRI to achieve an activable conformation.

In the context of the present ambiguity as described above, I have examined the interaction of HRI and Hsp90 using specific antibody probes. HRI or Hsp90 were immunoprecipitated by the corresponding monoclonal antibodies, from rabbit reticulocyte lysates under various conditions of HRI activation. The immunoprecipitates were then probed with both anti-HRI and anti-Hsp90 antibodies by Western blot. Results obtained from these analyses demonstrated that Hsp90 interacts with HRI and forms a complex, particularly during stress conditions and that HRI in such a complex is highly active. These results therefore indicate that Hsp90 may mediate regulation of protein synthesis by activating HRI during stress condition.

2. Materials and methods

2.1 Materials

Materials used for HRI purification were obtained from sources described previously (Trachsel *et al* 1978). Chemicals used for gel electrophoresis, hemin, creatine phosphate, creatine phosphokinase, ATP, N-ethylmaleimide (NEM), GSSG, 4-chloro-1-naphthol, secondary antibodies,

and other fine chemicals were purchased from Sigma Chemicals (USA). Protein G Sepharose 4 Fast Flow was purchased from Pharmacia-LKB Biotechnology Inc (USA). Radioactive chemicals were obtained from DuPont-New England Nuclear and from BRIT (Bhabha Atomic Research Centre, Mumbai).

HRI was purified from rabbit reticulocyte lysates as described elsewhere (Pal *et al* 1991). Initiation factor eIF-2 was purified from the 0.5 M salt-wash fraction of reticulocyte ribosomes as described elsewhere (Matts *et al* 1983). HeLa Hsp90 protein and anti-Hsp90 antibodies were gifts from Dr W Welch, UCSF, USA, and they were prepared as described by Rose *et al* (1989).

2.2 Protein synthesis and *in situ* phosphorylation of HRI in reticulocyte lysates

Rabbit reticulocyte lysates were prepared in the laboratory. Protein synthesis was carried out in 25 μ l reaction mixtures under various conditions as detailed in the legend. Protein synthesis was measured by incorporation of [14 C]leucine into proteins as described elsewhere (Pal *et al* 1991). In case of phosphorylation of HRI in the lysates *in situ*, protein synthesis was carried out in the presence of unlabelled leucine as described above. During incubation, reaction mixtures were pulsed with 20 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol) at 0–10 min.

2.3 Immunoprecipitation

[γ - 32 P]ATP-labelled protein synthesis reaction mixtures were diluted in phosphate buffered saline (PBS), pH 7.4, containing 50 mM NaF and 5 mM EDTA, and immunoprecipitated with either anti-HRI (Pal *et al* 1991) or anti-Hsp90 monoclonal antibodies. Non-immune sera were used as controls. Incubation with the antibodies was done overnight at 4°C followed by purification of the immunoprecipitates by incubating (1 h, 4°C) with protein G Sepharose 4 Fast Flow suspended in PBS containing 50 mM NaF and 5 mM EDTA. Immunoprecipitates were washed extensively with PBS containing 50 mM NaF, 5 mM EDTA and 0.5% Triton X-100 (5 times), and finally with the same buffer without Triton X-100. Washed pellets and the supernatants were treated with Laemmli sample buffer and were analysed by SDS-PAGE (Laemmli 1970). Gels were either stained, dried and autoradiographed, or the proteins were transferred to nitrocellulose membranes which were then autoradiographed and processed for Western blot analysis.

2.4 Western blot analysis

Proteins separated on SDS polyacrylamide gels were transferred to nitrocellulose membranes (0.45 μ M; Schleicher and Schuell) according to Towbin *et al* (1979).

The membranes containing proteins were then processed for immunoreactions as described elsewhere (Pal *et al* 1988). In brief, membranes were saturated with 5% milk in PBS (4 h, room temperature) and incubated with primary antibodies overnight at 4°C and then with peroxidase-conjugated secondary antibodies containing 10% rabbit normal serum (4 h, room temperature). Following each antibody incubation, the membranes were washed in PBS (3x, 10 min). Colour development was performed with H₂O₂ and 4-chloro-1-naphthol. In case of reuse of the membrane for a second primary antibody, following the first colour development, the membrane was resaturated briefly and processed as described above.

2.5 Protein kinase assays, SDS-PAGE and autoradiography

Protein kinase assay mixtures (20 μ l) contained 20 mM Tris-HCl (pH 7.6), 40–60 mM KCl, 2 mM Mg(OAc)₂ and 20–50 μ M ([γ - 32 P]ATP (3000 Ci/mmol)). HRI samples were added to the reaction mixtures and incubated for 10 min at 30°C. Other additions are indicated in the figure legends. Reactions were terminated by adding Laemmli sample buffer and samples were treated at 100°C for 5 min. Proteins were separated by SDS-PAGE according to Laemmli (1970). Gels were stained with either Coomassie brilliant blue or silver nitrate, dried and autoradiographed by using Kodak X-OMAT AR film.

3. Results

3.1 Immunoprecipitation of HRI-Hsp90 complex from NEM-treated reticulocyte lysates by anti-HRI mAbs

In order to examine the interaction of HRI and Hsp90 *in situ*, reticulocyte lysate HRI, either latent or activated under various conditions of protein synthesis was labelled by [γ - 32 P]ATP and immunoprecipitated by anti-HRI monoclonal antibodies, mAB F and mAB A (Pal *et al* 1991). It is to be noted here that of the two monoclonal antibodies, mAB A has a weak affinity for HRI, and it recognizes HRI-Hsp90 complex. On the other hand, mAB F which is a high affinity anti-HRI antibody, does not seem to recognize HRI-Hsp90 complex. Immunoprecipitates were subjected to SDS-PAGE and the proteins were transferred to nitrocellulose membrane. The membrane was autoradiographed (figure 1A) and subsequently probed with anti-Hsp90 antibody (figure 1B) and mAB F (figure 1C) by immunoblotting.

The specificity of the HRI signal in the immunoprecipitates was judged by 3 criteria: (i) co-migration with [32 P]-labelled purified HRI (lane 1), (ii) characteristic patterns of activation of HRI during heme-deficiency (lanes 2 and 3) and NEM (lanes 6 and 7) and GSSG-

treatment (lanes 8 and 9) of lysates, and (iii) recognition by anti-HRI mAB F in the immunoblot (figure 1C). As seen in the autoradiogram (figure 1A), [32 P]-labelled HRI was detected in all the immunoprecipitates. The additional [32 P]-labelled bands in the immunoprecipitates of NEM-treated lysates in particular, are due to non-specific adsorption. However, when the immunoprecipitates were washed with buffer containing more than 0.5% Triton X-100, some of the bands disappeared with a concomitant decrease in the Hsp90 signal (data not shown), which suggests that the complex is not very stable. The weaker HRI signals in the immunoprecipitates of mAB A (figure 1A, lanes 3, 5, 7 and 9) were due to the low amount of antibody used, as evident in the amount of IgG (H) signals in the Western blot (figure 1B; lanes 3, 5, 7 and 9) and also due to its lower affinity for HRI; the characteristics of these monoclonal antibodies are described in detail elsewhere (Pal *et al* 1991). Interestingly, when the nitrocellulose membrane was reacted with anti-Hsp90 antibody (figure 1B), Hsp90 signal was detected only in the mAB A immunoprecipitate of NEM-treated lysates (figure 1B, lane 7). Therefore, under the conditions used, the HRI-Hsp90 complex was observed only in lysates treated with NEM. Furthermore, it is also evident that the mAB F was unable to precipitate such a complex.

3.2 *In vitro* activation of HRI by Hsp90 and their co-immunoprecipitation by mAB A

To further investigate the interactions of HRI and Hsp90 *in vitro*, purified HRI (~100 ng) was preincubated with

purified HeLa cell Hsp90 (200 ng) under various conditions, and the autokinase and eIF-2 α kinase activities were then examined by phosphorylation in the presence of [γ - 32 P]ATP and purified eIF-2 α followed by SDS-PAGE and autoradiography (figure 2A). HRI kinase activity was significantly increased by the addition of Hsp90 (figure 2A, compare lanes 1 and 2). Furthermore, Hsp90 was able to enhance HRI kinase activities even in the presence of hemin (figure 2A, compare lanes 3 and 4). In the presence of hemin, highly purified preparation of HRI was slightly activated by NEM (lane 5); the addition of Hsp90 resulted in a significant increase of HRI autophosphorylation as well as eIF-2 α phosphorylation (lane 6). The extent of increase in the phosphorylation signals induced by Hsp90 was determined by counting the incorporation of [32 P] in the HRI and eIF-2 α bands in the gel (see table 1). The Hsp90-induced activation of HRI was specific; no such activation was obtained with other proteins, namely, bovine serum albumin (data not shown).

In order to determine if the enhanced activation of HRI in the presence of Hsp90 is accompanied by complex formation between them, aliquots of the reaction mixtures as in figure 2A were used for immunoprecipitation analysis by the mAB A which seems to be more efficient in recognizing HRI-Hsp90 complex. Results of this experiment (figure 2B, C, lanes 1–6) clearly indicate that Hsp90 did form a complex with HRI *in vitro* and that this occurred independent of the incubation conditions. Furthermore, under the conditions used, it appeared that all of HRI was present as a HRI-Hsp90 complex since all of HRI (lane 1 in C) and Hsp90 (lane 7 in

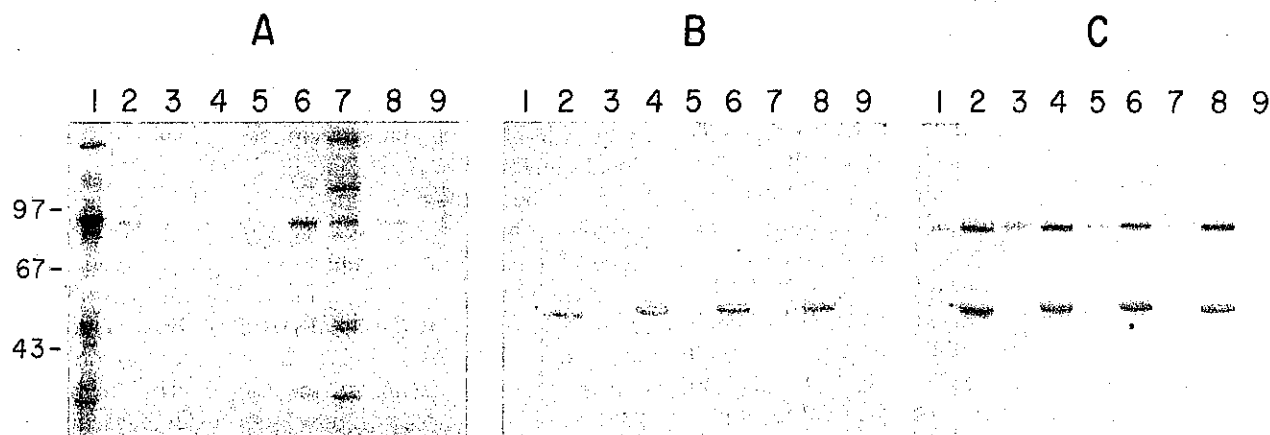


Figure 1. Immunoprecipitation of HRI-Hsp90 complex from reticulocyte lysates by anti-HRI mABs. [32 P]-labelled HRI was immunoprecipitated by anti-HRI mAB F and mAB A. Immunoprecipitates were transferred to nitrocellulose membrane which was autoradiographed (A) and Western blotted by anti-Hsp90 (B) and anti-HRI mAB F (C) antibodies. Lane 1, partially purified [32 P]-labelled HRI (standard); lanes 2–9, immunoprecipitates of mAB F (lanes 2, 4, 6 and 8) and mAB A (lanes 3, 5, 7 and 9). Immunoprecipitates contain HRI from protein synthesizing lysates under various conditions: lanes 2 and 3, heme-deficient; lanes 4 and 5, hemin-supplemented; lanes 6 and 7, NEM-treated; lanes 8 and 9, GSSG-treated. Note the presence of Hsp90 signal only in the mAB A immunoprecipitate of NEM-treated lysate (B, lane 7).

B and C) were recovered in the immune complexes (lanes 2, 4 and 6).

3.3 Association of HRI with Hsp90 during heat shock in reticulocyte lysates

It is known that HRI in reticulocytes or their lysates is activated by heat shock (Ernst *et al* 1982). In order to investigate whether Hsp90 forms a complex with HRI in lysates during heat shock, HRI activity *in situ* in the reticulocyte lysates under conditions of protein synthesis at normal temperature (30°C) as well as heat shock temperature (42°C) (figure 3A), was determined by the phosphorylation of added purified eIF-2. Aliquots at various time intervals were immunoprecipitated by anti-Hsp90 monoclonal antibody and the immunoprecipitates were immunoblotted with anti-HRI (mAB F) (figure 3B) to detect the presence of HRI in them.

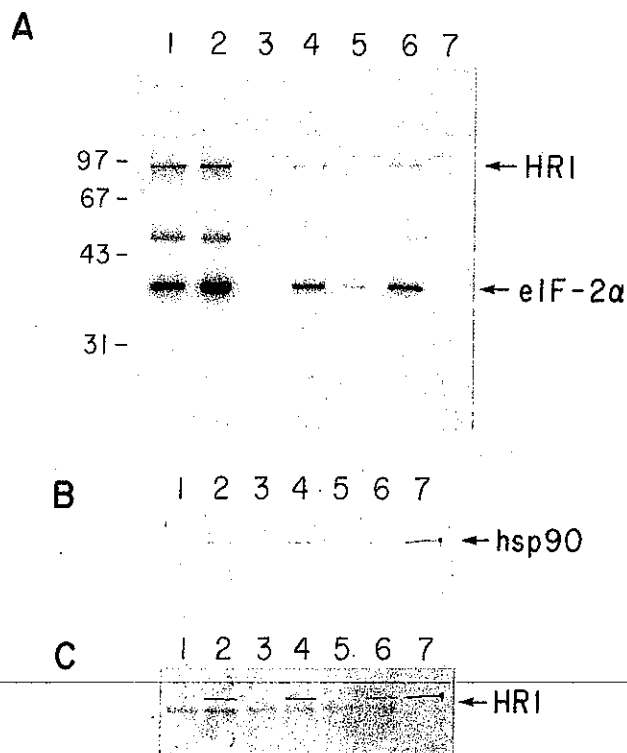


Figure 2. *In vitro* activation of HRI by Hsp90 and their co-immunoprecipitation by mAB A. HRI was pre-incubated in the presence or absence of Hsp90 (5 min, 30°C) and then phosphorylated in the presence of [γ -³²P]ATP and eIF-2 (5 min, 30°C). Aliquots of the same assays were analysed by SDS-PAGE and autoradiography (A), or immunoprecipitated with mAB A followed by immunoblotting with anti-Hsp90 antibody (B) and mAB F (C). Lane 1, HRI alone; lane 2, HRI + Hsp90; lane 3, HRI + hemin; lane 4, HRI + hemin + Hsp90; lane 5, HRI + hemin + NEM; lane 6, HRI + hemin + NEM + Hsp90. Hsp90 is used as standard in lane 7.

HRI activity was significantly higher in the heat shocked lysates at all the 3 time intervals studied (10, 30 and 45 min), as evident in both HRI autophosphorylation and eIF-2 α phosphorylation (figure 3A; lanes 7, 8 and 9). A considerable amount of HRI was found to be associated with Hsp90 in the lysates heat shocked for 45 min as seen in the Hsp90 immunoprecipitates (figure 3B, lane 7). Although lower amount of HRI was detected in the Hsp90 immunoprecipitates of lysates heat shocked for 10 and 30 min (figure 3B, lanes 5 and 6), no HRI was detected in the Hsp90 immunoprecipitates of hemin-supplemented lysates incubated at 30°C (figure 3B, lanes 2-4).

3.4 Synergistic effect of NEM-treatment and heat shock on HRI-Hsp90 association

In order to quantitate the amount of HRI as a Hsp90-HRI complex in the reticulocyte lysates under various conditions, sequential immunoprecipitation with anti-Hsp90 and anti-HRI (mAB F) antibodies followed by Western blotting was carried out. Hsp90 was immunoprecipitated by anti-Hsp90 antibody, from lysates incubated at 30°C as well as at 42°C in the presence and absence of NEM. The presence of HRI as a HRI-Hsp90 complex was determined by Western blot of the anti-Hsp90 immunoprecipitates with anti-HRI antibody (figure 4A). The supernatants left were further immunoprecipitated with anti-HRI mAB F followed by Western blot with mAB F to determine free HRI (figure 4B).

Immunoprecipitates of Hsp90 antibodies from heat shocked and NEM-treated lysates had a significantly greater amount of HRI associated with them than that associated in either heat shocked or NEM-treated lysates (figure 4A, lane 5 vs. lanes 3 or 4). Furthermore, this association was quantitative; most of the HRI was recovered in the Hsp90 immunoprecipitates as HRI-Hsp90 complex (figure 4A, lane 5), and almost no free HRI was detected in the supernatants (HRI immunoprecipitates; figure 4B, lane 4). The reverse was observed in the Hsp90 immunoprecipitates and supernatants (HRI immunoprecipitates) of reticulocyte lysates incubated at normal temperature (figure 4A, lane 2 and figure 4B,

Table 1. Effect of Hsp90 on HRI kinase activity.

HRI + supplements	[γ - ³² P]ATP incorporation (cpm)	
	HRI	eIF-2 α
HRI alone	1,892	6,526
HRI + Hsp90	2,611	9,856 (1.5-fold)
HRI + hemin	245	187
HRI + hemin + Hsp90	564	2,797 (14.95-fold)
HRI + hemin + NEM	242	403
HRI + hemin + NEM + Hsp90	540	2,623 (6.5-fold)
Hsp90 alone	111	71

lane 1). Therefore, NEM, not only had a synergistic effect during heat shock, but it also promoted an association of HRI with Hsp90 at normal temperature (30°C)

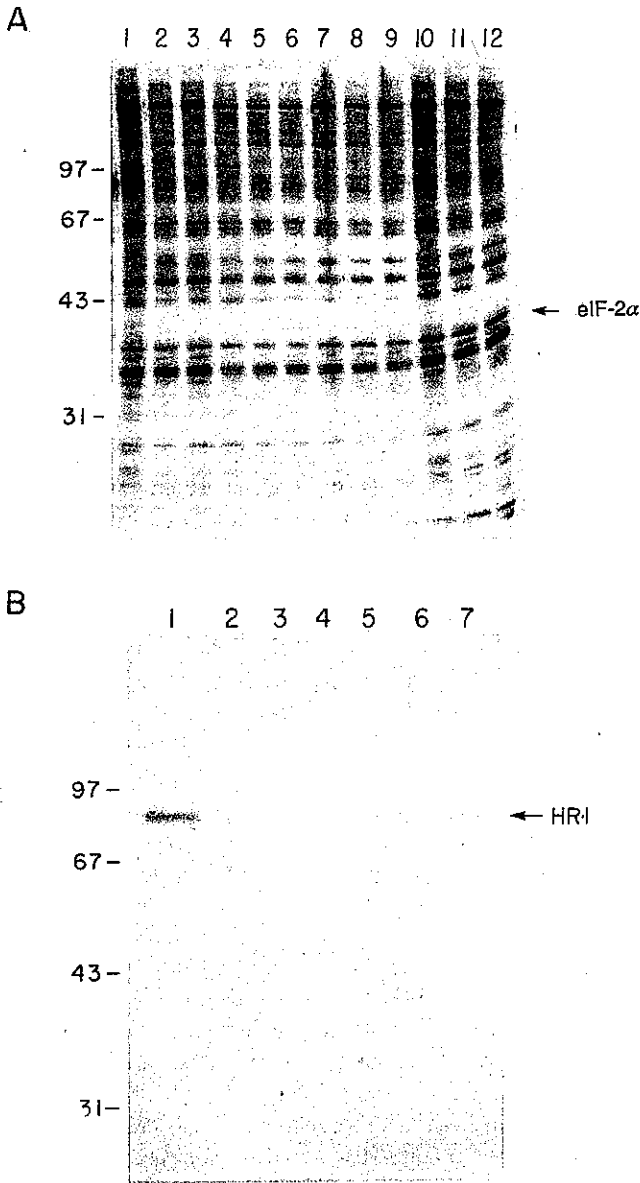


Figure 3. (A) Activation of HRI. HRI activity in the control or heat shocked lysates was measured by the phosphorylation of added purified eIF-2 (0.5 mg) under protein synthesizing conditions as described in § 2. Lanes 1–3, heme-deficient lysates at 30°C for 10 min, 30 min and 45 min, respectively; lanes 4–6, heme-supplemented lysates at 30°C for 10, 30 and 45 min, respectively; lanes 7–9, heme-supplemented lysates heat shocked at 42°C for 10, 30 and 45 min, respectively; lanes 10–12, heme-deficient lysates heat shocked at 42°C for 10, 30 and 45 min, respectively. (B) Association of Hsp90 with HRI. HRI in the lysates was immunoprecipitated by anti-Hsp90 antibody and then immunoblotted with mAB F. Lane 1, purified HRI; lanes 2–4, heme-supplemented lysates at 30°C for 10, 30 and 45 min, respectively; lanes 5–7, heme-supplemented lysates at 42°C for 10, 30 and 45 min, respectively.

since some amount of HRI was present in the Hsp90 immunoprecipitate (figure 4A, lane 3) and lesser amount of free HRI was observed compared to the control sample (figure 4B, lane 2 vs. lane 1). The overall Hsp90-HRI complex formation was more profound in NEM-treatment than during heat shock alone as indicated by the quantity of the free HRI left (figure 4B, compare lanes 2 and 3).

4. Discussion

Among many stress-unrelated functions of Hsp90, its role in regulation of protein synthesis is of great importance. The present study is an attempt to reveal the possible mechanism by which Hsp90 may regulate initiation of protein synthesis by performing both *in vitro* and *in situ* experiments in rabbit reticulocyte lysates. The results presented here demonstrate that Hsp90 is associated with HRI *in situ* in the reticulocyte lysates, upon NEM treatment at 30°C (figure 1) and during heat shock at 42°C (figure 3). Furthermore, it is also evident that HRI in the form of HRI-Hsp90 complex is highly active in terms of both autophosphorylation and eIF-2α kinase activity. The absence of detectable amount of Hsp90 associated with HRI activated during

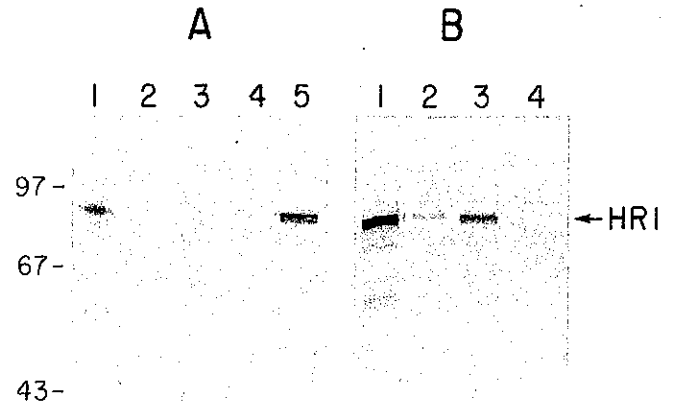


Figure 4. Synergistic effect of NEM-treatment and heat shock on HRI-Hsp90 association. (A) Hsp90 was immunoprecipitated by anti-Hsp90 antibody from heme-supplemented reticulocyte lysates at both 30°C and 42°C in the presence or absence of NEM for 30 min. The presence of HRI in these immunoprecipitates was detected by Western blot with anti-HRI mAB F. Lane 1, purified HRI (as standard); lanes 2–5, immunoprecipitates from lysates incubated at 30°C, in the absence (lane 2) or presence (lane 3) of NEM, and at 42°C, in the absence (lane 4) or presence (lane 5) of NEM. (B) The supernatants left after immunoprecipitation with anti-Hsp90 antibody were further immunoprecipitated with anti-HRI mAB F and immunoblotted (mAB F) to determine the amount of free HRI left in the supernatants. Lane 1, 30°C; lane 2, 30°C and NEM-treated; lane 3, 42°C; lane 4, 42°C and NEM-treated.

hemin-deficiency and GSSG-treatment (figure 1) suggests that either the mechanisms of HRI activation in these conditions are different from those during NEM-treatment or heat shock, or the amount of Hsp90-HRI complex if at all formed under those conditions is so small that it is barely detectable.

HRI is activated at heat shock temperature (42°C) in reticulocytes (Ernst *et al* 1982, and the present study), HeLa (De Benedetti and Baglioni 1986) and Ehrlich ascites cells (Scorsone *et al* 1987). The data reported here, therefore further establish that the heat shock induced activation of HRI is mediated by Hsp90. However, the exact mechanism of how Hsp90 stimulates catalytic activity of HRI thereby leading to its activation is yet to be elucidated. The other condition that led to activation of HRI was treatment of lysates with NEM, a sulfhydryl reagent. Since sulfhydryl reagents are known to induce heat shock response in eukaryotic cells (reviewed in Nover 1984), the mechanism of activation of HRI *in situ* by NEM may be similar to that during heat shock. Therefore, it is plausible that Hsp90 may be the common mediator for the activation of HRI during stress conditions. Although in this study only Hsp90-HRI interaction was of interest, a number of other proteins are also known to be present in HRI-Hsp90 complex (Matts *et al* 1992; Mendez and de Haro 1994; Xu *et al* 1997). In addition to Hsp90, these proteins, namely, HSP70, FKBP52 and p23 may also contribute to the regulation of HRI activity. Details of such influence need to be worked out in order to understand their role in *in vivo* function in terms of regulation of protein synthesis.

Of the two anti-HRI monoclonal antibodies used in the immunoprecipitation experiments, mAB F did not seem to recognize HRI-Hsp90 complex (figure 1). The inability of mAB F to immunoprecipitate HRI-Hsp90 complex from either NEM-treated or heat shocked lysates indicates that this antibody is unable to recognize HRI epitope in the HRI-Hsp90 complex, which is altered by Hsp90 binding. This suggests that the HRI epitope for this antibody is at or near the Hsp90 binding site. When Hsp90 binds to HRI, it prevents the binding of mAB F to HRI.

In vitro experiments (figure 2) demonstrated that the amount of HRI and Hsp90 used for the assays were all used up for HRI-Hsp90 complex formation in any of the conditions used. However, a very small amount of Hsp90 which is associated with HRI *in vivo* is sufficient to cause a great deal of HRI activation (figure 1). Thus, the extent of association between Hsp90 and HRI *in situ* in the lysates appeared to be different than that *in vitro*. This is perhaps due to involvement of other factors and also dependent on the total amount of Hsp90 available for interaction with HRI.

During heat shock, the amount of Hsp90 associated

with HRI is increased as a function of time (figure 3), and a concomitant increase in HRI phosphorylation is also observed. This increased formation of HRI-Hsp90 complex could be due to either the availability of more amount of Hsp90 synthesized during heat shock (Lindquist 1986) or due to post-translational modifications such as phosphorylation of Hsp90 during heat shock. Indeed, it has been shown that only the phosphorylated form of Hsp90 activates HRI *in vitro* (Szyszka *et al* 1989).

Activation of HRI in the reticulocyte lysates by NEM was higher than the activation by heat shock. This is consistent with the fact that more HRI-Hsp90 complex is formed upon NEM-treatment than that formed during heat shock (figure 4). A maximum association between Hsp90 and HRI was observed under conditions of simultaneous heat shock and NEM-treatment when almost no free HRI was detected in the lysates (figure 4). Thus, a concomitant increase in HRI activity and HRI-Hsp90 complex formation suggests that the activation of HRI in reticulocyte during heat shock is the result of the association of Hsp90 to HRI.

In conclusion, the data reported herein establish that Hsp90 may be instrumental in regulating protein synthesis by modulating the activity of the heme-regulated eIF-2 α kinase during stress response. Since Hsp90 is one of the abundant proteins in a cell, and is overexpressed during early embryogenesis (reviewed in Pal *et al* 1998), it is not unlikely that Hsp90 is involved in translation regulation under normal physiological conditions as well as during development and differentiation.

Acknowledgements

I wish to thank Mr Sanjay Anand and Mrs Manisha Joshi-Purandare for their help in preparation of this manuscript. A part of the work was carried out in the laboratory of Dr Irving M London in collaboration with Dr J-J Chen, at Massachusetts Institute of Technology, USA. Financial supports from the Council of Scientific and Industrial Research (Grant No. 37/822/93-EMR-II), and from the University Grants Commission (Grant No. F. 3-8/95 (SR-II), New Delhi, are duly acknowledged.

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