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# Dual Role for Hsc70 in the Biogenesis and Regulation of the Heme-Regulated Kinase of the α Subunit of Eukaryotic Translation Initiation Factor 2

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**The heme-regulated kinase of the α subunit of eukaryotic initiation factor 2 (HRI) is activated in rabbit reticulocyte lysate (RRL) in response to a number of environmental conditions, including heme deficiency, heat shock, and oxidative stress. Activation of HRI causes an arrest of initiation of protein synthesis. Recently, we have demonstrated that the heat shock cognate protein Hsc70 negatively modulates the activation of HRI in RRL in response to these environmental conditions. Hsc70 is also known to be a critical component of the Hsp90 chaperone machinery in RRL, which plays an obligatory role for HRI to acquire and maintain a conformation that is competent to activate. Using de novo-synthesized HRI in synchronized pulse-chase translations, we have examined the role of Hsc70 in the regulation of HRI biogenesis and activation. Like Hsp90, Hsc70 interacted with nascent HRI and HRI that was matured to a state which was competent to undergo stimulus-induced activation (mature-competent HRI). Interaction of HRI with Hsc70 was required for the transformation of HRI, as the Hsc70 antagonist clofibric acid inhibited the folding of HRI into a mature-competent conformation. Unlike Hsp90, Hsc70 also interacted with transformed HRI. Clofibric acid disrupted the interaction of Hsc70 with transformed HRI that had been matured and transformed in the absence of the drug. Disruption of Hsc70 interaction with transformed HRI in heme-deficient RRL resulted in its hyperactivation. Furthermore, activation of HRI in response to heat shock or denatured proteins also resulted in a similar blockage of Hsc70 interaction with transformed HRI. These results indicate that Hsc70 is required for the folding and transformation of HRI into an active kinase but is subsequently required to negatively attenuate the activation of transformed HRI.**

The heme-regulated inhibitor (HRI) of protein synthesis in rabbit reticulocyte lysate is activated in response to a host of environmental conditions, including heme deficiency, heat shock, oxidative stress, and the presence of denatured proteins (reviewed in references 9, 10, 27, 31, 33, and 36). HRI specifically phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2α). Phosphorylated eIF-2α arrests protein synthesis at the level of initiation by sequestering eIF-2B, the guanine nucleotide exchange factor required for the recycling of eIF-2 · GDP, in a poorly dissociable complex (36, 39).

The biogenesis of HRI into an active heme-regulatable kinase is a complex phenomenon which proceeds through several intermediate stages. Using synchronized pulse-chase translations, we have identified several intermediates of HRI that are generated during its folding and activation (55). After its release from ribosomes in hemin-supplemented rabbit reticulocyte lysate (RRL), newly synthesized HRI (early-folding intermediates of HRI) matures to a stage where it is competent of transforming into an active kinase (mature-competent HRI). While mature-competent HRI is not an active kinase, its potential to become an active kinase can be unmasked by *N*-ethylmaleimide (NEM) treatment. NEM activates HRI by covalently modifying sensitive sulfhydryls of HRI which play a role in regulating HRI activity (9, 11). Thus, the conformation of mature-competent HRI can be distinguished from that of early-folding intermediates of HRI, as NEM treatment of this population of HRI molecules does not result in their activation (55).

In heme-deficient RRL, a portion of the mature-competent HRI transforms via autophosphorylation into an active heme-regulatable eIF-2α kinase (transformed HRI). Transformed HRI exhibits a slower electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Addition of hemin suppresses the activity of transformed HRI without inducing changes in its phosphorylation status (repressed HRI). In addition to these defined populations of HRI, transformed HRI becomes more highly activated upon prolonged incubation in heme-deficient RRL or upon treatment with NEM. The further activation of HRI under these conditions correlates with its hyperphosphorylation (hyperphosphorylated HRI), which makes HRI less responsive to inhibition by hemin (17, 31, 53).

HRI interacts with several heat shock proteins in RRL, including Hsp90, Hsc70, and their associated cohorts FKBP52 and p23 (41, 57). Hsp90 interacts with nascent HRI cotranslationally, and this interaction persists after release of newly synthesized HRI from ribosomes in hemin-supplemented RRL (55). Furthermore, we have demonstrated that a functional interaction between Hsp90 and HRI is obligatory for HRI to acquire and maintain a conformation that is competent to become transformed into a stable, heme-regulatable kinase. However, after its transformation, HRI does not interact with Hsp90, and its regulation by hemin and stability are not Hsp90 dependent.

HRI also interacts with Hsc70. Earlier work suggests that the interaction of Hsc70 with HRI negatively modulates HRI activation. Sensitivity of HRI to activate in response to heme

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deficiency or to heat and oxidative stress correlated with levels of Hsc70 present in different lysate preparations (37). Activation of HRI in response to heat shock and denatured proteins was accompanied by dissociation of HRI from Hsc70 (38). Furthermore, addition of purified Hsc70 inhibited the activation of HRI in response to heme deficiency (22, 53) and in response to heat and oxidative stress in hemin-supplemented RRL (53). Hsc70 appeared to act by inhibiting the hyperphosphorylation of HRI which occurs upon the activation of transformed HRI and causes HRI to become progressively more resistant to inhibition by heme. Hsc70 did not inhibit transformation of HRI, indicating a specific regulatory role for Hsc70 on HRI activation.

HRI that is endogenous to RRL represents a heterogeneous mixture of kinase molecules. As such, many of the details with respect to the stage at which Hsc70 interacts with HRI during its biogenesis and activation, and the functional significance of these interactions remains to be clarified. In this report, we have studied the role of Hsc70 on the maturation and activation of HRI that was synthesized de novo in RRL. The results indicated that Hsc70 plays a dual role in the regulation HRI: (i) an essential positive role for kinase folding, maintenance, and transformation; and (ii) a negative role in attenuating kinase activation in response to hemin and stress conditions.

## MATERIALS AND METHODS

**De novo synthesis and maturation of HRI.** Coupled transcription-translation of HRI and $His_7$-HRI were initiated in nuclease-treated RRL (TnT RRL; Promega) at 30°C in the absence of [$^{35}$S]Met for 15 min (preliminary experiments indicated that HRI synthesis began in TnT lysates between 10 and 15 min of incubation). At 15 min, a pulse of [$^{35}$S]Met (460 μCi/ml) was given. After 4 min of radiolabeling, 1 volume of TnT protein synthesis mix containing [$^{35}$S]Met-labeled HRI ([$^{35}$S]$His_7$-HRI) was mixed with 4 volumes of normal heme-deficient or hemin-supplemented (10 μM hemin) protein synthesis mixes (55) containing non-nuclease-treated RRL and the protein synthesis initiation inhibitors edeine (10 μM) and/or aurintricarboxylic acid (60 μM). [$^{35}$S]$His_7$-HRI was then incubated for 60 min at 30°C. HRI synthesis was found to be completed after 8 to 12 min of chase, with no further incorporation of [$^{35}$S]Met (55).

The degree of transformation of [$^{35}$S]$His_7$-HRI that is observed in experiments varies between lots of RRL used for synthesis and maturation of HRI. The concentration of exogenous hemin that is optimum for suppressing activation of endogenous HRI and maintaining protein synthesis varies between lots of RRL. The degree of HRI transformation observed with the above protocol is dependent on hemin concentration (55). Lots of RRL that required little exogenous hemin to be added to maintain protein synthesis, presumably because their concentration of endogenous heme is high, transformed HRI poorly (55a). The second shift in HRI mobility on SDS-PAGE that occurs upon the hyperphosphorylation of HRI is also more clearly seen in experiments using lots of RRL that transforms HRI more efficiently. Lots of RRL also vary significantly in their chaperone content (37), which is likely to contribute to differences in transformation efficiency.

**Assay of the kinase activity of [$^{35}$S]$His_7$-HRI adsorbed to Ni-NTA resin.** $Ni^{2+}$-nitrilotriacetic acid coupled to agarose (Ni-NTA resin; Qiagen) was equilibrated with adsorption buffer (50 mM Tris-HCl [pH 7.5], 10 mM imidazole). RRL mixes containing [$^{35}$S]$His_7$-HRI were clarified by centrifugation at 10,000 rpm for 5 min before adsorption to Ni-NTA resin. [$^{35}$S]$His_7$-HRI from 25 μl of RRL reaction mixes were bound to the resin (10 μl) for 1 h on ice, followed by three washes with 500 μl of buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM imidazole. Assays for the kinase activity of [$^{35}$S]$His_7$-HRI bound to Ni-NTA resin were performed for 4 min at 30°C as described elsewhere (55). Samples were analyzed by SDS-PAGE (10% gel), followed by transfer to a polyvinylidene difluoride (PVDF) membrane and autoradiography as described previously (26). Autophosphorylation of HRI was assayed by the incorporation of [$^{32}$P]$P_i$ into HRI during eIF-2α kinase assays incubated with [γ-$^{32}$P]ATP. $^{32}$P-labeled HRI and eIF-2α were detected by quantitatively quenching $^{35}$S emissions with three intervening layers of previously developed X-ray film.

**Immunoadsorption.** Preparation of goat anti-mouse immunoglobulin G cross-linked to agarose, binding of anti-Hsc70 antibody BB70 (or nonimmune control antibody), and coimmunoadsorption of HRI with Hsc70 were carried out as previously described (38, 41). Clarified RRL mixes (20 to 25 μl) containing [$^{35}$S]HRI were used for immunoadsorption. After 60 min of binding on ice, immunopellets were washed three times with 500 μl of wash buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1% Tween 20, 10 mM monothioglycerol), and the immunopellets were eluted with SDS-PAGE sample buffer. Proteins present in immunopellets, and supernatants were separated on SDS-PAGE (10% gel) and transferred to a PVDF membrane. [$^{35}$S]HRI was detected by autoradiography.

**Cotranslational association of Hsc70 and Hsp90 with HRI.** TnT RRL lysates, containing or lacking (control) plasmid template coding for HRI, were labeled with [$^{35}$S]Met as described above. After 18.5 min of synthesis, the protein synthesis mix was diluted with 2 volumes of ice cold buffer containing 20 mM Tris-HCl (pH 7.5), 1 M KCl, and either 2.5 mM magnesium acetate to maintain polyribosome integrity or 10 mM EDTA to disrupt polyribosomes and dissociate nascent chains. As an additional control, nascent chains were released from polyribosomes after 15-min synthesis by treatment of translation mixes with 1 mM puromycin for 5 min at 30°C prior to dilution with buffer. Diluted translations were layered on top of 15 to 40% sucrose gradients containing buffers and salts as described above and centrifuged for 4.5 h at 40,000 rpm in an AH650 rotor. The supernatant was removed, and the ribosomal pellets were dissolved in SDS sample buffer. Proteins present in ribosomal pellets were separated by SDS-PAGE on a 10% gel and transferred to a PVDF membrane. Hsc70 and Hsp90 were detected by Western blotting.

**Protein synthesis and eIF-2α phosphorylation in RRL.** Protein synthesis was carried out at 30°C in standard RRL reaction mixtures with the addition of [$^{14}$C]leucine as described previously (16, 32). Hemin-supplemented lysates contained 20 μM hemin-HCl. Protein synthesis was determined by measuring the incorporation of [$^{14}$C]leucine into the acid-precipitable protein at 30°C in standard RRL reaction mixtures. eIF-2α phosphorylations in 2 μl of protein synthesis mixes were analyzed as previously described by Western blotting of one-dimensional vertical isoelectric focusing slab gels, using a 1:1,000 dilution of anti-eIF-2α monoclonal ascites fluid (42, 48).

**Assay for effect of clofibric acid on eIF-2B guanine nucleotide exchange activity.** eIF-2B activity was measured as described previously (39). Briefly, protein synthesis mixes were incubated with or without clofibric acid for 20 min at 30°C. Protein synthesis mix (50 μl) was then mixed with 130 μl of ice-cold dilution buffer (40 mM Tris HCl [pH 7.4], 100 mM KCl, 50 mM KF, 2 mM magnesium acetate, 10% glycerol, 40 μM GDP) and 20 μl of preformed eIF-2 · [$^{3}$H]GDP complex. Reaction mixes were then incubated at 30°C for 2 min. Exchange assays were stopped by the addition of 1 ml of ice-cold wash buffer, followed by filtration of the reaction mixture through nitrocellulose filters (HAWP 02500; Millipore) which rapidly bind the remaining eIF-2 · [$^{3}$H]GDP complex. Filters were then washed with an additional 15 ml of ice-cold wash buffer to remove any unbound [$^{3}$H]GDP.

## RESULTS

**Interaction of Hsc70 with HRI during HRI biogenesis and activation.** Hsc70 interacts with nascent polypeptide chains cotranslationally (4, 20). While Hsp90 does not commonly interact with polypeptides during their synthesis (4, 20), we have observed that Hsp90 also interacts with HRI cotranslationally (55). Studies examining the sequence of events that occur during chaperone-mediated reconstitution of steroid hormone binding activity have indicated that an obligate interaction of Hsc70 with steroid hormone receptors (SHRs) precedes the formation of stable complexes between SHRs and Hsp90 (reference 46 and references therein). To study the interaction of Hsc70 with HRI, we synthesized [$^{35}$S]HRI de novo in RRL and determined the interaction of Hsc70 with HRI folding and activation intermediates.

The cotranslational interaction of HRI with Hsc70 was examined in TnT RRL programmed with or without HRI template (20, 55). Ribosomes actively synthesizing HRI were isolated by centrifugation through a 15 to 40% sucrose gradients, and the ribosome pellet was analyzed for the presence of Hsc70 by SDS-PAGE and Western blotting. As reported earlier (55), Hsp90 was detected in the ribosomal pellets containing bound nascent HRI polypeptide chains (Fig. 1A, lane 3). Like Hsp90, Hsc70 was detected in the ribosomal pellets containing bound nascent HRI polypeptide chains (lane 3). We detected little Hsc70 in ribosome pellets isolated in the presence of EDTA (lane 4) and none in ribosome pellets isolated from TnT RRL that was not programmed with template (lanes 1 and 2) or in polysome pellets that were treated with 1 mM puromycin to release nascent HRI chains (Fig. 1B, lane 2). Therefore, occurrence of Hsc70 in polysomal pellets was specific for the presence of nascent HRI.

To verify the cotranslational interaction of Hsc70 with nas-

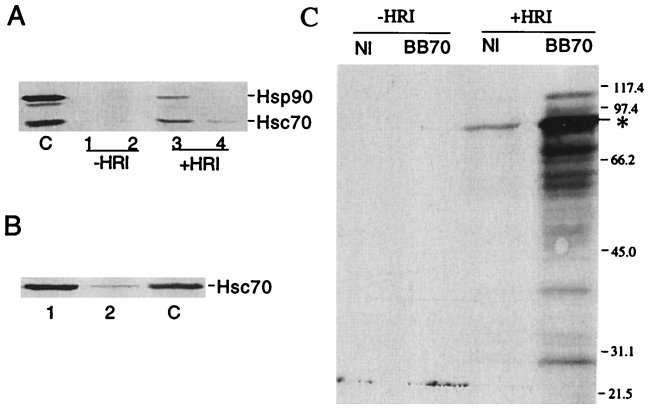


FIG. 1. Cotranslational interaction of Hsc70 with HRI. TnT RRLs were programmed with (+HRI) or without (−HRI) HRI template for 18.5 min at 30°C. (A) Translation mixtures were separated on 15 to 40% sucrose gradients in the presence of either 2.5 mM $Mg^{2+}$ (lanes 1 and 3) or 10 mM EDTA (lanes 2 and 4). (B) In addition, translation mixes with HRI template were either treated (lane 2) or not treated (lane 1) with 1 mM puromycin for 5 min at 30°C after 15 min synthesis to release the nascent chains and then separated on 15 to 40% sucrose gradients. Polysomal pellets were dissolved in SDS sample buffer and analyzed by SDS-PAGE. Hsc70 and Hsp90 were detected by Western blot analysis with anti-Hsc70 antiserum N-27 and anti-Hsp90 antiserum 84/86. C, 1 μl of RRL applied as a standard. (C) Nascent HRI polypeptides from the translation mixtures with (+HRI) and without (−HRI) HRI template were immunoprecipitated with BB70 anti-Hsc70 antibodies in the presence of 10 mM EDTA as described in Materials and Methods. [$^{35}$S]HRI was detected by autoradiography. *, full-length HRI. NI, immunoadsorptions done with nonimmune control antibody. Sizes are indicated in kilodaltons.

cent HRI, we examined whether nascent [$^{35}$S]HRI was coimmunoadsorbed by anti-Hsc70 antibodies. Nascent [$^{35}$S]HRI polypeptides were released from the polysomes by treatment with EDTA, and chaperone-associated polypeptides were isolated by coadsorption with anti-Hsc70 antibodies. Nascent HRI polypeptide chains with estimated molecular masses of ~30 kDa or greater were observed to interact with Hsc70 in an immunospecific manner (Fig. 1C, lane 4), confirming the cotranslational association of Hsc70 with HRI. Control translations lacking HRI template (lane 2) further confirmed the presence of nascent HRI polypeptides in the immunopellets.

To further characterize the association of Hsc70 with HRI, we examined the interaction of Hsc70 with newly synthesized HRI following its release from the ribosomes (Fig. 2). HRI continued to interact with Hsc70 after completion of HRI synthesis. This interaction was unaffected by hemin, as equivalent amounts of HRI were coimmunoprecipitated with anti-Hsc70 antibodies in the presence or absence of hemin (Fig. 2, lanes 3 and 4). The interaction of Hsc70 with HRI continued after 60 min of incubation in the presence of hemin (lane 7). When HRI was matured in a heme-deficient RRL, a portion of the HRI transformed, as indicated by the presence of a [$^{35}$S]HRI band with a slower electrophoretic mobility (lanes 6 and 8). Hsc70 continued to interact with both untransformed and transformed HRI (lane 8). The maintenance of an interaction between Hsc70 and transformed HRI supports the possibility that this interaction regulates the activity of HRI.

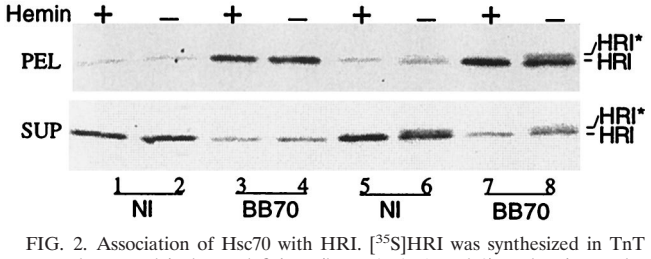


FIG. 2. Association of Hsc70 with HRI. [$^{35}$S]HRI was synthesized in TnT RRL and matured in heme-deficient (lanes 2, 4, 6, and 8) or hemin-supplemented (lanes 1, 3, 5, and 7) RRL for 8 (lanes 1 to 4) or 60 (lanes 5 to 8) min. Aliquots of each sample (20 μl) were immunoadsorbed with the anti-Hsc70 antibody BB70 or nonimmune (NI) control antibody. Proteins in the immunopellets (PEL) were analyzed by SDS-PAGE and autoradiography. HRI*, slow-mobility form of HRI (transformed HRI); SUP, supernatant.

**Role of Hsc70 during activation of HRI in response to stress conditions.** We have previously presented evidence that Hsc70 not only suppresses HRI activation in response to heme deficiency (53) but also negatively modulates HRI activation in response to heat and oxidative stress (38, 53). However, its not clear how Hsc70 suppresses the activation of HRI. This question is complicated by the facts that HRI exists in situ as a heterogeneous mixture of folding and activation intermediates and that the relationship between the molecular forms of HRI activated during heme deficiency and those which are activated in response to stress in hemin-supplemented RRL remains to be clarified. In an attempt to understand the mechanism of suppression of HRI activity by Hsc70, we characterized (i) the molecular forms of HRI that are activated during heme deficiency and stress and (ii) the interaction of Hsc70 with HRI under these conditions.

To characterize the molecular forms of HRI activated in response to stress, the effect of heat shock on HRI activation was studied in synchronized populations of HRI synthesized de novo. [$^{35}$S]$His_7$-HRI was synthesized by pulse-chase in TnT RRL and incubated in hemin-supplemented RRL containing 10 μM hemin to generate mature-competent HRI. Alternatively, HRI was incubated in heme-deficient RRL to generate transformed HRI, and/or 10 μM hemin was then added to transformed HRI to suppress its activity (repressed HRI). Mature-competent, transformed, and repressed HRI were incubated at 30°C (control) or 42°C (heat shock). After 20 min incubation, [$^{35}$S]$His_7$-HRI was adsorbed to Ni-NTA resin and kinase activity was assayed (Fig. 3A). HRI that lacked the $His_7$ tag was similarly analyzed to provide a measurement of the nonspecific binding of endogenous HRI and [$^{35}$S]HRI to the resin (lanes 7 and 8).

Consistent with earlier observations (55), (i) mature-competent HRI lacked eIF-2α kinase activity (Fig. 3A, lane 1), as indicated by the absence of eIF-2α phosphorylation above that observed in the controls for nonspecific binding (lane 8); (ii) transformed HRI was active, as indicated by the increased autophosphorylation and eIF-2α phosphorylation (lane 2) above that observed in the control for nonspecific binding (lane 7); and (iii) the kinase activity of transformed HRI was repressed by the addition of hemin (repressed HRI) (lane 3 versus lane 2). Incubation of mature-competent [$^{35}$S]$His_7$-HRI at 42°C (heat shock) did not change its autokinase or the eIF-2α kinase activities (lane 4) compared to the activities of mature-competent [$^{35}$S]$His_7$-HRI incubated at 30°C (lane 1). The autokinase and eIF-2α kinase activities of [$^{35}$S]$His_7$-HRI that had been transformed in heme-deficient RRL were further increased at 42°C (lane 5) compared to [$^{35}$S]$His_7$-HRI incubated at 30°C (lane 2). In addition, the autokinase and eIF-2α kinase activities of repressed [$^{35}$S]$His_7$-HRI doubled at 42°C in the presence of hemin (lanes 6) compared to the control incubated at 30°C (lane 3). Thus, transformation of HRI was essential for stress-induced activation of HRI, as mature-competent HRI did not activate upon incubation at 42°C. These results indicate that transformed HRI in heme-deficient RRL and repressed HRI in hemin-supplemented RRL were activated in response to heat shock.

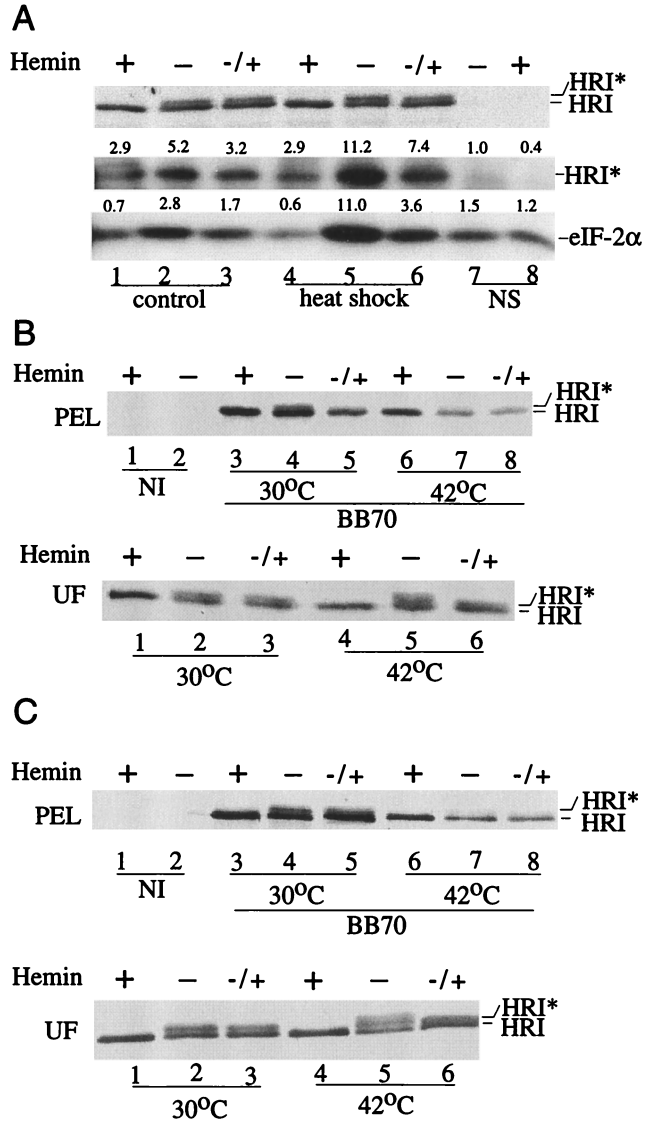


FIG. 3. Effect of heat shock on activation of HRI and the interaction of Hsc70 with HRI. (A) [$^{35}$S]His$_7$-HRI (lanes 1 to 6) and HRI lacking the His$_7$ tag (lanes 7 and 8) were synthesized in TnT RRL. [$^{35}$S]His$_7$-HRI and [$^{35}$S]HRI were then incubated in hemin-supplemented (mature-competent; +) or heme-deficient (transformed; −) RRL or incubated in heme-deficient RRL for 45 min followed by addition of 10 μM hemin (repressed; −/+) for 10 min. At the end of 55 min of incubation, RRLs were incubated at either 30°C (control; lanes 1 to 3, 7, and 8) or 42°C (heat shock; lanes 4 to 6) for 20 min. Affinity purification of [$^{35}$S]His$_7$-HRI on Ni-NTA resin and kinase assays were done as described in Materials and Methods. [$^{35}$S]His$_7$-HRI was detected by direct autoradiography (top), while [$^{32}$P]HRI (middle), and eIF-2α (bottom) were detected by quenching $^{35}$S emissions with three intervening layers of previously exposed films. The numbers are the amount of [$^{32}$P]HRI (top) and [$^{32}$P]eIF-2α (bottom) quantified by scanning densitometry and expressed as optical density × square millimeters. (55). HRI*, transformed HRI with slower electrophoretic mobility; NS, nonspecific. (B and C) De novo synthesis and maturation of [$^{35}$S]HRI into mature-competent (+), transformed (−), and repressed (−/+) conformations followed by control (30°C) and heat shock (42°C) treatments were done as described above. After the heat shock treatment, RRLs were either treated (C) or not treated (B) with apyrase (1 U/10 μl of RRL mix) for 10 min on ice before the immunoprecipitation. [$^{35}$S]HRI was immunoprecipitated either with anti-Hsc70 antibody BB70 (lanes 3 to 8) or nonimmune (NI) control antibody (lanes 1 and 2) as described in Materials and Methods. [$^{35}$S]HRI present in the immunopellets (PEL) and in the unfractionated RRL before the immunoprecipitations (UF) were detected by autoradiography.

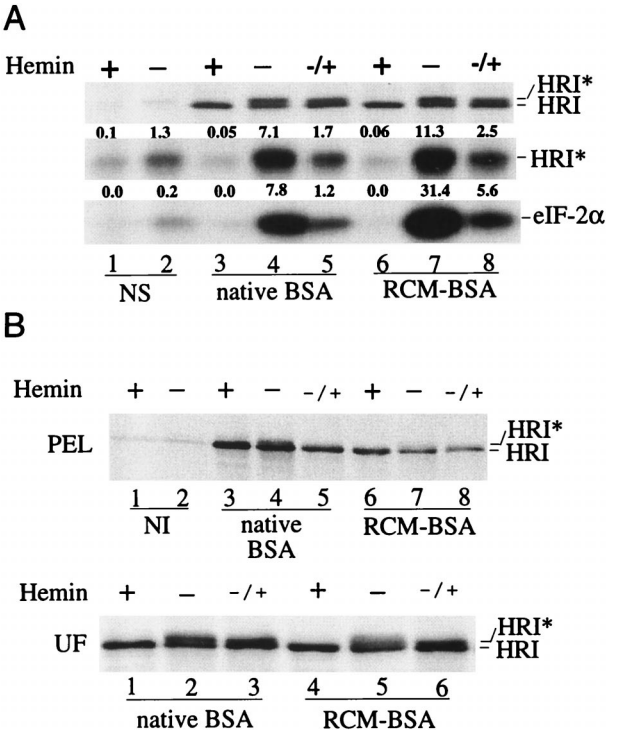


FIG. 4. Effect of RCM-BSA on activation of HRI and interaction of Hsc70 with HRI. De novo synthesis of [$^{35}$S]His$_7$-HRI (lanes 3 to 8) and [$^{35}$S]HRI (lanes 1 and 2) in TnT RRL and maturation into mature-competent (+), transformed (−), and repressed (−/+) conformations were done in hemin-supplemented and heme-deficient RRLs as described for Fig. 3. RRLs were then incubated with 1 μg of either native BSA (lanes 1 to 5) or RCM-BSA (lanes 6 to 8) per ml for 20 min. Following these incubations, affinity purification of HRI, kinase assays, and detection of [$^{35}$S]His$_7$-HRI (A, top), [$^{32}$P]HRI (middle), and eIF-2α (bottom) were done as described for Fig. 3 or RRL was immunoadsorbed with either anti-Hsc70 antibody BB70 (B, lanes 3 to 8) or nonimmune control antibody (lanes 1 and 2). [$^{35}$S]HRI present in the immunopellets (PEL) and in the unfractionated RRL before the immunoprecipitations (UF) were detected by autoradiography. NS, nonspecific.

To further characterize the molecular forms of HRI that are activated in response to stress, we examined the effect of a model denatured protein (reduced-carboxymethylated bovine serum albumin [RCM-BSA]) on the activation of HRI synthesized de novo. The activation of HRI during heat shock has been proposed to be due to stress-induced accumulation of denatured proteins which block the interaction of Hsc70 with HRI (38, 53). Consistent with this hypothesis addition of model denatured proteins to hemin-supplemented RRL causes the activation of HRI (38, 53). Addition of RCM-BSA to RRL containing populations of [$^{35}$S]His$_7$-HRI synthesized de novo was observed to mimic the effect of heat shock on HRI activation (Fig. 4A). Mature-competent [$^{35}$S]His$_7$-HRI remained as an inactive kinase upon the addition of either RCM-BSA (Fig. 4A, lane 6) or native BSA (Fig. 4A, lane 3) to hemin-supplemented RRL. However, addition of RCM-BSA to hemin-supplemented RRL containing repressed HRI caused a marked increase in both the autokinase and eIF-2α kinase activities of [$^{35}$S]His$_7$-HRI (lane 8) compared to the control, native BSA (lane 5). Like heat shock, RCM-BSA further activated [$^{35}$S]His$_7$-HRI in heme-deficient RRL containing transformed HRI (lane 7).

**Correlation between the loss of Hsc70 interaction with HRI and HRI activation.** To further characterize the mechanism by which stress-induced activation of HRI occurs, we examined

the interaction of Hsc70 with mature-competent, transformed, and repressed [$^{35}$S]HRI in stressed RRL (Fig. 3B and 4B). Previously, we observed that the activation of HRI in response to heat shock or the addition of RCM-BSA to RRL correlated with loss of the interaction between Hsc70 and HRI (38, 53). Mature-competent, transformed, or repressed [$^{35}$S]HRI was incubated at 30 or 42°C (heat shocked) as described above, and the ability of anti-Hsc70 antibody BB70 to coimmunoadsorb the various forms of [$^{35}$S]HRI was then examined. Both mature-competent and transformed [$^{35}$S]HRI were coimmunoadsorbed with Hsc70 from heme-deficient RRL which was incubated under control conditions (Fig. 3B, lane 4). Heat shock decreased the interaction of Hsc70 with both these forms of [$^{35}$S]HRI. SDS-PAGE of samples taken from unfractionated RRL prior to immunoprecipitation indicated that heat shock treatment of heme-deficient RRL caused the hyperphosphorylation of transformed [$^{35}$S]HRI, which was evident by a further mobility shift of transformed [$^{35}$S]HRI (Fig. 3B, UF, lane 5 versus lane 2). While both mature-competent and transformed HRI were present in the supernatants, primarily untransformed [$^{35}$S]HRI was coimmunoadsorbed from heat-shocked RRL. Following heat shock, little transformed [$^{35}$S]HRI or repressed [$^{35}$S]HRI was coadsorbed with Hsc70 from heme-deficient (lane 7) or hemin-supplemented (lane 8) RRL, respectively. The continued interaction of untransformed HRI with Hsc70 during heat shock likely reflects the fact that untransformed HRI is unstable and readily denatures and aggregates (55, 57).

Of significant interest was the observation that repressed [$^{35}$S]HRI was not coimmunoadsorbed with anti-Hsc70 antibodies from hemin-supplemented control RRL (Fig. 3B, lane 5), unless the RRL was treated with apyrase to hydrolyze ATP to ADP before the immunoprecipitations (Fig. 3C, lane 5). Exchange of ADP bound to Hsc70 for ATP is required to induce the rapid dissociation of Hsc70 from polypeptide substrates (reviewed in references 3 and 24). Heat shock similarly reduced the amount of repressed HRI that was coimmunoadsorbed with anti-Hsc70 antibodies after apyrase treatment of RRL (Fig. 3C, lane 8). These observations suggest that the lack of Hsc70 interaction with transformed HRI and/or repressed HRI seems to be important for activation of HRI during heat shock.

Similar to heat shock, RCM-BSA resulted in the loss of Hsc70 interaction with transformed HRI concomitant with the enhanced activation of HRI. The ability of RCM-BSA to bind and sequester Hsc70 resulted in a proportional decrease in the amount of both mature-competent and transformed [$^{35}$S]HRI that was coimmunoadsorbed from RRL by anti-Hsc70 antibodies (Fig. 4B, native BSA versus RCM-BSA). While transformed [$^{35}$S]HRI was coadsorbed with Hsc70 from heme-deficient RRL containing native BSA (Fig. 4B, PEL, lane 4), little transformed [$^{35}$S]HRI was coadsorbed with Hsc70 from heme-deficient RRL in the presence of RCM-BSA (Fig. 4B, PEL, lane 7). Like heat shock, RCM-BSA treatment of heme-deficient RRL caused the hyperphosphorylation of transformed [$^{35}$S]HRI, which was evident by a further mobility shift of transformed [$^{35}$S]HRI (Fig. 4B, UF, lane 5 versus lane 2). In the absence of apyrase treatment, repressed [$^{35}$S]HRI did not coimmunoadsorb with anti-Hsc70 antibodies when incubated with native BSA.

**Effect of the Hsc70 binding drug clofibric acid on HRI activation and HRI interaction with Hsc70.** To further confirm the regulatory role of Hsc70 on HRI activation, we studied the effect of Hsc70 binding drug clofibric acid on [$^{35}$S]His$_7$-HRI activation. Clofibric acid specifically binds to Hsc70 at or near its ATP binding site (1, 30). The hypothesis that clofibric acid

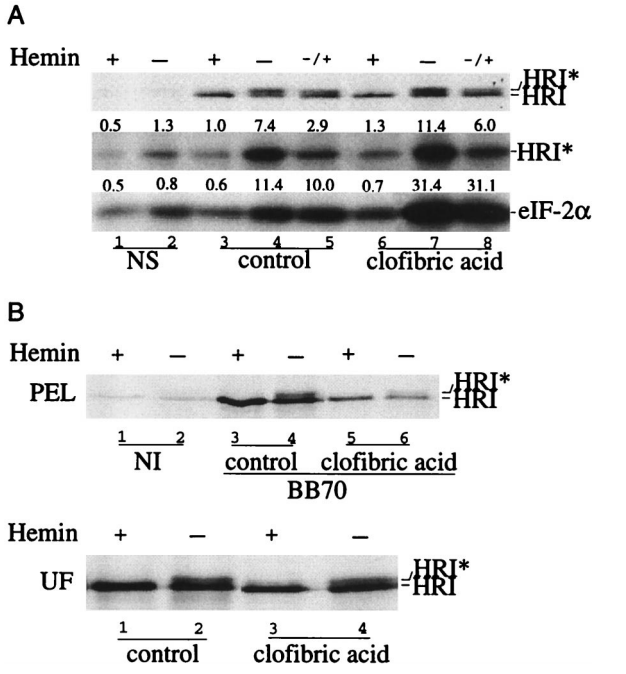


FIG. 5. Effect of clofibric acid on HRI activation and interaction of Hsc70 with HRI. De novo synthesis of [$^{35}$S]His$_7$-HRI in TnT RRL and maturation of [$^{35}$S]His$_7$-HRI into mature-competent (+), transformed (−), and repressed (−/+) conformations in hemin-supplemented and heme-deficient RRL were done as described for Fig. 3. At the end of 55 min of incubation, samples were incubated for 20 min with (lanes 6 to 8) or without (lanes 1 to 5) 15 mM clofibric acid. (A) Analysis of the kinase activity of affinity-purified [$^{35}$S]His$_7$-HRI (lanes 3 to 8) and nonspecific controls (lanes 1 and 2) and detection of [$^{35}$S]His$_7$-HRI (top), [$^{32}$P]HRI (middle), and eIF-2α (bottom) were done as described for Fig. 3. NS, nonspecific. (B) De novo synthesis and maturation of [$^{35}$S]HRI into mature-competent (+) and transformed (−) conformations followed by clofibric acid treatment were done as described above. Immunoadsorptions with anti-Hsc70 antibody BB70 (lanes 3 to 6) and nonimmune (NI) control antibody (lanes 1 and 2) and the detection of [$^{35}$S]HRI present in the immunopellets (PEL) and in the unfractionated RRL (UF) were done as described for Fig. 3.

is a specific chaperone antagonist is supported by the observations that (i) clofibric acid inhibits the ability of chaperones present in RRL to facilitate the renaturation of thermally denatured luciferase (51) and (ii) clofibric acid-induced inhibition of luciferase renaturation correlates with inhibition of the binding of Hsc70 to denatured luciferase (52).

To further characterize the regulatory role of Hsc70 on HRI activation, we studied the effect of clofibric acid on the activity of the various forms of [$^{35}$S]His$_7$-HRI synthesized de novo in RRL. Mature-competent, transformed, and repressed [$^{35}$S]His$_7$-HRI were synthesized in synchronized pulse-chase translations as described above. The RRLs were then treated with 15 mM clofibric acid for 20 min. [$^{35}$S]His$_7$-HRI was affinity purified on Ni-NTA resin and assayed for kinase activity (Fig. 5A). The effect of clofibric acid on HRI activation was similar to that of heat shock. Clofibric acid had no effect on the kinase activities of mature-competent [$^{35}$S]His$_7$-HRI in hemin-supplemented RRL compared to control RRL (no treatment with clofibric acid) (Fig. 5A, lane 3 versus lane 6). Similar to heat shock and RCM-BSA treatments, the kinase activity of transformed [$^{35}$S]His$_7$-HRI increased markedly with clofibric acid treatment compared to the untreated control (lane 4 versus lane 7). Clofibric acid treatment also increased the autokinase and eIF-2α kinase activities of repressed [$^{35}$S]His$_7$-HRI three fold over the activities of the untreated control (lane 5 versus lane 8).

The effect of clofibric acid on the interaction of Hsc70 with mature-competent and transformed [$^{35}$S]HRI in heme-deficient RRL was also examined (Fig. 5B). After treatment of RRL with 15 mM clofibric acid for 20 min, no transformed [$^{35}$S]HRI was coimmunoadsorbed with Hsc70 from RRL in the presence of clofibric acid (Fig. 5B, lane 6 versus lane 4). While anti-Hsc70 antibodies did coadsorb untransformed (mature-competent) [$^{35}$S]HRI with Hsc70 from clofibric acid-treated RRL (lanes 5 and 6), the amount of HRI that was coadsorbed was markedly reduced compared to nontreated controls (lanes 3 and 4). However, when immunoresins were washed under more stringent conditions (buffer containing 150 mM NaCl), clofibric acid was observed to block the interaction of Hsc70 with both untransformed and transformed HRI (see below). Thus, while heat shock and RCM-BSA could have effects on RRL in addition to their inhibitory effect on Hsc70 function that could contribute to HRI activation, the correlation between clofibric acid-induced activation of transformed HRI and the clofibric acid-induced inhibition of the interaction of Hsc70 with transformed HRI further supports a regulatory role for the interaction of Hsc70 with transformed HRI.

To confirm the specific effects of clofibric acid as a chaperone antagonist on HRI activation, we studied the effect of clofibric acid on (i) protein synthesis, eIF-2α phosphorylation, and eIF-2B activity, hallmarks of HRI activation in RRL; and (ii) the activity of purified HRI. Addition of 15 mM clofibric acid to RRL inhibited protein synthesis, affecting the initial rate of protein synthesis and gradually causing an arrest of translation (Fig. 6). Titration of clofibric acid into RRL indicated that the concentration of clofibric acid that inhibited protein synthesis by 50% ($IC_{50}$; 8 mM) (not shown) correlated well with the $IC_{50}$ for clofibric acid-induced inhibition of the renaturation of luciferase (11 mM) (51).

To characterize the mechanism of inhibition of initiation of translation in clofibric acid-treated RRL, we analyzed the phosphorylation status of eIF-2α by vertical slab gel isoelectric focusing. Western blot analysis indicated that clofibric acid treatment increased eIF-2α phosphorylation markedly (Fig. 6, insert). Guanine nucleotide exchange assays were subsequently performed to determine whether the change in eIF-2α phosphorylation was of a magnitude significant enough to affect the activity of eIF-2B. eIF-2B activity was inhibited by 75% in clofibric acid-treated RRL (2,206 cpm of [$^{3}$H]GDP exchanged from eIF-2 in 2 min) relative to control RRL (8,275 cpm of [$^{3}$H]GDP exchanged from eIF-2 in 2 min). These results are consistent with the hypothesis that inhibitors of Hsc70 cause activation of endogenous HRI in RRL. In contrast, the Hsp90-specific inhibitor geldanamycin has no effect on protein synthesis or eIF-2α phosphorylation when added to either hemin-supplemented or heme-deficient RRL (25, 55). Consistent with these observations, transformed HRI does not interact with Hsp90 (55), such that the regulation of the activation and repression of transformed HRI occurs through a mechanism that is not dependent on the interaction of transformed HRI with Hsp90.

To further confirm that the effect of clofibric acid on HRI activation was through its effect on Hsc70, and not a direct effect on the kinase, we studied the effect of clofibric acid on activation of purified HRI that was free of associated chaperones (Fig. 6B). Clofibric acid did not affect HRI kinase activity when incubated with the purified HRI in vitro. Autokinase activity of HRI incubated with 10 mM clofibric acid, the concentration which inhibited protein synthesis ($IC_{50} \sim 8$ mM) and luciferase renaturation ($IC_{50} \sim 11$ mM) (51), was same as for the control with no drug treatment (Fig. 6B, lane 1 versus lane 4). However, at 15 mM clofibric acid, HRI kinase activity

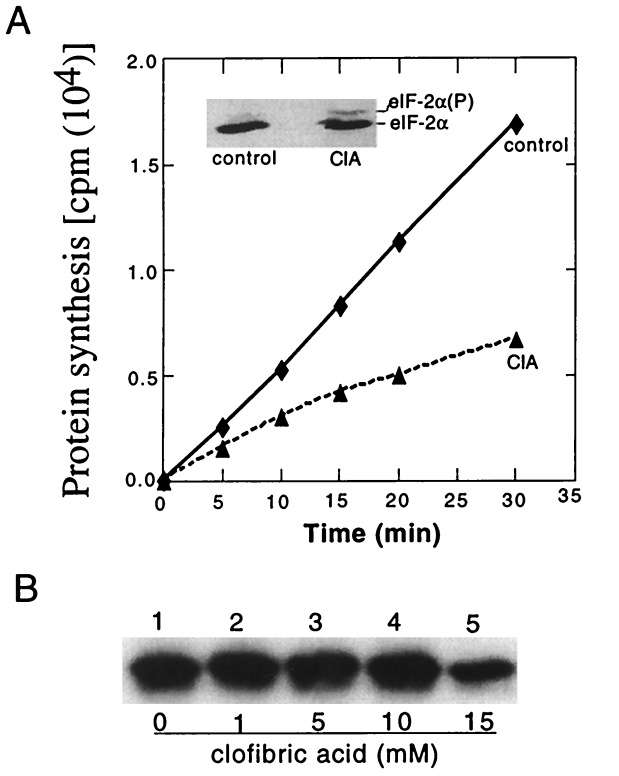


FIG. 6. Effect of clofibric acid on protein synthesis and eIF-2α phosphorylation in RRL. (A) Hemin-supplemented protein synthesis mixes were incubated at 30°C for 30 min in the presence (CIA) or absence (control) of 15 mM clofibric acid. Aliquots (3 μl) were taken at the times indicated to determine the incorporation of [$^{14}$C]leucine into acid-precipitable protein. After 30 min of incubation, a 3-μl aliquot was taken to determine the level of eIF-2α phosphorylation by vertical slab gel isoelectric focusing followed by Western blotting (inset). eIF-2α(P), phosphorylated eIF-2α. (B) The kinase activity of purified HRI incubated with increasing concentrations of clofibric acid was determined by using the same buffer and incubation conditions as used to assay affinity purified HRI. [$^{32}$P]HRI was detected by autoradiography.

was reduced. Thus, the effect of clofibric acid on HRI hyperactivation was not a direct effect of clofibric acid on the kinase.

**Effect of clofibric acid on HRI folding.** Hsc70 is required for the assembly of stable chaperone complexes between SHRs and Hsp90 and the acquisition of steroid hormone binding activity (46). The interaction of Hsc70 with SHRs is obligate and precedes the binding of Hsp90. Since Hsc70 was found to interact with HRI nascent polypeptides cotranslationally (Fig. 1) and early folding intermediates (Fig. 2), studies were carried out to determine whether Hsc70 also has an obligate positive role in the maturation of HRI.

To determine the role of Hsc70 in facilitating the folding and maturation of HRI, the effect of clofibric acid on the transformation of newly synthesized [$^{35}$S]$His_7$-HRI was examined (Fig. 7B). Clofibric acid was given to nascent [$^{35}$S]$His_7$-HRI immediately after the ribosomal runoff. After 60 min of incubation, [$^{35}$S]$His_7$-HRI was affinity purified on Ni-NTA resins and assayed for transformation (mobility shift on SDS-PAGE) and kinase activity (Fig. 7B) followed by Western blotting with anti-Hsc70 monoclonal antibody N-27 to detect the interaction of Hsc70 with [$^{35}$S]$His_7$-HRI (Fig. 7A). In clofibric acid-treated heme-deficient RRL, the mobility shift of [$^{35}$S]$His_7$-HRI on SDS-PAGE that is characteristic of HRI transformation was not observed (Fig. 7B, lane 4 versus 6). Consistent with the lack of [$^{35}$S]$His_7$-HRI transformation, [$^{35}$S]$His_7$-HRI affinity puri-

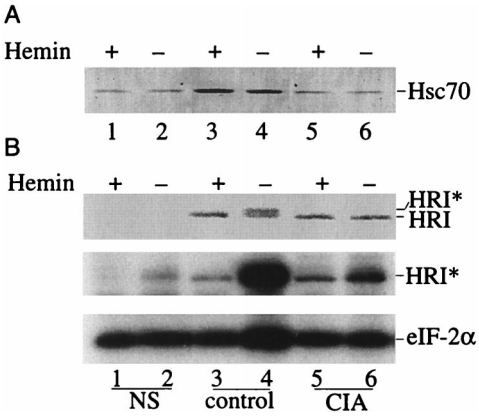


FIG. 7. Effect of clofibric acid on the folding of nascent HRI and its interaction with Hsc70. [$^{35}$S]His$_7$-HRI (lanes 3 to 8) and HRI lacking the His$_7$ tag (lanes 1 and 2) were synthesized in TnT RRL. [$^{35}$S]His$_7$-HRI and [$^{35}$S]HRI were then incubated in heme-deficient (−) or hemin-supplemented (+) RRL in the presence (lanes 5 and 6) or absence (lanes 1 to 4) of 15 mM clofibric acid for 60 min at 30°C. After 60 min of incubation, samples were adsorbed to Ni-NTA resin and assayed for eIF-2α kinase activity as described in Materials and Methods, separated by SDS-PAGE, and transferred to a PVDF membrane. (A) Hsc70 was detected by Western blot analysis using anti-Hsc70 antibody N-27 according to standard protocols. (B) Autoradiograms show [$^{35}$S]His$_7$-HRI (top), [$^{32}$P]HRI (middle), and eIF-2α (bottom) phosphorylation. NS, nonspecific binding to the Ni-NTA resin from RRL expressing [$^{35}$S]HRI lacking the His$_7$ tag under identical conditions.

fied from the clofibric acid-treated heme-deficient RRL had little autokinase activity and no eIF-2α kinase activity (Fig. 7B, lane 4 versus 6). Furthermore, while interaction of Hsc70 with HRI was observed in both hemin-supplemented RRL containing mature-competent [$^{35}$S]His$_7$-HRI and heme-deficient RRL containing a mixture of mature-competent and transformed [$^{35}$S]His$_7$-HRI (Fig. 7A, lanes 3 and 4), no interaction of Hsc70 with newly synthesized [$^{35}$S]His$_7$-HRI was observed in RRL treated with clofibric acid above the level that represented nonspecifically bound Hsc70 (Fig. 7A, lanes 5 and 6 versus lanes 1 and 2). Thus, clofibric acid disrupted Hsc70's interaction with [$^{35}$S]His$_7$-HRI in both hemin-supplemented and heme-deficient RRL, resulting in inhibition of [$^{35}$S]His$_7$-HRI transformation and activation. These results support the implication that Hsc70 plays a positive role in folding HRI.

Since Hsc70 is required for Hsp90-dependent acquisition of steroid hormone binding activity of SHRs (46), we examined the effect of clofibric acid on the interaction of Hsp90 with HRI. Consistent with our understanding of SHR-chaperone complex formation (46), little Hsp90 was detected in association with HRI following clofibric acid treatment, which correlated with a loss in Hsc70 binding (Fig. 8A). Treatment of nascent HRI with Hsp90-binding drug geldanamycin inhibited the transformation of HRI and caused the loss of Hsp90 interaction (55) (Fig. 8B) but did not affect the interaction of Hsc70. Thus, the data indicated that the interaction of Hsc70 with HRI precedes Hsp90 and that Hsc70 plays an important role in the assembly of chaperone complexes on substrate proteins.

**Effect of clofibric acid on activation of mature-competent HRI.** In hemin-supplemented RRL, mature-competent HRI is unstable and needs chaperone support to maintain its competence to be activated (55). However, unlike early-folding intermediates, mature-competent HRI is folded to a conformation that can be activated by NEM (55). The maintenance of mature-competent HRI requires the physical association of Hsp90 with mature-competent HRI, and disruption of this

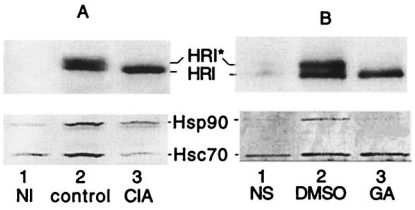


FIG. 8. Effects of clofibric acid and geldanamycin on the interaction of Hsc70 and Hsp90 with HRI. [$^{35}$S]His$_7$-HRI was synthesized in TnT RRL, followed by maturation for 60 min at 30°C in heme-deficient RRL treated (lane 3) or not treated (lane 1 and 2) with 15 mM clofibric acid (CIA) (A) or 10 μg of geldanamycin (GA) per ml (B) as described in Materials and Methods. [$^{35}$S]His$_7$-HRI was affinity purified on anti-His antibodies (A) or Ni-NTA agarose (B), and the copurifying chaperones were detected by Western blotting. NS, nonspecific; NI, nonimmune controls; DMSO, dimethyl sulfoxide.

interaction with geldanamycin treatment inhibits the transformation and activation of mature-competent HRI in response to heme deficiency (55). Similar to Hsp90, Hsc70 interaction persisted with mature-competent HRI (Fig. 2) (55). To test the hypothesis that Hsc70 also plays an obligate positive role in maintaining the competence of untransformed mature HRI, we studied the effect of clofibric acid on transformation and activation of mature-competent [$^{35}$S]His$_7$-HRI (Fig. 9). [$^{35}$S]His$_7$-HRI was synthesized in synchronized pulse-chase translations and matured to its competent conformation by incubating in hemin-supplemented RRL for 50 min. Then 15 mM clofibric acid was given for 10 min or was not. Subsequently reactions were mixed with fresh hemin-supplemented or heme-deficient RRL (1:2, vol/vol) containing or lacking 15 mM clofibric acid. These mixtures were incubated for an additional 45 min, followed by affinity purification of [$^{35}$S]His$_7$-HRI on Ni-NTA resins and kinase assays.

Mature-competent [$^{35}$S]His$_7$-HRI remained inactive in hemin-supplemented RRL, as indicated by (i) the lack of the transformation-associated mobility shift of [$^{35}$S]His$_7$-HRI on SDS-PAGE (Fig. 9, upper panel), (ii) the lack of autokinase activity (middle panel), and (iii) the marked reduction in eIF-2α kinase activity (lower panel). Consistent with the earlier observations (Fig. 5A), clofibric acid had no effect on activation of mature-competent [$^{35}$S]His$_7$-HRI in hemin-sup-

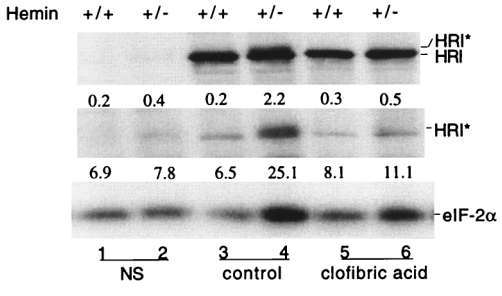


FIG. 9. Effect of clofibric acid on activation of mature-competent HRI in response to heme deficiency. HRI lacking the His$_7$ tag (lanes 1 and 2) and His$_7$-HRI (lanes 3 to 6) were synthesized and matured in hemin-supplemented RRL for 50 min. Clofibric acid (15 mM) (lanes 5 and 6) or water (lanes 1 to 4) was then added, and the incubations were continued for 10 min. Samples were then mixed (1:2, vol/vol) with fresh heme-deficient (lanes 2, 4, and 6) or hemin-supplemented (lanes 1, 3, and 5) RRL that contained (lanes 5 and 6) or did not contain (lanes 1 to 4) 15 mM clofibric acid. After an additional 45-min incubation, [$^{35}$S]His$_7$-HRI was adsorbed to Ni-NTA resin and assayed for autokinase and eIF-2α kinase activities. Samples were separated by SDS-PAGE and transferred to a PVDF membrane. Autoradiograms show [$^{35}$S]His$_7$-HRI (top) [$^{32}$P]HRI (middle), and eIF-2α (bottom) phosphorylation. NS, nonspecific binding to the Ni-NTA resin from RRL expressing [$^{35}$S]HRI lacking the His$_7$ tag.

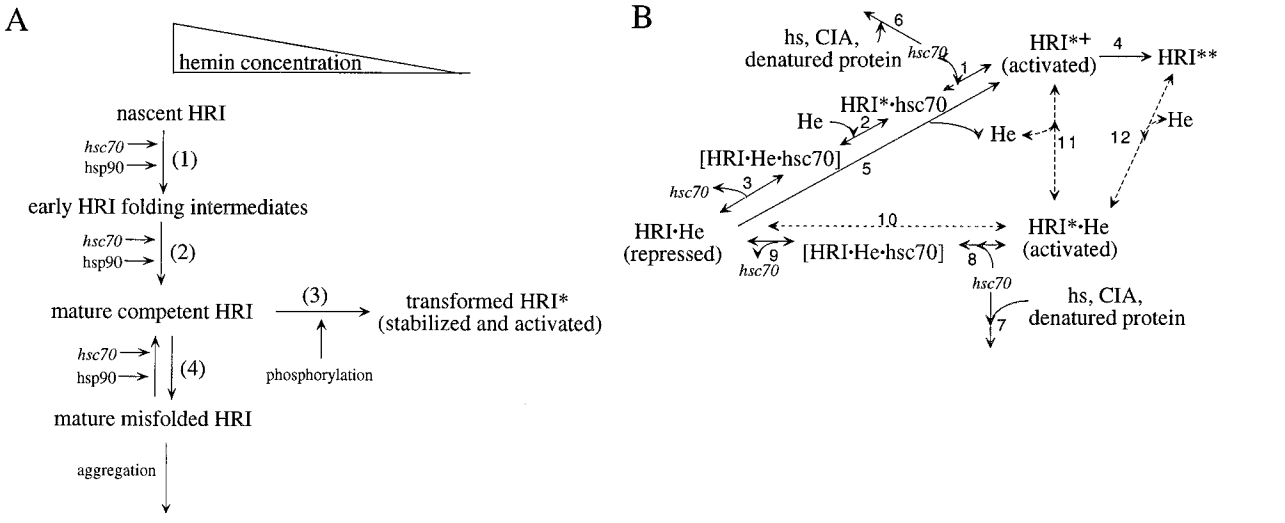


FIG. 10. Model for the role of Hsp90 and Hsc70 in the maturation, transformation, and activity of newly synthesized HRI in RRL. (A) Positive role of Hsc70 and Hsp90 in folding, maturation, and transformation of HRI. Vertical arrows indicate conversion of HRI from one conformation to another during the maturation of HRI to a form that is competent to activate. Horizontal arrows indicate conversion of HRI from one conformation to another during transformation and activation of HRI. Arrows pointing from Hsc70 and Hsp90 indicate that these chaperones physically interact with HRI while facilitating the step in HRI maturation and transformation as evidenced by the functional importance of these interactions. Italicized text is the present data indicating the role of Hsc70 for HRI maturation and transformation. (B) Role of Hsc70 in negatively attenuating the activation of transformed HRI in response to heme deficiency and stress. hs, heat shock. CIA, clofibric acid; HRI*$^+$, transformed HRI free of Hsc70; HRI**, hyperphosphorylated HRI; He, hemin. Dashed lines indicate possible mechanisms that are not addressed by data in the present study.

plemented RRL compared to the control (Fig. 9, lane 3 versus lane 5). In the absence of clofibric acid treatment, mature-competent [$^{35}$S]His$_7$-HRI was activated when incubated in heme-deficient RRL. Activation was evident from the transformation of [$^{35}$S]His$_7$-HRI and increased autophosphorylation and eIF-2$\alpha$ phosphorylation (lane 4). When clofibric acid was included in heme-deficient RRL, the transformation and activation of [$^{35}$S]His$_7$-HRI was inhibited despite its prior maturation to a competent conformation in the absence of the drug (lane 4 versus lane 6). These results indicated that like Hsp90, Hsc70 also plays a positive role in the maintenance of competence of mature HRI until its transformation and activation.

## DISCUSSION

Using [$^{35}$S]His$_7$-HRI synthesized de novo in synchronized pulse-chase translations, we studied the role of Hsc70 in the biogenesis and activation of HRI. The results reported above indicate that Hsc70 plays two distinct roles: (i) a positive role in folding, maintenance, and transformation of HRI (Fig. 1, 2, 7, and 9) and (ii) a negative role in modulating the activity of transformed HRI (Fig. 3 to 5). Based on these results and our previous observations regarding the positive role of Hsp90 in the maturation and transformation of HRI, we extended our previous model for the effects of HRI/chaperone interactions to include the role of Hsc70 (Fig. 10) and discuss the model below.

**(i) Positive role of Hsc70 during HRI biogenesis.** In its first role, Hsc70 functions as a partner of Hsp90 in de novo folding, maturation, maintenance, and transformation of HRI. Consistent with observations that a large fraction of polypeptides associate with Hsc70 during their synthesis (4, 15, 20, 54), Hsc70 interacted cotranslationally with nascent HRI during its synthesis on polyribosomes (Fig. 1) (Fig. 10A, 1). However, in contrast to the transient interaction that is observed for the interaction of Hsc70 with most newly synthesized polypeptides (4, 15, 20, 54), Hsc70 continues to interact with early-folding intermediates after their release from polyribosomes (Fig. 2) (Fig. 10A, 2). Interaction of Hsc70 appears to be required to establish the stable association of Hsp90 with newly synthesized HRI which, in contrast to most polypeptides examined to date, also occurs cotranslationally (Fig. 1) (55). This conclusion is supported by the ability of clofibric acid, an Hsc70 antagonist (1), to disrupt the interaction of Hsc70 with newly synthesized HRI (Fig. 7 and 8). Clofibric acid treatment also prevented the recovery of Hsp90 in HRI-chaperone heterocomplexes (Fig. 8). In contrast, the Hsp90 binding drug geldanamycin resulted in the loss of the interaction of Hsp90 with HRI but not in the loss of Hsc70 (Fig. 8). These observations are consistent with the findings that chaperone-mediated reconstitution of steroid hormone binding activity requires an obligate interaction of Hsc70 with SHRs that precedes the formation of stable complexes between SHRs and Hsp90 (46).

Hsc70 and Hsp90 act in concert to mature early-folding HRI intermediates (Fig. 10A, 2) and maintain mature-competent HRI (Fig. 10A, 4) prior to its transformation (Fig. 10A, 3). When the interaction of newly synthesized HRI with Hsc70 and/or Hsp90 was prematurely interrupted by treatment with clofibric acid (Fig. 7) or geldanamycin (55), respectively, early-folding intermediates were not matured into a conformation such that they were competent to activate in response to heme deficiency (Fig. 7) (55) or NEM treatment (55). Furthermore, treatment with clofibric acid (Fig. 9) or geldanamycin (55) compromised the competence of previously matured HRI to subsequently activate in response to heme deficiency (Fig. 9) (55). Thus, the occurrence of Hsc70 and Hsp90 in HRI-chaperone heterocomplexes is functionally significant, with both chaperones playing an obligate positive role for HRI attaining and maintaining a state of competence to activate.

There are few studies in the literature regarding the involvement of Hsc70 or its cellular homologs in the biogenesis of other protein kinases. Recently, the association of HSP70-2 with the Cdc2 kinase has been demonstrated to facilitate the

formation of a heterodimer between Cdc2 and cyclin B1, leading to changes in Cdc2 phosphorylation and kinase activity (14). HRI is known to be functional as a homodimer (8). However, neither geldanamycin nor clofibric acid appears to inhibit the maturation or transformation of HRI by preventing HRI dimerization (55a).

**(ii) Negative role of Hsc70 in attenuating HRI activation.** Subsequent to transformation and activation induced by heme deficiency, HRI no longer associates with Hsp90 (55), but Hsc70 continues to interact with transformed HRI. It is not clear from the present data whether the Hsc70 that was in a dynamic association with mature-competent HRI continues to interact with HRI after its phosphorylation and transformation (Fig. 10A, 3), or whether new Hsc70 reassociates with transformed HRI after the dissociation of Hsp90. At this stage, Hsc70 plays a negative role in attenuating the activity of transformed HRI. This conclusion is supported by several observations: (i) Hsc70 continues to interact with transformed HRI (Fig. 2), (ii) addition of purified Hsc70 reduces the activation of transformed HRI in response to heme deficiency both in situ and in vitro (22, 53), (iii) blockage of the interaction of Hsc70 with transformed HRI in heme-deficient RRL by heat shock or RCM-BSA treatment leads to the enhanced activation of transformed HRI's autokinase and eIF-2α kinase activities (Fig. 3 and 4); (iv) addition of the Hsc70 antagonist clofibric acid similarly enhances the autokinase and eIF-2α kinase activities of transformed HRI and blocks Hsc70's interaction with transformed HRI (Fig. 5), and (v) addition of purified Hsc70 enhances the sensitivity of transformed HRI to hemin-induced inactivation both in situ and in vitro (22, 53). Since, hemin-induced inactivation of transformed HRI occurs without changes in its electrophoretic mobility [Fig. 10B, HRI · He (repressed)] (55) (Fig. 3 to 5), neither Hsc70 nor hemin appears to act by stimulating the removal of phosphates from the phosphorylation sites responsible for the transformation of HRI.

Hsc70 appears to negatively attenuate the activation of transformed HRI by decreasing its autokinase activity, thus preventing its hyperphosphorylation. We have previously shown that Hsc70 does not cause a reduction in transformed HRI activity in heme-deficient RRL by inhibiting the transformation of mature-competent HRI (53). Rather Hsc70 reduced the autokinase activity of transformed HRI both in vitro and in situ under heme-deficient conditions and inhibited HRI hyperphosphorylation (22, 53). In this report, the enhanced activation of HRI that occurred in heme-deficient RRL in response to heat shock, RCM-BSA, or clofibric acid treatment correlated with the loss of interaction of Hsc70 with transformed HRI (Fig. 3 to 5). The enhancement of transformed HRI's autokinase activity induced by heat shock or addition of RCM-BSA resulted in the hyperphosphorylation of HRI to the extent that an additional band with slower electrophoretic mobility was visible after analysis by SDS-PAGE (Fig. 3 and 4). Thus, we proposed that the ability of Hsc70 to bind HRI and inhibit its autokinase activity negatively attenuates the activation state of transformed HRI, since hyperphosphorylated HRI (Fig. 10B, HRI**) is markedly less responsive to inhibition by hemin (17, 31).

Hsc70 interaction with repressed HRI was observed only if RRL was treated with apyrase prior to coadsorption of HRI with anti-Hsc70 antibodies (Fig. 3C). Apyrase treatment of cell lysates stabilizes the binding of Hsc70 (and its homologs) to polypeptide substrates (4, 38, 41), and we previously observed that apyrase treatment of RRL stabilized the interaction of Hsc70 with endogenous HRI in hemin-supplemented RRL (41). Furthermore, immunoadsorbed HRI can be stripped of associated Hsc70 upon incubation in buffer containing ATP and 0.5 M NaCl (57). These observations suggest that an ATP-dependent dissociation of Hsc70 from HRI occurs upon the repression of the activity of transformed HRI following repletion of RRL with hemin.

Hsc70 also acts to negatively attenuate the activation of HRI in response to stress. We previously showed that addition of Hsc70 to hemin-supplemented RRL inhibited the activation of HRI induced by heat shock and RCM-BSA (53). In this report, we demonstrate that heat shock or the addition of RCM-BSA or clofibric acid does not activate HRI in hemin-supplemented RRL containing only mature-competent HRI (Fig. 3 to 5). Thus, the molecular form of HRI that is activated in hemin-supplemented RRL in response to these treatments appears to be repressed HRI. Furthermore, heat shock decreased the amount of repressed HRI that was coimmunoadsorbed with Hsc70 from hemin-supplemented RRL which was treated with apyrase prior to immunoadsorption of Hsc70 (Fig. 3C). Thus, heat-induced activation of repressed HRI in hemin-supplemented RRL correlated with the disruption of the interaction of Hsc70 with repressed HRI.

**Working model for the negative attenuation of transformed HRI activation by Hsc70.** Three possible mechanisms (see below) through which the dynamic interaction of transformed HRI with heme and Hsc70 could act to regulate transformed HRI activity synergistically are presented in Fig. 10B. The alternate pathways need not be mutually exclusive and reflect our current uncertainty as to the molecular mechanism by which Hsc70 attenuates the activation of HRI. The common feature of these possible pathways is that the interaction of Hsc70 with activated HRI induces a conformational change within HRI that facilitates the repression of HRI's kinase activity. (i) Under conditions of heme deficiency, the binding of Hsc70 to transformed HRI may directly suppress its autokinase activity, preventing the hyperphosphorylation of transformed HRI (Fig. 10B, 1). (ii) The binding of Hsc70 to transformed HRI may increase the binding affinity of HRI for heme, enhancing the ability of the limiting concentration of heme present in heme-deficient RRL to suppress HRI activation (Fig. 10B, 2). (iii) The binding of Hsc70 to transformed HRI which has reassociated with heme may facilitate a conformational change in HRI that is required for heme-induced inhibition of HRI activity (Fig. 10B, 3). In addition, it is not clear from the present study to what degree, if any, Hsc70 can act to suppress the activity of HRI after its has become hyperphosphorylated (Fig. 10B, 4, HRI**).

Furthermore, a certain proportion of the HRI in hemin-supplemented RRL will be in a derepressed (activated) state, however transiently, due to HRI's reversible interaction with heme (Fig. 10B, 5). Stress-induced sequestration of Hsc70 (Fig. 10B, 6 and 7) would result in a deficiency in Hsc70 available to suppress HRI's autokinase activity as described above. In the absence of bound Hsc70 to suppress its autokinase activity, derepressed HRI would autophosphorylate and become hyperphosphorylated, leading to increasing resistance to inhibition by heme. Thus, by utilizing reiterative cycles of interaction with both heme and Hsc70 to regulate HRI, a reticulocyte could attenuate the activation state of HRI to reflect both the degree of heme deficiency and stress that it is experiencing.

Another possible mechanism that our data cannot currently exclude is that repressed HRI, at some frequency, can undergo a conformational change that activates its kinase activity without the dissociation of its bound heme (Fig. 10B, 10). Stress-induced sequestration of Hsc70 would reduce the availability of Hsc70 to reverse such a conformational change, leading to

the accumulation of active HRI (Fig. 10B, 8 and 9). Autophosphorylation or heme dissociation might contribute to the further activation of this pool of HRI (Fig. 10B, 11 and 12). Furthermore, some stress conditions, such as heat shock, might increase the frequency at which such an activating conformational change occurs. Such a conformational change might involve disulfide bond formation or sulfhydryl rearrangements, since a functional thioredoxin/thioredoxin reductase reducing system is required to maintain HRI in a repressed state in hemin-supplemented RRL (reference 40 and references therein), and sulfhydryl reactive compounds are well known to induce HRI activation (10).

The actual mechanism by which Hsc70 attenuates HRI activation in response to stress will undoubtedly be more complex than the schemes presented in Fig. 10B, since the ATPase, polypeptide binding, and ATP/ADP nucleotide exchange activities of Hsp/Hsc70 are regulated through its interactions with cochaperones (i.e., DnaJ homologs [7, 19], p48 [Hip] [29, 45], p60 [Hop] [49], and BAG-1 [50]). Eukaryotic homologs of DnaJ modulate the interaction of Hsp90/Hsc70 with polypeptides through their abilities to both bind polypeptide targets and stimulate the ATPase activity of Hsp70 (7, 13, 19). The DnaJ homologs HDJ-1 (Hsp40) and HDJ-2/YDJ-1 stimulate Hsp/Hsc70-mediated protein renaturation in vitro (18, 47). Western blot analysis has indicated that rabbit homologs of HDJ-1 and HDJ-2 are coimmunoadsorbed with endogenous RRL HRI (23). We are currently investigating whether either of these DnaJ homologs interacts with specific forms of HRI. Thus, heat shock and RCM-BSA are unlikely to act specifically on Hsc70 alone, since the ability of stress-induced denatured protein to bind cochaperones that modulate Hsc70 function is likely to contribute to the mechanism of stress-induced activation of HRI.

Possible involvement of Hsp/Hsc70 in the regulation of other protein kinases has been discussed in recent publications. The Hsp/Hsc70 cochaperone BAG-1, which has inhibitory effects on Hsc70 chaperone activity in vitro (28, 50), has been observed to be present in complexes with activated Raf-1 kinase (56). Elevated levels of Hsp72 has been demonstrated to prevent stress-induced activation of Jun N-terminal kinase (21). The $P58^{IPK}$ cellular inhibitor of the double-stranded RNA-activated eIF-2α protein kinase PKR is a DnaJ homolog that modulates Hsp70 activity (44). It has been postulated that $P58^{IPK}$ is a cochaperone that possibly directs Hsp/Hsc70 to alter the conformation of PKR, thus inhibiting kinase function (44). Thus, specific cochaperones may regulate the ability of Hsp/Hsc70 to modulate kinase function. However, unlike the case for HRI, no direct interaction between Hsp90/Hsc70 and these kinases has been reported.

Thus, besides the well-characterized role for HRI in coordinating globin synthesis in maturing reticulocytes with heme availability, HRI should also be considered to be a stress-regulated eIF-2α kinase. Hsp70 and its homologs have been postulated to act as sensors of the buildup of abnormal proteins after heat shock and other stresses (2, 12). We have proposed that regulation of HRI activation through its dynamic interaction with Hsc70 represents a mechanism through which the rate of protein synthesis can be coordinated with other cellular processes that require Hsc70 (or possibly other Hsp70 family members), such as the ability to fold, assemble, and transport newly synthesized proteins or to renature proteins damaged by stress (37, 38, 53). Any condition that adversely affects processes modulated by Hsc70 would titrate Hsc70 from HRI, activate HRI, and down-regulate protein synthesis. Similarly, activation of PKR in response to cellular stresses that lead to accumulation of misfolded proteins in the endoplasmic reticulum is suppressed upon accumulation of elevated levels of the Hsp70 homolog grp78 in the endoplasmic reticulum (6). The ability to turn off protein synthesis in response to adverse environmental conditions is likely to be of particular importance, since newly synthesized proteins are particularly prone to denaturation and inhibitors of protein synthesis alone have been shown to protect cells from hyperthermic killing (34, 35). The possible importance of HRI as a stress-regulated kinase is further augmented by recent reports that firmly establish the fact that HRI is expressed in nonerythroid cells (5, 43).

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