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Hsp90 Is Obligatory for the Heme-regulated eIF-2α Kinase to Acquire and Maintain an Activable Conformation*

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The heme-regulated eukaryotic initiation factor 2α (eIF-2α) kinase (HRI) interacts with hsp90 *in situ* in rabbit reticulocyte lysate (RRL). In this report, we have examined the role of hsp90 in the maturation of newly synthesized HRI in both hemin-supplemented and heme-deficient RRL. Analysis of translating polyribosomes indicated that hsp90 interacts with nascent HRI cotranslationally. Coimmunoadsorption of HRI with hsp90 by the 8D3 anti-hsp90 antibody indicated that this interaction persisted after release of newly synthesized HRI from ribosomes. Incubation of HRI in heme-deficient lysate resulted in the transformation of a portion of the HRI polypeptides into an active heme-regulatable eIF-2α kinase that exhibited slower electrophoretic mobility. Transformation of HRI was dependent on autophosphorylation, and transformed HRI was resistant to aggregation induced by treatment of RRL with *N*-ethylmaleimide. Transformed HRI did not coimmunoadsorb with hsp90, and regulation of the activity of transformed HRI by hemin was not hsp90-dependent. The hsp90 binding drug geldanamycin disrupted the interaction of hsp90 with HRI and inhibited the maturation of HRI into a form that was competent to undergo autophosphorylation. Additionally geldanamycin inhibited the transformation of HRI into a stable heme-regulatable kinase. These results indicate that hsp90 plays an obligatory role in HRI acquiring and maintaining a conformation that is competent to become transformed into an aggregation-resistant activable kinase.

In rabbit reticulocyte lysate (RRL),[1] initiation of protein synthesis is regulated by heme-regulated inhibitor (HRI), a protein kinase that specifically phosphorylates the 38-kDa α-subunit of eukaryotic initiation factor-2, eIF-2 (reviewed in Refs. 1–5). In the eIF-2·GTP·Met-tRNA$_f$ ternary complex, GTP is hydrolyzed during the last step of the initiation of translation. Subsequent exchange of GDP for GTP to recycle eIF-2 is catalyzed by eIF-2B. Phosphorylation of the α-subunit of eIF-2 by activated HRI results in the binding and sequestration of eIF-2B in a poorly dissociable complex (reviewed in Refs. 6–8). This unavailability of eIF-2B results in the accumulation of eIF-2 in complexes with GDP, thus inhibiting the formation of the ternary complex and arresting initiation of protein synthesis.

In RRL, HRI is activated by a number of conditions as follows: heme deficiency, heat shock, sulfhydryl reagents, oxidants, glucose deficiency, and ethanol (reviewed in Ref. 7). Activation of HRI in response to heme deficiency is thought to proceed through a number of stages. HRI is initially present as an inactive pro-inhibitor. During heme deficiency, HRI is thought to be activated to a form that is completely reversible by hemin. Heme-reversible HRI is subsequently converted into an intermediate form whose activity is partially inhibited by hemin. Finally, after prolonged incubation in the absence of heme, HRI progresses to a heme-irreversible form (reviewed in Refs. 3 and 7). The relationship between the molecular forms of HRI activated in response to heme deficiency and forms of active HRI generated in hemin-supplemented lysate is not well understood.

As HRI is activated, it becomes progressively more phosphorylated (9). While this process in known to involve autophosphorylation, the possibility that HRI activation may involve phosphorylation of HRI by other protein kinases has also been proposed (10). Activation of HRI may also involve sulfhydryl oxidation or sulfhydryl/disulfide bond rearrangements (3, 11, 12). Covalent modification of some critical HRI sulfhydryl groups by *N*-ethylmaleimide causes hyperphosphorylation and irreversible activation of HRI (13).

In addition to these posttranslational modifications, activation of HRI correlates with dissociation from specific chaperones (14–17). In RRL, HRI occurs in heteromeric complexes containing the heat shock proteins hsp90, hsc70, and their associated cohorts FKBP52 and p23[2] (14, 18). The interaction of the hsp90 chaperone machinery with HRI appears to be dynamic rather than static[2] (18). The association of hsp90 with HRI is enhanced in the presence of hemin (18) and requires the presence of Mg^{2+} and ATP.[2] These observations have led to hypotheses proposing that interaction of hsp90 with HRI in the presence of hemin inhibits HRI activation and that the dissociation of hsp90 from HRI causes HRI activation (15). However, our recent results do not support these hypotheses.[2] Rather, the interaction of hsp90 with HRI appears to stabilize HRI and protect it from denaturation. This positive role for hsp90 is more consistent with the known function of hsp90 as a molec-

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[1] The abbreviations used are: RRL, non-nuclease-treated rabbit reticulocyte lysate supplemented with ATP-regenerating system, required salts, buffers, and protein synthesis initiation inhibitors; HRI, heme-regulated inhibitor; hsp90, heat shock protein 90; NEM, *N*-ethylmaleimide; eIF, eukaryotic initiation factor; Ni-NTA resin, Ni^{2+}-nitrilotriacetic acid coupled to agarose; TnT RRL, reticulocyte lysate with coupled transcription and translation; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

[2] Z. Xu, J. K. Pal, V. Thulasiraman, H. P. Hahn, J. J. Chen, and R. L. Matts, submitted for publication.

ular chaperone of certain signal transduction proteins (reviewed in Ref. 19).

In this study, we have used the hsp90-specific inhibitor, geldanamycin, to investigate the role of hsp90 in the regulation of HRI in RRL. Our findings indicate that (i) hsp90 plays an obligatory role as a molecular chaperone in the maturation[3] of newly synthesized HRI into a conformation that is competent to autophosphorylate and transform[3] into a heme-regulatable kinase; and (ii) continuous chaperone support is required to maintain HRI in this competent conformation prior to its transformation.

EXPERIMENTAL PROCEDURES

Construction of Vectors for the Expression of His_7-tagged HRI—The *Nco*I-*Eco*RI fragments from the pSP64 plasmids containing the coding sequence of wild type (20) and the K199R mutant (21) of HRI were cloned into the *Bgl*II site of pSP64T (22). A short synthetic double-stranded DNA sequence with *Nco*I overhangs (5′ C ATG CAT CAC CAT CAC CAT CAC CA 3′ and 3′ GTA GTG GTA GTG GTA GTG GTG TAC 5′) coding for Met-His_7 residues was then inserted into the HRI *Nco*I site. The reading frame was confirmed by DNA sequencing. Different plasmids used in the study are wild type HRI in pSP64T with no His-tag, HRI; wild type HRI with His-tag in pSP64T, His_7-HRI; and K199R mutant with His tag in pSP64T, His_7-K199R HRI.

De Novo Synthesis and Maturation of HRI—Coupled transcription/translation of HRI and His_7-HRI were initiated in nuclease-treated rabbit reticulocyte lysate (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 460 μCi/ml [^{35}S]Met 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 (11) 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 8–12 min of chase with no further incorporation of [^{35}S]Met.

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), 20 mM sodium molybdate, and 10 mM imidazole). RRL mixes containing [^{35}S]His_7-HRI were adjusted to 20 mM sodium molybdate, incubated for 3 min on ice, and centrifuged 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 was bound to the resin (10 μl) for 1 h on ice, followed by 3 washes with 500 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM sodium molybdate, and 50 mM imidazole (wash buffer). 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 (14). Samples were analyzed by 10% SDS-PAGE, followed by transfer to PVDF membrane and autoradiography as described previously (23). Autophosphorylation of HRI was assayed by the incorporation of $^{32}P_i$ 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] emission with two intervening layers of previously developed x-ray film.

Detection of hsp90 Associated with His_7-HRI Adsorbed to Ni-NTA Resin—The same membranes used for kinase assays were used for Western blot analysis. The interaction of hsp90 with His_7-HRI was detected using anti-hsp90 84/86 peptide polyclonal antiserum (provided by Dr. Stephen Ullrich, NCI) and standard protocols (18). Expression of wild type HRI with no His-tag under similar conditions was used as control for nonspecific binding to Ni-NTA resin in all the studies.

Immunoadsorption—Preparation of goat anti-mouse IgM cross-linked to agarose, binding of 8D3 anti-hsp90 antibody or nonimmune control to goat anti-mouse-agarose, and coimmunoadsorption of HRI with hsp90 were carried out as described previously (18). Briefly 5 μl of RRL mixes containing [^{35}S]HRI were adjusted to 20 mM sodium molybdate. After 90 min of binding on ice, immunopellets were washed 3 times with 500 μl of TBS (50 mM Tris-HCl (pH 7.5), and 150 mM NaCl), and the immunopellets were eluted with SDS sample buffer. Proteins present in immunopellets and supernatants were separated on 10% SDS-PAGE and transferred to PVDF membrane. [^{35}S]HRI was detected by autoradiography.

Cotranslational Association of hsp90 with HRI—TnT RRL lysates programmed with either luciferase, HRI, or no template 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), 25 mM KCl, and either 2.5 mM magnesium acetate or 10 mM EDTA. Diluted translations were layered on top of 15–40% sucrose gradients containing buffers and salts as 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 on 10% SDS-PAGE and transferred to PVDF membrane. Hsp90 was detected by Western blotting as described above.

HPLC Analysis of the Molecular Size of HRI—De novo synthesis and maturation of [^{35}S]HRI in heme-deficient and hemin-supplemented RRL by pulse-chase were performed as described above. At the end of 45 min maturation, RRL was treated with 1 mM NEM for 15 min followed by analysis of 40 μl of protein synthesis mix on a Pharmacia HR 10/30 Superdex 200 column pre-equilibrated at 4 °C in HPLC buffer containing 25 mM HEPES·NaOH, 25 mM sodium glycerophosphate, 2 mM EDTA, 0.5% Tween, 10% (w/v) glycerol, and 100 mM KCl (pH 7.4). Fractions (0.2 ml) were collected and analyzed by 10% SDS-PAGE, autoradiography, and densitometry as described (23).

RESULTS

Maturation of [^{35}S]His_7-HRI in Hemin-supplemented and Heme-deficient RRL—Because the activation of HRI in RRL appears to be a multistage process, the endogenous HRI present in RRL may represent a heterogeneous population of molecules. Thus, pulse-chase [^{35}S]Met labeling of synchronized translations of an N-terminal His_7-tagged HRI, His_7-HRI were used to study the maturation and activation of a homogeneous population of HRI molecules in RRL. Following initial synthesis and radiolabeling, [^{35}S]His_7-HRI was subsequently incubated in hemin-supplemented or heme-deficient RRL, adsorbed to Ni-NTA resin, and separated by SDS-PAGE (Fig. 1). HRI that lacked the His_7-tag was similarly analyzed to provide a measure of nonspecific binding of endogenous HRI and [^{35}S]HRI to the resin (Fig. 1, *A–B*, *lanes 1* and *8*).

In hemin-supplemented RRL, [^{35}S]His_7-HRI occurred as an apparently homogeneous population of molecules, represented by the detection of a single ^{35}S-labeled polypeptide (Fig. 1*A*). In contrast, incubation of [^{35}S]His_7-HRI in heme-deficient RRL produced a heterogeneous population of [^{35}S]His_7-HRI molecules, 50% that had a slower electrophoretic mobility than that observed for [^{35}S]His_7-HRI in heme-supplemented RRL (Fig. 1*B*). The proportion of HRI undergoing the electrophoretic shift was proportional to hemin concentration (Fig. 1*C*). No further increase in the amount of the slow mobility form of [^{35}S]His_7-HRI was produced following incubations in the heme-deficient RRL for up to 2 h (not shown). The presence of the His_7-tag had no effect on the electrophoretic mobility of HRI (not shown). The slow moving [^{35}S]His_7-HRI comigrated with active, autophosphorylated HRI purified from rabbit reticulocyte lysate and labeled by incubation with [γ-^{32}P]ATP *in vitro* (Fig. 1*B, lane 9*). Thus, HRI was posttranslationally modified in heme-deficient lysate, resulting in reduced electrophoretic mobility, and this mobility shift may have been related to events occurring during HRI activation.

To determine whether the shift in HRI mobility correlated with acquisition of activity, kinase assays were carried out on [^{35}S]His_7-HRI adsorbed to Ni-NTA resin (Fig. 2). The eIF-2α kinase activity adsorbed to the Ni-NTA resin was specific to the adsorption of the His_7-tagged HRI, as little eIF-2α kinase activity was recovered from control adsorption of reactions containing HRI lacking the His_7-tag (Fig. 2, *lanes 1–4*). In hemin-

[3] In this report, we use the term maturation to refer to the process of folding newly synthesized HRI into a conformation that is competent to autophosphorylate and activate in response to heme deficiency (matured HRI). We use transformation to refer to the process of autophosphorylation and subsequent events that converts HRI into a stable, active, and heme-regulatable eIF-2α kinase with altered electrophoretic mobility on SDS-PAGE (transformed HRI).

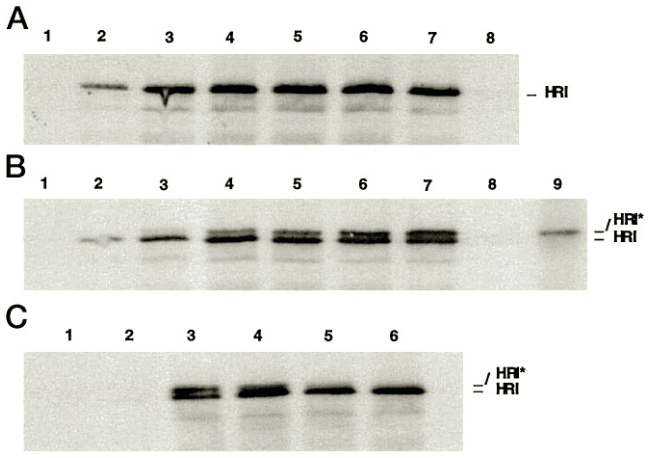

FIG. 1. **Pulse-chase analysis of [^{35}S]His$_7$-HRI maturation in heme-deficient and hemin-supplemented RRL.** His$_7$-HRI and HRI were translated at 30 °C in TnT RRL for 15 min followed by a 4-min pulse of [^{35}S]Met. [^{35}S]His$_7$-HRI was incubated in normal non-nuclease-treated hemin-supplemented (10 μM hemin) (*A*) or heme-deficient (*B*) RRL by mixing 1 volume of TnT translations with 4 volumes of normal RRL. At different time points (min), 25 μl of reaction mixes were adsorbed to Ni-NTA resin, and the adsorbed proteins were analyzed on 10% SDS-PAGE and autoradiography. [^{35}S]His$_7$-HRI matured in hemin-supplemented lysate (*A*), and heme-deficient lysate (*B*) for 0, 10, 20, 30, 45, and 60 min (*lanes 2–7*), respectively. Nonspecific binding of HRI lacking the His$_7$-tag matured for 0 (*lane 1*) and 60 min (*lane 8*) to Ni-NTA resin. *Lane 9* of *B* is purified HRI incubated with [γ-^{32}P]ATP for 4 min at 30 °C. *C*, [^{35}S]His$_7$-HRI translated in TnT RRL as above and chased in normal RRL containing 0 (*lane 3*), 2.5 (*lane 4*), 5 (*lane 5*), and 10 μM hemin (*lane 6*). *Lanes 1* and *2* are nonspecific binding of HRI lacking the His$_7$-tag to the resin after its maturation in the presence of 0 and 10 μM hemin, respectively. *HRI**, slow mobility form of HRI.

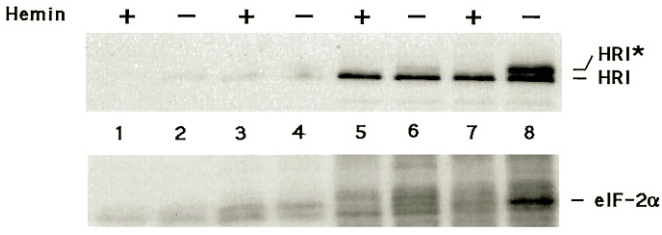

FIG. 2. **eIF-2α kinase activity of [^{35}S]His$_7$-HRI adsorbed to Ni-NTA resin.** His$_7$-HRI and HRI lacking the His$_7$-tag were synthesized and matured in heme-deficient and hemin-supplemented RRL as described in Fig. 1. The ability of [^{35}S]His$_7$-HRI adsorbed to Ni-NTA resin (*upper panel*) to phosphorylate purified eIF-2α (*lower panel*) was determined in incubations at 30 °C for 4 min as described under "Experimental Procedures." *Lanes 1–4* represent nonspecific binding of non-(His$_7$)-tagged HRI to Ni-NTA resin and kinase activity. The eIF-2α kinase assays were carried out on [^{35}S]His$_7$-HRI adsorbed to Ni-NTA resin after an 8 (*lanes 1, 2, 5*, and *6*) or 60 min (*lanes 3, 4, 7*, and *8*) incubation of [^{35}S]His$_7$-HRI in hemin-supplemented (+) and heme-deficient (−) RRL. *HRI**, slow mobility form of HRI.

supplemented and heme-deficient lysates, no eIF-2α kinase activity (Fig. 2, *lane 5* and *6, lower panel*) and little of the slow mobility form of [^{35}S]His$_7$-HRI (Fig. 2, *lane 5* and *6, upper panel*) were present after a short 8-min incubation of pulse-labeled [^{35}S]His$_7$-HRI. After a 60 min incubation of [^{35}S]His$_7$-HRI in heme-deficient RRL, both the mobility shift (Fig. 2, *lane 8, upper panel*) and enhanced eIF-2α kinase activity (Fig. 2, *lane 8, lower panel*) were observed. In hemin-supplemented RRL, [^{35}S]His$_7$-HRI remained inactive even after 60 min of incubation, as the eIF-2α kinase activity detected was equal to the background activity (Fig. 2, *lanes 5* and *7, lower panel*). These results indicate that the mobility shift of HRI in heme-deficient RRL correlated with acquisition of eIF-2α kinase activity.

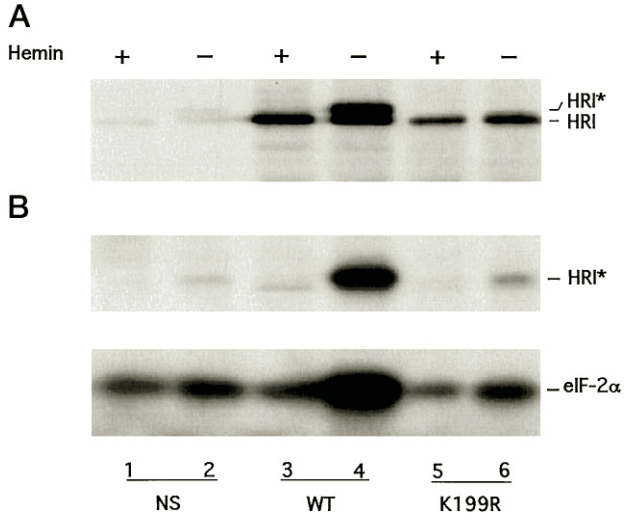

FIG. 3. **Maturation of wild type and K199R mutant of HRI.** His$_7$-HRI (*lanes 3* and *4*), His$_7$-K199R HRI (*lanes 5* and *6*), and HRI lacking the His-tag (*lanes 1* and *2*) were synthesized and matured in heme-deficient (−) and hemin-supplemented (+) RRL for 60 min, as described in Fig. 1. Samples were then adsorbed to Ni-NTA resin, and the adsorbed materials were assayed for the ability to autophosphorylate and phosphorylate purified eIF-2α. ^{32}P-Labeled HRI and eIF-2α were detected by exposing the membrane with intensifying screens and 2 layers of intervening blank film to quench ^{35}S emissions. *A*, detection of [^{35}S]HRI by direct autoradiography. *B*, detection of autophosphorylation (*upper panel*) and eIF-2α phosphorylation (*lower panel*). *WT*, wild type; *NS*, nonspecific.

To test the hypothesis that acquisition of reduced electrophoretic mobility and eIF-2α kinase activity required the autophosphorylation of HRI, we studied the electrophoretic mobility of the inactive K199R mutant of HRI, which does not undergo autophosphorylation (21). [^{35}S]His$_7$-HRI and [^{35}S]His$_7$-K199R HRI were incubated in hemin-supplemented and heme-deficient RRL for 60 min, adsorbed to Ni-NTA resin, and assayed for kinase activity (Fig. 3). In contrast to wild type [^{35}S]His$_7$-HRI (Fig. 3*A, lane 4*), incubation of [^{35}S]His$_7$-K199R HRI in heme-deficient RRL did not produce kinase molecules with reduced electrophoretic mobility (Fig. 3*A, lane 6*). The [^{35}S]His$_7$-K199R HRI was inactive in autophosphorylation (Fig. 3*B, lane 6, upper panel*) and did not increase eIF-2α phosphorylation above background levels representing nonspecifically bound activity (Fig. 3*B, lane 6 versus lane 2, lower panel*). These findings indicated that intramolecular autophosphorylation of HRI was required for its activation.

Association of hsp90 with HRI—Physical interactions of HRI with hsp90 are well documented (14, 15, 18). To determine if hsp90 associates with HRI prior to its release from the ribosomes, polyribosomes were isolated from translation mixtures programmed with either HRI, luciferase, or no template. Consistent with previous results (24), hsp90 was not detected in the polysomal pellets programmed with luciferase (Fig. 4*B, lane 2*). Also, hsp90 was not detected in the no-template control translation (Fig. 4*A, lane 2*). In contrast, hsp90 was specifically detected in the polyribosomal pellets isolated from translations programmed with HRI template (Fig. 4, *A–B, lane 4*). This association of hsp90 with the ribosomal pellet was not observed when peptide chains were released from the ribosomes by treatment with EDTA (Fig. 4, *A–B, lane 3*). However, EDTA did not destabilize the interaction of hsp90 with HRI in RRL (not shown). These results demonstrated that hsp90 associated with nascent HRI cotranslationally.

We next examined whether the association of hsp90 with HRI was maintained after release of newly synthesized HRI from the ribosomes. Coimmunoadsorption of HRI by anti-hsp90

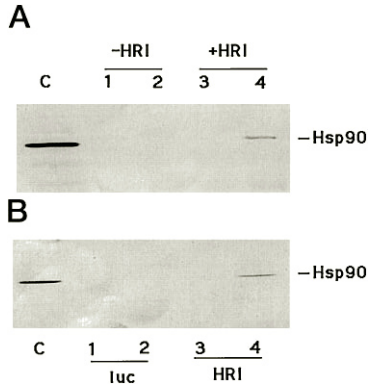

FIG. 4. **Cotranslational association of hsp90 with HRI.** TnT RRLs were programmed with (+*HRI*) or without (−*HRI*) HRI template (*A*), or with luciferase (*luc*) and HRI templates (*B*) for 18.5 min at 30 °C. Translation mixtures were separated on 15–40% glycerol gradients in the presence of either 2.5 mM Mg^{2+} (*lanes 2* and *4*) or 10 mM EDTA (*lanes 1* and *3*). Polysomal pellets were dissolved in SDS sample buffer and analyzed by SDS-PAGE. Hsp90 was detected by Western blot analysis with 84/86 anti-hsp90 antisera. *C*, 1 μl of RRL applied as standard.

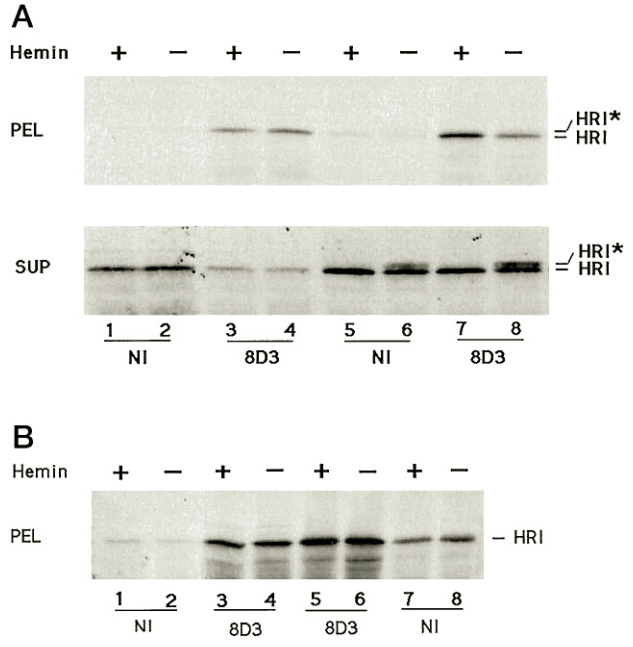

FIG. 5. **Association of hsp90 with wild type and the K199R mutant of HRI.** [^{35}S]HRI (*A*) and [^{35}S]K199R HRI (*B*) were synthesized in TnT RRL and matured in heme-deficient and hemin-supplemented RRL for 8 (*lanes 1–4*) or 60 min (*lanes 5–8*). Aliquots of each sample (5 μl) were immunoadsorbed with the 8D3 anti-hsp90 antibody or non-immune (*NI*) control antibody. Proteins in supernatant (*SUP*) and immunopellets (*PEL*) were analyzed by SDS-PAGE and autoradiography. *HRI**, slow mobility form of HRI.

antibodies following an 8-min chase of translations demonstrated that the association of [^{35}S]HRI with hsp90 was maintained even after HRI's release from ribosomes (Fig. 5*A, lanes 3* and *4*). This interaction persisted even after 60 min of incubation in heme-supplemented RRL (Fig. 5*A, lane 7*). In contrast, incubation in heme-deficient RRL resulted in the recovery of less HRI as an hsp90·HRI complex. While both the fast and slow mobility forms of [^{35}S]HRI were produced in heme-deficient RRL after 60 min incubation (Fig. 5*A, SUP, lane 8*), only the fast mobility form of [^{35}S]HRI was coimmunoadsorbed by anti-hsp90 antibodies (Fig. 5*A, PEL, lane 8*). Thus, HRI did not associate with hsp90 following HRI autophosphorylation and transformation.

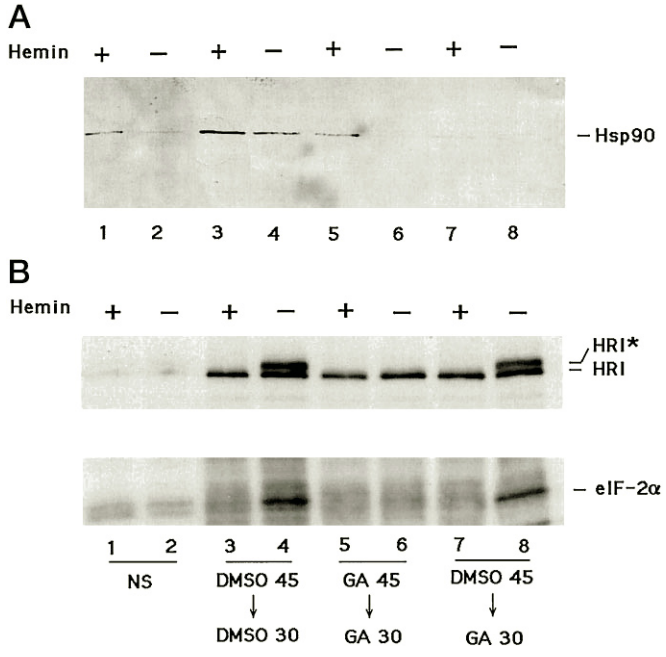

FIG. 6. **Effect of geldanamycin on the interaction of hsp90 with HRI and on HRI activation.** ^{35}S-Labeled His_7-HRI (*lanes 3–8*) and HRI lacking the His-tag (*lanes 1* and *2*) were synthesized in TnT RRL. [^{35}S]His_7-HRI and [^{35}S]HRI were then incubated in heme-deficient (−) or hemin supplemented (+) RRL in the presence of Me_2SO (*DMSO*) (*lanes 3* and *4*) or geldanamycin (10 μg/ml) (*lanes 5* and *6*) for 75 min at 30 °C. In addition, [^{35}S]His_7-HRI was first incubated in hemin-supplemented (+) or heme-deficient (−) RRL for 45 min in the presence of Me_2SO and then subsequently treated with 10 μg/ml geldanamycin for a further 30 min (*lanes 7* and *8*). After 75 min of incubation, samples were adsorbed to Ni-NTA resin. Bound material was assayed for eIF-2α kinase activity as described under "Experimental Procedures," separated by SDS-PAGE, and transferred to PVDF membrane. *A*, Hsp90 was detected by Western blot analysis using anti-hsp90 antisera following standard protocols. *B*, autoradiograms: (*upper panel*) [^{35}S](His7)HRI; (*lower panel*) eIF-2α phosphorylation. *NS*, nonspecific binding to the Ni-NTA resin from RRL expressing [^{35}S]HRI lacking the His_7-tag.

To confirm this conclusion, we examined the interaction of hsp90 with the [^{35}S]K199R HRI mutant (Fig. 5*B*). Similar to wild type [^{35}S]HRI, hsp90 association with newly synthesized [^{35}S]K199R HRI mutant was maintained posttranslationally after an 8-min chase in heme-deficient and hemin-supplemented RRL (Fig. 5*B, lanes 3* and *4*). However, in contrast to active HRI, the [^{35}S]K199R HRI mutant coimmunoadsorbed with hsp90 to the same extent in heme-deficient or hemin-supplemented RRL at the end of the 60-min incubation (Fig. 5*B, lanes 5* and *6*). Thus, transformation of HRI into an hsp90-free form required autophosphorylation.

Effects of Geldanamycin on the Interaction of hsp90 with HRI—To determine whether hsp90 played a direct role in the maturation and transformation of HRI, we utilized the hsp90 inhibitor, geldanamycin (25). At the concentrations used in this study, the drug vehicle Me_2SO had no effect on the following: (i) the association of hsp90 with HRI; (ii) protein synthesis; (iii) interaction of chaperones with substrate proteins (*e.g.* thermally denatured luciferase (26)); or (iv) eIF-2α kinase activity.

Hsp90 coadsorbed with His_7-HRI on Ni-NTA resins, and this coadsorption was enhanced in hemin-supplemented RRL relative to heme-deficient RRL (Fig. 6*A, lane 3* and *4*). In the presence of geldanamycin, the amount of hsp90 recovered during adsorption of hsp90·His_7-HRI complexes was equal to the amount of hsp90 that nonspecifically bound to the resin (Fig. 6*A, lanes 1* and *2 versus lanes 5* and *6*). Additionally, geldanamycin inhibited the coadsorption of hsp90·His_7-HRI complexes

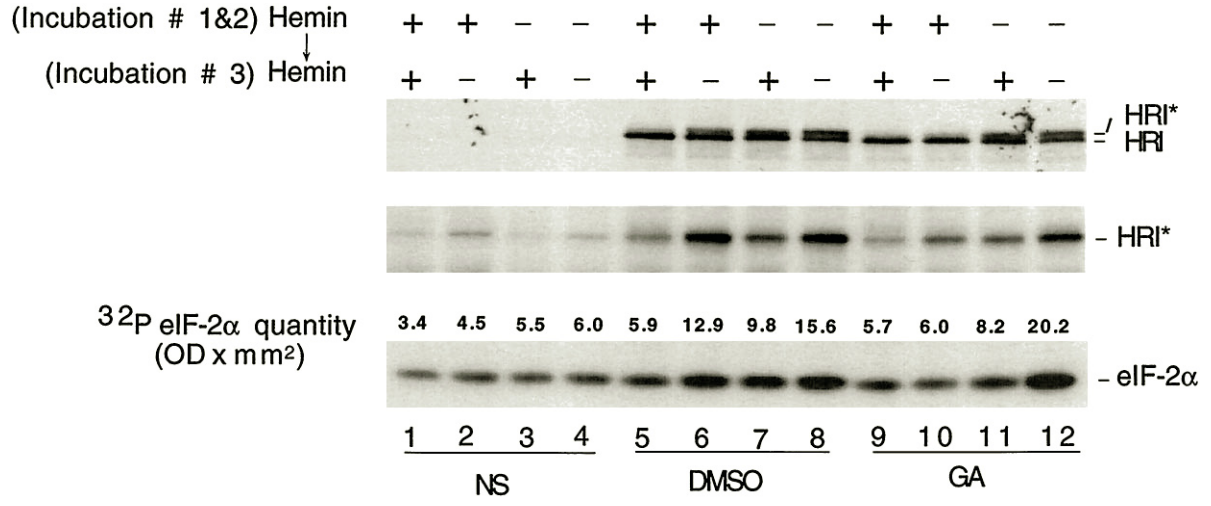

FIG. 7. **Effect of geldanamycin on activation of matured HRI in response to hemin.** HRI lacking the His-tag (*lanes 1–4*) and His_7-HRI (*lanes 5–12*) were translated and matured in heme-deficient (*lanes 3, 4, 7, 8, 11,* and *12*) or hemin-supplemented RRL (*lanes 1, 2, 5, 6, 9,* and *10*) for 50 min (*Incubation 1*). Either Me_2SO (*DMSO*) (*lanes 1–8*) or 10 μg/ml geldanamycin (*lanes 9–12*) were then added, and the incubations were continued for 10 min (*Incubation 2*). Samples were then mixed (1:2, v/v) with fresh heme-deficient (*lanes 2, 4, 6, 8, 10*, and *12*) or hemin-supplemented (*lanes 1, 3, 5, 7, 9*, and *11*) RRL containing Me_2SO (*lanes 1–8*) or 10 μg/ml geldanamycin (*lanes 9–12*). After an additional 60 min incubation (*Incubation 3*), [^{35}S]His_7-HRI was adsorbed to Ni-NTA resin and assayed for autokinase (*middle panel*) and eIF-2α kinase (*lower panel*) activity. Samples were separated by SDS-PAGE and transferred to PVDF membrane. Electrophoretic mobility of [^{35}S]His_7-HRI is presented in *upper panel*. *NS*, material nonspecifically bound to the Ni-NTA resin and kinase activity from RRL expressing [^{35}S]HRI lacking the His_7-tag (*lanes 1–4*).

from either heme-deficient or hemin-supplemented RRL even after an initial 45-min incubation of HRI in the absence of the drug (Fig. 6*A, lanes 7* and *8*). These results indicated that geldanamycin disrupted the association of hsp90 with both newly synthesized and matured His_7-HRI in heme-deficient and hemin-supplemented RRL.

Effects of Geldanamycin on the Maturation of HRI—To determine whether the interaction of hsp90 with HRI is essential for HRI maturation, we examined the effects of geldanamycin on the transformation of newly synthesized HRI into an active kinase. When newly synthesized [^{35}S]His_7-HRI was incubated in heme-deficient lysate, the drug vehicle Me_2SO did not inhibit HRI transformation into the slow mobility form (Fig. 6*B, lane 4, upper panel*) and did not inhibit HRI acquisition of eIF-2α kinase activity (Fig. 6*B, lane 4, lower panel*). In contrast, when newly synthesized [^{35}S]His_7-HRI was incubated in geldanamycin-treated heme-deficient RRL, geldanamycin specifically inhibited HRI transformation into the slow mobility form (Fig. 6*B, lane 6, upper panel*) and inhibited HRI acquisition of eIF-2α kinase activity (Fig. 6*B, lane 6, lower panel*). The pharmacologically inactive benzoquinoid ansamycin, geldampicin (25), had no effect on [^{35}S]His_7-HRI maturation, activation, nor the interaction of HRI with hsp90, indicating that the effects of geldanamycin were specific (not shown). Geldanamycin had no direct inhibitory effect on [^{35}S]His_7-HRI activity when added directly to the kinase assays (not shown). Geldanamycin did not reverse the electrophoretic mobility shift (Fig. 6*B, lane 8, upper panel*) or eIF-2α kinase activity (Fig. 6*B, lane 8, lower panel*) once such was acquired in heme-deficient lysate lacking geldanamycin, indicating that the geldanamycin effects did not reflect the reversal of HRI activation nor the suppression of active HRI by geldanamycin. These results demonstrated that geldanamycin-sensitive hsp90 activity is required for newly synthesized HRI to become an active kinase.

Effect of Geldanamycin on the Activation of Mature HRI—The experiments described above demonstrated an essential positive role for geldanamycin-inhibitable hsp90 function in *de novo* folding and activation of HRI in heme-deficient RRL. However, interactions between hsp90 and HRI persist in hemin-supplemented RRL (Fig. 5 and Ref. 18). This persistence suggested that in hemin-supplemented RRL, hsp90 maintained or provided mature HRI molecules with the competence to activate. To test this hypothesis, [^{35}S]His_7-HRI was subjected to three successive incubations in RRL lacking or containing hemin and in which hsp90 function was or was not inhibited by geldanamycin. [^{35}S]His_7-HRI was initially incubated in hemin-supplemented RRL lacking geldanamycin for 50 min to mature the HRI (incubation 1). Subsequently, hsp90 function was or was not inhibited by the addition of geldanamycin, and the RRL reactions were incubated an additional 10 min (incubation 2). The purpose of incubation 2 was to disrupt hsp90's post-maturational maintenance of HRI during this time. After incubation 2, aliquots of these reactions were mixed with fresh RRL (1:2, v/v) containing or lacking hemin, and these reactions were incubated an additional 60 min to assay HRI's competence to transform and activate (incubation 3). These activating/transforming RRLs (lacking or containing hemin) used for incubation 3 contained the same concentration of geldanamycin or Me_2SO as were used for incubation 2. Subsequent to these successive incubations, we examined the electrophoretic mobility (Fig. 7, *top panel*), autophosphorylation (Fig. 7, *middle panel*), and eIF-2α kinase activity (Fig. 7, *bottom panel*) of [^{35}S]His_7-HRI recovered in Ni-NTA resin adsorption pellets. Background kinase activity that was nonspecifically bound to the Ni-NTA resin was determined in assays of activity recovered from untreated RRLs that were programmed with HRI lacking the His_7-tag (Fig. 7, *NS*, *lanes 1–4*). Additionally, these experiments were designed to confirm that hsp90 was neither necessary to maintain the activity of transformed HRI nor to inhibit HRI activity in the presence of hemin.

In the absence of geldanamycin, [^{35}S]His_7-HRI became activated in response to heme deficiency (Fig. 7, *lane 5 versus lane 6*). However, in the presence of geldanamycin, [^{35}S]His_7-HRI was not activated in response to heme deficiency (Fig. 7, *lane 9* and *10 versus lane 5* and *6*), despite initial *de novo* maturation of [^{35}S]His_7-HRI by hsp90 in the absence of geldanamycin. Geldanamycin did not inhibit previously activated [^{35}S]His_7-HRI (Fig. 7, *lane 8 versus lane 12*) nor did geldanamycin activate the matured [^{35}S]His_7-HRI (Fig. 7, *lane 5 versus lane 9*) in

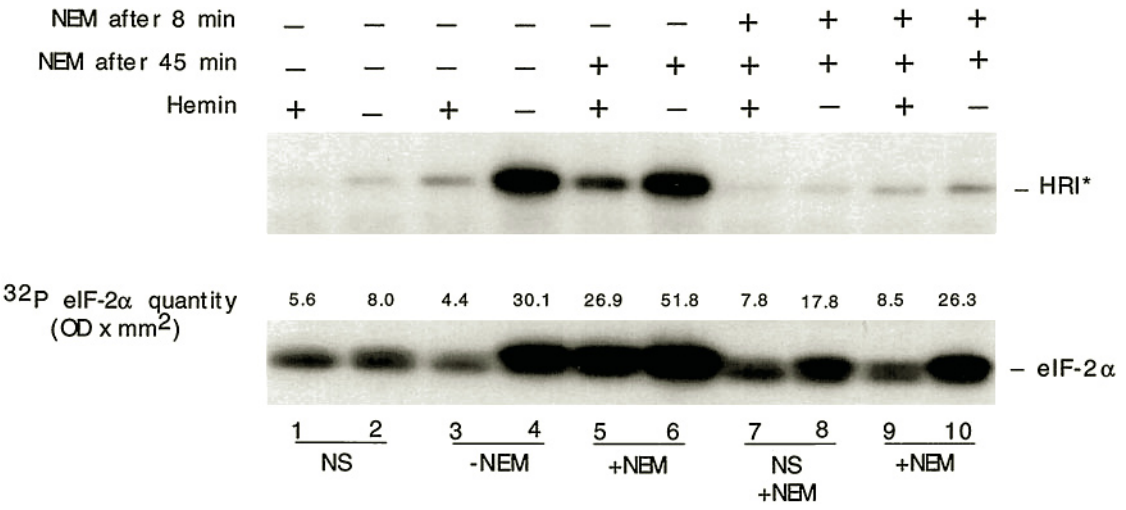

FIG. 8. **Effect of NEM on maturation and activation of HRI.** His_7-HRI and HRI without His-tag were translated and matured in hemin-supplemented (+) and heme-deficient (−) RRL. The RRLs received no further treatment (*lanes 1–4*) or were treated with NEM (1 mM) either after 8 min (*lanes 7–10*) or 45 min (*lanes 5* and *6*) of maturation. At the end of 60 min incubation equal amounts of [^{35}S]His_7-HRI were adsorbed to Ni-NTA resin, and kinase assays were performed. Autophosphorylation of HRI (*upper panel*) and eIF-2α phosphorylation (*lower panel*) were detected by autoradiography as described under "Experimental Procedures." *NS*, nonspecific activity with HRI lacking the His-tag under similar conditions.

hemin-supplemented RRL. Thus, geldanamycin-inhibitable hsp90 function was required for mature HRI to be competent to activate, even after a prior 50 min of maturation in hemin-supplemented RRL. Furthermore, geldanamycin did not inibit heme-mediated inactivation of previously activated [^{35}S]His_7-HRI (Fig. 7, *lanes 7* and 8 *versus lanes 11* and *12*).

The Effect of N-Ethylmaleimide (NEM)—The results from the above experiment indicated that geldanamycin-inhibitable hsp90 function was required for HRI to be competent to activate. To characterize mature but inactive molecules of HRI, NEM was used to unmask the kinase potential of HRI molecules independently of the presence of hemin (1, 3). NEM activation of HRI was examined in assays of HRI autophosphorylation (Fig. 8, *upper panel*) and eIF-2α phosphorylation (Fig. 8, *lower panel*) using equal amounts of [^{35}S]His_7-HRI adsorbed to Ni-NTA resin. [^{35}S]His_7-HRI was not activated when hemin-supplemented lysate was treated with NEM 8 min after the arrest of HRI synthesis (Fig. 8, *lane 9*). Similarly, in hemin-deficient RRL, NEM treatment of immature newly synthesized HRI (treatment 8 min after arrest of HRI synthesis) induced little autophosphorylation (Fig. 8, *lane 10 versus lane 8, upper panel*) and eIF-2α kinase activity (Fig. 8, *lane 10 versus lane 8, lower panel*) over activity recovered in a nonspecific fashion (Fig. 8, *lane 10 versus lane 8*). In addition, the slow mobility form of [^{35}S]His_7-HRI characteristic of HRI folding into an active kinase was not observed (not shown). Thus, HRI needed to be folded to be activated by NEM.

Following a 45-min maturation in hemin-supplemented lysate, a portion of the mature [^{35}S]His_7-HRI molecules were activated by NEM in the presence of hemin, as indicated by increased levels of autophosphorylation (Fig. 8, *lane 5, upper panel*) and eIF-2α phosphorylation (Fig. 8, *lane 5, lower panel*) over those observed in non-NEM-treated reactions (Fig. 8, *lane 3*) and over levels nonspecifically bound to the Ni-NTA resin (Fig. 8, *lanes 1* and *7*). These results indicated that at the end of 45 min incubation in hemin-supplemented RRL, [^{35}S]His_7-HRI was matured to a form that was competent to be activated. However, only a small portion of [^{35}S]His_7-HRI was activated upon NEM treatment, since NEM inactivates the chaperone machinery of RRL[4] in addition to activating HRI by derivatization of sensitive sulfhydryl groups (1, 3). As further controls for NEM activation, HRI was also matured in heme-deficient RRL and treated with NEM. NEM hyperactivated HRI previously activated in heme-deficient lysate (Fig. 8, *lane 6 versus lane 4*).

The Effect of Inactivation of the Chaperone Machinery of RRL on the Stability of HRI—NEM inactivates the ability of the RRL chaperone machinery to renature thermally denatured firefly luciferase.[4] To determine if prolonged interaction of hsp90 with the fast mobility form of HRI (Fig. 5) plays a role in stabilizing the structure of HRI, we examined the effect of NEM treatment on the stability of [^{35}S]HRI synthesized in pulse-chase translations in RRL. HPLC gel exclusion chromatography of [^{35}S]HRI indicated that HRI was present as multiple molecular weight species (Fig. 9). The slow mobility form of HRI generated in heme-deficient RRL was present as a more discrete peak of material (Fig. 9*C*). When NEM treatment was given after a 45-min incubation in heme-deficient and hemin-supplemented RRL, the fast mobility form of HRI from both heme-deficient and hemin-supplemented RRL aggregated and was observed to elute in the void volume ($M_r > 1.3 \times 10^6$) (Fig. 9, *A–B*). Only the slow mobility form that was present in NEM-treated heme-deficient RRL eluted normally (Fig. 9*C*) and was active in phosphorylating eIF-2α (not shown). Thus, in RRL, only HRI that had been autophosphorylated and transformed (fast mobility form) was resistant to denaturation and aggregation following inactivation of chaperone machinery by NEM. This was the population of HRI that did not physically nor functionally associate with hsp90.

DISCUSSION

Hsp90's support of HRI function was studied using a homogeneous population of [^{35}S]His_7-HRI synthesized in synchronized pulse-chased translations. This population was distinguished from endogenous HRI via the recombinant addition of an His_7-tag. Using this approach, and the hsp90 inhibitor geldanamycin, we have delineated two essential roles for HRI as follows: (i) hsp90 is required to fold nascent HRI into an active/activable kinase; and (ii) hsp90 is required for maintenance and stabilization of kinase structure prior to HRI activation. These essential and positive roles of hsp90 are clearly incompatible with other models suggesting that hsp90 functions to suppress HRI activity (15).

[4] V. Thulasiraman and R. L. Matts, unpublished observations.

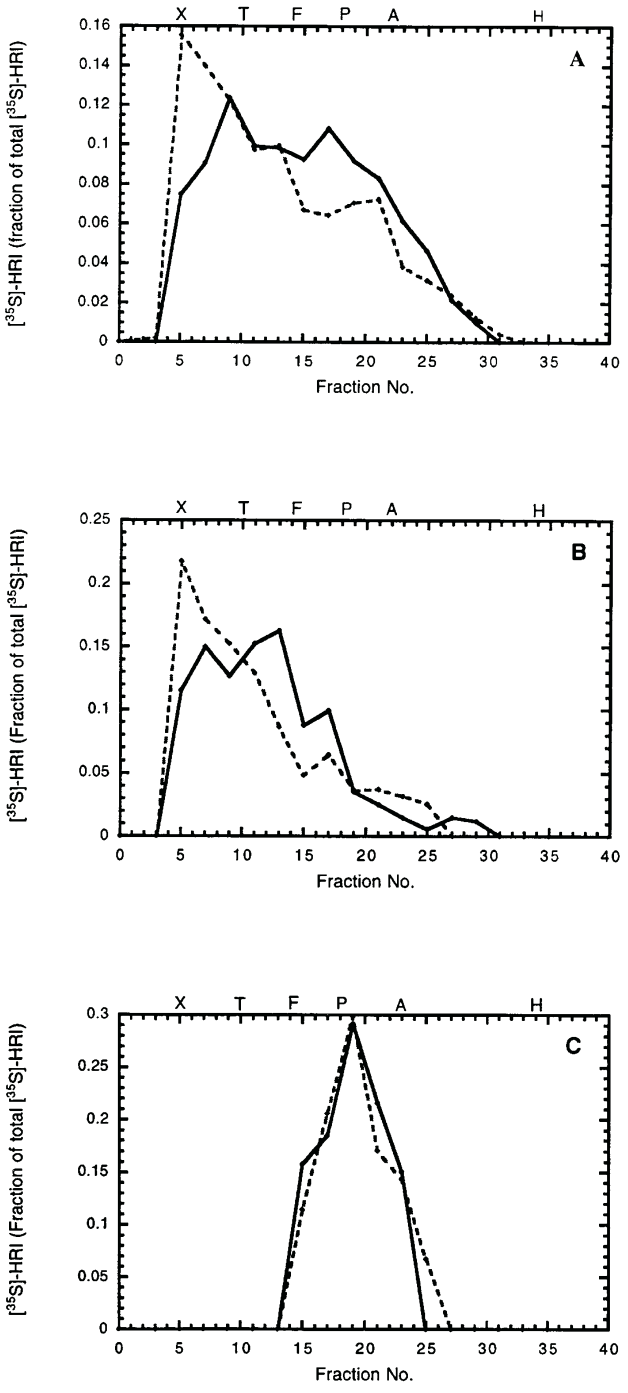

FIG. 9. **Effect of NEM on the aggregation of [^{35}S]HRI matured in heme-deficient and hemin-supplemented RRL.** [^{35}S]HRI was translated and matured in heme-deficient or hemin-supplemented RRL for 45 min as described in Fig. 1. RRLs were then incubated in the presence (- - -) or absence (—) of 1 mM NEM for 15 min. Subsequently protein synthesis mixes were fractionated by HPLC size exclusion chromatography, and alternate fractions were analyzed by SDS-PAGE on 10% gels and autoradiography. Relative [^{35}S]HRI in each fraction was quantified by scanning densitometry. *A*, [^{35}S]HRI matured in hemin-supplemented RRL. *B*, fast mobility form of [^{35}S]HRI matured in heme-deficient RRL. *C*, slow mobility form of [^{35}S]HRI matured in heme-deficient RRL. Elution of molecular weight standards is noted at the *top* of the graph. *H*, hemoglobin, 64 kDa; *A*, aldolase, 150 kDa; *P*, pyruvate kinase, 240 kDa; *F*, ferritin, 440 kDa; *T*, thyroglobulin, 669 kDa; *X*, excluded volume of the column (M_r >1.3 × 10^6).

Based on our results, we present a model for the positive role of hsp90 in maturation of HRI and its transformation into a stable, active heme-regulated kinase (Fig. 10). According to the model, hsp90 binds to nascent HRI during synthesis of HRI on polyribosomes (Fig. 4). This cotranslational interaction of hsp90 with HRI implies that hsp90 facilitates the *de novo* folding of HRI. However, the role of hsp90 in folding of nascent polypeptides appears to be substrate-specific, since hsp90 does not appear to be associated with nascent luciferase (Fig. 4, and Refs. 23 and 24), and the hsp90 inhibitor geldanamycin has no effect on the folding of newly synthesized luciferase.[5]

After the release of HRI from ribosomes (Fig. 10, *early HRI folding intermediates*), hsp90 continues to interact with HRI (Fig. 5). However, these early HRI folding intermediates are not active (Fig. 2) and cannot be activated by NEM treatment of either hemin-supplemented or heme-deficient RRL (Fig. 8). Thus, at this stage of maturation, HRI is not folded into a conformation that is competent to undergo autophosphorylation and activation. Hsp90 function in subsequent maturation is inferred from the physical association at this point and is consistent with the inhibition by geldanamycin of subsequent maturation of these molecules.

Upon further incubation, early HRI folding intermediates mature into a competent form (Fig. 10, *mature competent HRI*), as indicated by the acquisition by HRI molecules of the ability to be unmasked by NEM (Fig. 8, *lane 5 versus lane 3*). However, physical and functional interactions between HRI and hsp90 persist at this time since HRI previously matured in geldanamycin-free hemin-supplemented RRL fails to activate upon subsequent incubations in RRL containing geldanamycin (Fig. 7). This observation suggests that matured competent HRI becomes misfolded in the absence of hsp90 support (Fig. 10, *matured misfolded HRI*). In the model, mature misfolded HRI is presented as being off the *de novo* folding pathway. However, it is equally possible that "mature misfolded HRI" might be part of normal HRI folding intermediates. Regardless, in addition to its role in *de novo* folding, the sustained interaction of hsp90 with HRI in the presence of hemin (Fig. 5) plays an important role in maintaining HRI in a competent conformation prior to its activation, autophosphorylation, and transformation.

In the absence of functional chaperone machinery, mature misfolded HRI aggregates. NEM inactivates the capacity of RRL chaperone machinery to renature and maintain the activity of firefly luciferase.[4] NEM treatment causes the aggregation of mature-but-inactive [^{35}S]HRI (Fig. 9). Thus, besides the general requirement for hsp90 to fold HRI into a competent conformation, hsp90 also functions to protect competent HRI from denaturation while HRI awaits stimuli that induce its activation.

Competent HRI is activated in response to heme deficiency. Initial events leading to the activation of HRI likely occur while HRI is still bound to hsp90 or may involve reiterative cycles of binding and release of HRI by the hsp90 chaperone machinery. We have recently observed that active hsp90-bound HRI can be detected.[2] We thus postulate an active but unstable transition state during HRI transformation and a potential role for hsp90 during this step of the activation process (Fig. 10, *unstable active HRI*).

Phosphorylation events play an obligatory role in HRI activation in response to heme deficiency. The proportion of the HRI molecules that are transformed into the stable slow mobility form of HRI is dependent on hemin concentration (Fig. 1*C*). In the absence of the ability to catalyze autophosphorylation, the reduced electrophoretic mobility characteristic of activation (Fig. 2) is not observed with the inactive K199R HRI

[5] V. Thulasiraman, S. D. Hartson, W. Huang, P. Burn, and R. L. Matts, submitted for publication.

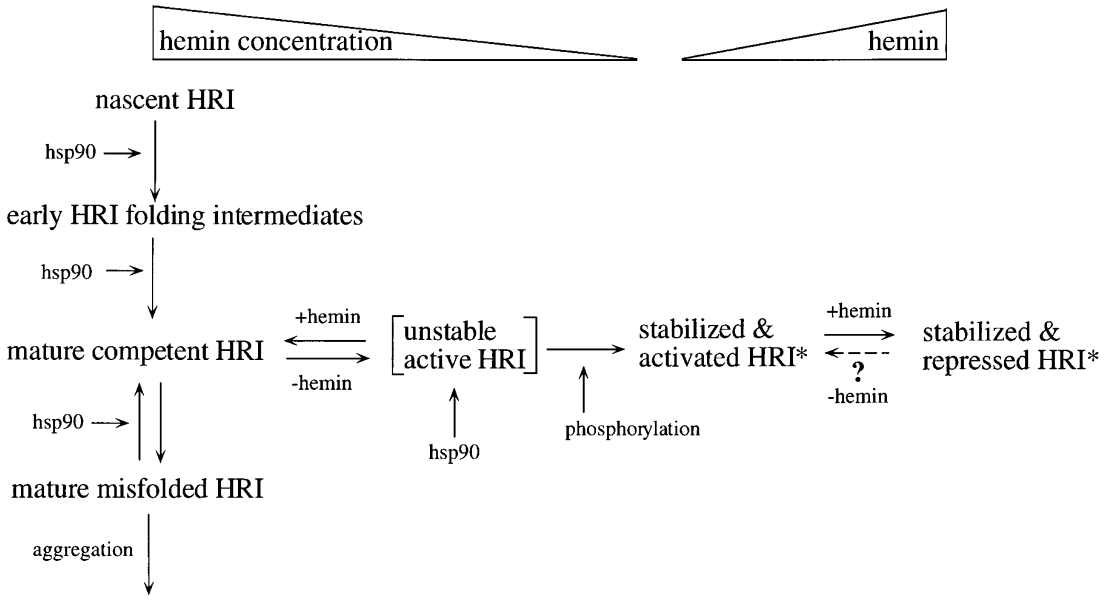

FIG. 10. **Model for the positive role of hsp90 in the maturation and transformation of newly synthesized HRI in RRL.** *Vertical arrows* indicate conversion of HRI from one conformation to another during the maturation of HRI to a form that is competent to activate. *Horizontal arrows* indicate conversion of HRI from one conformation to another during transformation of HRI. *Arrows* pointing from hsp90 indicate that hsp90 may have a direct role in facilitating the event during HRI maturation and transformation. *+hemin,* events occurring in hemin-supplemented RRL. *−hemin*, events occurring in heme-deficient RRL. *HRI**, slow mobility form of HRI. *?* indicates that reactivation of repressed HRI in response to heme deficiency has not been tested.

mutant (Fig. 3). This result indicates that autophosphorylation is a prerequisite for activation in response to heme deficiency since active endogenous HRI is present. However, the number and sequence of subsequent phosphorylation events that reduce the electrophoretic mobility of HRI and lead to its complete activation and stabilization is not clear from the present data. Phosphorylation of HRI by other kinases in RRL has also been proposed (*e.g.* casein kinase II (10)).

Phosphorylation of HRI stabilizes the kinase structure such that it no longer needs hsp90 to support its function. This conclusion is supported by the several observations: (i) the slow mobility form of HRI does not associate with hsp90 (Fig. 5); (ii) the inactive K199R mutant, which can not autophosphorylate, remains bound to hsp90 in heme-deficient RRL (Fig. 6); (iii) geldanamycin has no inhibitory effect on previously matured and activated HRI activity in heme-deficient lysate (Figs. 6 and 7); (iv) the transformed slow mobility form of HRI is stable and resistant to aggregation in NEM-treated RRL (Fig. 10*C*).

Although hsp90 functions to protect mature HRI from denaturation and to maintain its competence to activate in response to heme deficiency, subsequent hemin regulation of transformed HRI is hsp90-independent. The activity of transformed HRI, which is not associated with hsp90, is still responsive to hemin (Fig. 7). Additionally, while geldanamycin blocks the interaction of hsp90 with HRI, it does not inhibit the ability of hemin to repress the activity of transformed HRI (Fig. 7). Furthermore, hemin-induced repression of the activity of transformed HRI is not accompanied by reassociation of hsp90 with HRI (not shown). In addition, the ratio of the slow to fast mobility forms of [^{35}S]HRI matured and transformed in heme-deficient RRL did not change upon subsequent return of the [^{35}S]HRI to hemin-supplemented RRL (Fig. 7). Thus, although the activation of HRI kinase activity by hemin is reversible, the transformation of HRI, as judged by its electrophoretic mobility shift on SDS-PAGE, is not. We have not yet investigated whether transformed HRI that has been repressed by hemin can reactivate in response to heme deficiency.

The model presented for HRI maturation and activation may represent a heuristic model for other hsp90-dependent kinases as well. Hsp90 plays an essential positive role in the function of the viral protein tyrosine kinase p60^{src} (27) and a cellular member of this family, p56^{lck} (28). The nature of this role is to support kinase folding (28). This role appears to be manifested, in part, during the biogenesis of these kinases (28–30). However, hsp90 occurs in complexes with numerous other kinases, and these complexes do not appear to be specific to nascent molecules (28, 31–36). Thus, in addition to its role in the kinase biogenesis, hsp90 may support the maintenance folding of kinases subsequent to their *de novo* folding. Consistent with these observations, hsp90 mediates the potential of both newly synthesized and matured molecules of HRI.

However, the dependence of kinase molecules on hsp90 machinery may be conditional. The interaction of Cdk4 with hsp90 and the 50-kDa hsp90 cohort cdc37 appears to be specific for that population lacking the essential cyclin subunit (33). Similarly, the interaction of hsp90 with raf appears to occur at specific time points in the cell cycle (37). Additionally, although p56^{lck} requires hsp90 to maintain kinase function in RRL, hsp90-bound p56^{lck} appears to represent a small portion of the total p56^{lck} population occurring in T cells (28). Thus, we have previously postulated that in addition to facilitating *de novo* folding, hsp90 may provide reiterative support of kinase structure prior to specific stabilizing events (28). This model is consistent with a model postulating reiterative hsp90-mediated support of the progesterone receptor to maintain hormone binding competence until such binding stabilizes the structure of the receptor (38). For HRI, transformation to an hsp90-independent state involves autophosphorylation induced by heme deficiency. Thus, hsp90's previously documented roles in kinase biogenesis and in maintenance of protein structure can be integrated in light of the model presented here for the pathway of HRI maturation and activation. This model for HRI maturation, transformation, and activation postulates overlapping pathways of kinase folding and regulation.

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