Importance of the C-Terminal Domain of Harc for Binding to Hsp70 and Hop as Well as Its Response to Heat Shock†

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ABSTRACT: Hsp90 is a molecular chaperone that acts in concert with Hsp70 to mediate the folding of many important regulatory proteins (e.g., protein kinases) into functional conformations. The chaperone activity of Hsp90 is primarily regulated by its cochaperones. For example, the Hsp90 cochaperone Cdc37 recruits Hsp90 to protein kinases as well as inhibiting its ATPase activity to promote the binding of Hsp90 to protein kinases. Harc is a structurally related Hsp90 cochaperone with a three-domain structure in which the middle domain binds Hsp90. In contrast to Cdc37 though, Harc also binds to Hsp70 and Hop (Hsp70/Hsp90 organizing protein). Here we demonstrate that deletion of the C-terminal domain of Harc abolished the binding of Hsp70 and Hop and reduced the affinity of Hsp90 binding to Harc. Significantly, the C-terminal domain of Harc bound Hsp70, but it did not bind Hop or Hsp90. Size exclusion chromatography of cell lysates revealed that Hop only formed a complex with Harc in the presence of Hsp90 and Hsp70, consistent with a model in which the interaction of Hop with Harc is mediated via the binding of Hop to Harc-bound Hsp90 and Hsp70. Notably, heat shock resulted in a marked decrease in the solubility of Harc, a response that was further augmented by the deletion of the C-terminal domain of Harc. This latter finding is especially interesting given that bioinformatics analysis indicated that cells may express splice variants of Harc that encode C-terminally truncated Harc isoforms. Together, these findings indicate that the C-terminal domain of Harc is a key determinant of its cochaperone functions.

The heat shock response is an evolutionarily conserved mechanism that exists to protect cells from a diverse range of environmental stress factors, including elevated temperature, oxidative agents (e.g., reactive oxygen metabolites), heavy metals, and toxins (*1*−*4*). Several pathological conditions (e.g., ischemia, infection, and inflammation) are also known to induce a heat shock response in mammalian cells (*5*). The heat shock response is characterized by the increased expression of a group of proteins collectively termed heat shock proteins (Hsp's).[1] Some heat shock proteins (e.g., Hsp90, Hsp70, and Hsp60) function as molecular chaperones and protect cells from stress by limiting the extent of protein damage. More specifically, they limit incorrect protein folding and aggregation as well as facilitate the refolding of proteins. Heat shock proteins are also involved in the ubiquitination and proteasomal degradation of unfolded or incorrectly folded proteins (*6*−*9*). Significantly though, many heat shock proteins are constitutively expressed under normal growth conditions and play important roles in maintaining normal cellular homeostasis (*2*, *10*).

Hsp90 and Hsp70 are abundant and highly conserved molecular chaperones that act in concert with other chaperones (e.g., Hsp40) and cochaperones (e.g., Cdc37) to mediate the folding of client proteins into functional conformations. Detailed mechanistic studies of the folding of steroid receptors into functional conformations have yielded a generalized paradigm for protein folding by Hsp90 and Hsp70 (*11*). In this model, a so-called "early" protein folding complex is formed by the binding of Hsp70 and Hsp40 to the client protein (*12*, *13*). The Hsp70/Hsp90 organizing protein (Hop) then stabilizes the interaction of Hsp90 with Hsp70 and the client protein, resulting in the formation of an "intermediate complex" (*12*−*14*). Further maturation of the complex is accompanied by the loss of Hsp70 and Hop from the complex and the interaction of other Hsp90 cochaperones (e.g., p23) and immunophilins, such as the FK506-binding protein FKBP52, with Hsp90 and its client protein (*15*−*18*).

The majority of the proteins that are dependent on Hsp90 for their folding into functional conformations are either protein kinases or steroid hormone receptors (*11*). Elucidation of the mechanisms underlying the selectivity of Hsp90 for particular client proteins has generated considerable interest. It is now emerging that cochaperones play central roles in governing the binding of Hsp90 to client proteins. This is likely to be achieved by the cochaperone recruiting Hsp90 to the client protein and/or regulating the ATPase activity of Hsp90, thereby governing its affinity for the protein. For example, the recognition of some protein kinases (e.g., Src-

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[1] Abbreviations: GFP, green fluorescent protein; Hop, Hsp70/Hsp90 organizing protein; Hsp, heat shock protein; FKBP, FK506-binding protein.

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family kinases) by Hsp90 is dictated by Cdc37 (*19*, *20*). Cdc37 has a three-domain structure in which the N-terminal domain binds client protein kinases, while the middle domain binds Hsp90 (*21*−*25*). These features of Cdc37 enable it to recruit Hsp90 to client protein kinases. Further studies have established that the binding of Cdc37 to the N-terminal domain of Hsp90 inhibits the ATPase activity of Hsp90 and consequently may lock Hsp90 in a conformation that favors the binding of protein kinases (*26*, *27*).

Harc is an Hsp90 cochaperone that is structurally related to Cdc37 but whose precise function is currently unknown (*22*). However, the structural similarity between Harc and Cdc37 suggests that Harc may function to regulate the binding of a specific class of proteins by Hsp90. Whereas Cdc37 bound Src-family kinases and Raf-1 in transfected cells, Harc did not bind these protein kinases but did form a complex with Hsp90, Hsp70, and Hop (*22*). We recently reported that Harc formed homodimers when expressed in cells; furthermore, heterodimerization of Harc with Cdc37 was observed when both proteins were coexpressed (*24*). Here, we have further dissected the regulation of Harc; in particular, we have focused on the role of the C-terminal domain of Harc in regulating the binding of Hsp90, Hsp70, and Hop to Harc, as well as in regulating the response of Harc to heat shock.

EXPERIMENTAL PROCEDURES

Reagents. Cell culture medium and supplements, fetal calf serum (FCS), and precast SDS−PAGE gels were from Invitrogen. The HRP-conjugated and agarose-conjugated anti-FLAG monoclonal (M2) antibodies were obtained from Sigma. The rabbit polyclonal anti-Hsp90 antibody (PA3-013) was from Affinity BioReagents, Inc., while the mouse monoclonal anti-Hsp90 and anti-Hop antibodies were purchased from BD Biosciences. The mouse monoclonal anti-Hsp70 antibody (BB70) was a generous gift from Dr. David Toft (Mayo Clinic, Rochester). Complete protease inhibitors and FuGENE 6 transfection reagent were from Roche, and size exclusion chromatography standards were obtained from Bio-Rad.

Expression Vectors. Mammalian expression vectors encoding N-terminal FLAG-tagged versions of human Harc, HarcD12, Cdc37, Cdarc, and Hac37 were as previously described (*22*−*24*). The vector pEF-HA−Harc, which expresses an N-terminal HA-tagged version of Harc, was created by excising the cDNA insert from pEF-FLAG−Harc with *Mlu*I and subcloning the fragment into the corresponding site in the vector pEF-HA. An expression vector encoding FLAG−HarcD3 (Lys-267 to Val-337) was generated by PCR using the following primer pair: 5′-ACG CGT AAG TCA AGA GTA AGA CTT TAT TCT CAA-3′ (forward) and 5′-ACG CGT TTA TAC AGT GTC CAT CAT TTT GGG TTC-3′ (reverse). The PCR product generated was digested with *Mlu*I and subcloned into pEF-FLAG. The vector pEGFP-FLAG−Harc, which expresses FLAG-tagged Harc fused to the N-terminus of enhanced green fluorescent protein (i.e., FLAG−Harc−GFP), was generated by PCR using the following primer pair: 5′-GAA TTC CCA CCA TGG CGC GCC AGG ACT ACA AGG ACG AC-3′ (forward) and 5′-GGG CCC TAC AGT GTC CAT CAT TTT GGG TTC ATC ATC TTC-3′ (reverse). The PCR product generated was digested with *Eco*RI and *Apa*I followed by subcloning into pEGFP-N2 (Clontech). A vector encoding FLAG-tagged Harc domain 3 fused to the N-terminus of GFP (i.e., FLAG−HarcD3−GFP) was made similarly by PCR with the primer pair 5′-GAA TTC CCA CCA TGG CGC GCC AGG ACT ACA AGG AC-3′ (forward) and 5′-GGG CCC TAC AGT GTC CAT CAT TTT GGG TTC ATC ATC-3′ (reverse). The vector pGEX-Harc, which encodes a GST−Harc fusion protein, was created by PCR using the primer pair 5′-GAA TTC GAA CAA CCG TGG CCG CCT CCG GGA-3′ (forward) and 5′-GCG GCC GCT TAT ACA GTG TCC ATC ATT TTG-3′ (reverse). The PCR product generated was digested with *Eco*RI and *Not*I followed by subcloning into pGEX-4T-1. A vector encoding Lys-267 to Val-337 of Harc fused to the C-terminus of GST (i.e., GST−HarcD3) was made similarly by PCR with the primer pair 5′-GAA TTC AAG TCA AGA GTA AGA CTT TAT TCT CAA-3′ (forward) and 5′-GCG GCC GCT TAT ACA GTG TCC ATC ATT TTG-3′ (reverse).

Cell Culture and Transfections. HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 2 mM GlutaMax-1 and grown at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transfected using FuGENE 6 reagent according to the manufacturer's instructions and lysed 24−48 h post-transfection (*22*, *23*).

Cell Lysis, Immunoprecipitation, and Western Blotting. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then incubated in 20 mM Hepes (pH 7.4), 2 mM EGTA, 0.05% Nonidet P-40 (NP-40), and Complete protease inhibitors for 15 min on ice. The cells were scraped from the tissue culture dishes and subjected to homogenization using a tight-fitting dounce homogenizer. The cell lysates were centrifuged at 13000*g* for 10 min at 4 °C, the supernatants retained, and then the protein concentrations measured using a Bio-Rad protein assay kit. The concentrations of NP-40 and NaCl in the cleared cell lysates were adjusted to the levels required (as indicated in the figure captions) prior to immunoprecipitation. FLAG-tagged proteins were immunoprecipitated from cell lysates by incubating 250−1000 μg of protein from each cell lysate with agarose-conjugated anti-FLAG antibodies for 1 h (heat shock experiments) or 3−4 h (all other experiments) at 4 °C with continual mixing. The immunoprecipitates were washed four times with the buffers indicated in the figure captions. Cell lysates and immunoprecipitates were subjected to electrophoresis on 10% SDS−PAGE gels. The proteins were then transferred to Immobilon-P membranes followed by Western blotting.

Trypsin Sensitivity Assay. FLAG−Harc and FLAG−HarcD12 were immunoprecipitated from cell lysates using anti-FLAG beads. After being washed four times with lysis buffer containing 0.5% NP-40 and either 100 mM NaCl or 1 M NaCl, the immunoprecipitates were washed once with 20 mM Hepes (pH 7.4). The immunoprecipitates were then resuspended in 40 mM ammonium bicarbonate (pH $\approx$ 8.5) containing the concentrations of trypsin indicated in the figure caption and incubated for 30 min at 37 °C. Digestion was stopped by the addition of 5× Laemmli SDS−PAGE sample buffer and heating for 10 min at 95 °C, after which time the samples were subjected to SDS−PAGE on 14%

gels. The gels were then stained using a SilverQuest kit (Invitrogen).

GST−Harc Fusion Proteins: Expression, Purification, and Binding Assays. The vectors pGEX-4T-1, pGEX-Harc, and pGEX-HarcD3 were introduced into competent BL21 *Escherichia coli* bacteria, and the expression of the GST fusion proteins was induced by the addition of isopropyl β-thiogalactopyranoside. The fusion proteins were then purified by standard procedures with glutathione−Sepharose beads (*28*). Binding assays were conducted by incubating 20 μL of glutathione−Sepharose beads containing ~2 μg of bound GST fusion protein with aliquots of cell lysate containing 1 mg of protein for 4 h at 4 °C with continual mixing. The beads were washed four times with lysis buffer containing 100 mM NaCl and either 0.05% or 0.5% NP-40, after which they were subjected to Western blotting.

Size Exclusion Chromatography. Transfected HEK293T cells were lysed in buffer containing 0.1% NP-40. Following clarification by centrifugation and filtration through a 0.2 μm syringe filter, 0.25 mL aliquots of the cell lysates were applied to a Superose-6 column (HR 10/30, GE Healthcare) fitted to an Agilent 1100 series HPLC system that had been equilibrated with 20 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EGTA, and 10% glycerol. Elution was performed at a flow rate of 0.2 mL/min, and fractions were collected each minute. The column was calibrated with thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa). For immunoprecipitation assays, 0.15 mL of the indicated column fractions was subjected to immunoprecipitation with anti-FLAG beads as described above.

Heat Shock Treatment. Tissue culture dishes were sealed with Parafilm and placed in a 43 °C water bath for the required length of time. The cells were then gently washed with ice-cold PBS and lysed for 5 min with 20 mM Hepes (pH 7.4), 2 mM EGTA, 100 mM NaCl, 0.1% NP-40, 10% glycerol, and Complete protease inhibitors. The cell lysates were centrifuged at 13000*g* for 10 min at 4 °C and the supernatants retained. Aliquots of the NP40-soluble supernatants, which contained equal amounts of protein, were diluted with 1/5 volume of 5× Laemmli SDS−PAGE sample buffer and heated for 5 min at 95 °C. The NP40-insoluble cell pellets were solubilized in 1× Laemmli sample buffer (volume of the sample buffer = 125% volume of the NP-40 lysis buffer) for 10 min at 95 °C. Equal volumes of the NP40-soluble and NP40-insoluble fractions were analyzed by Western blotting.

RESULTS

The C-Terminal Domain of Harc Is Important for Complex Formation with Hop and Hsp70. Harc is an Hsp90 cochaperone that, while structurally related to Cdc37, is likely to serve a unique but still undefined role in Hsp90-mediated protein folding. We had previously shown that Harc formed a complex with Hsp90, Hsp70, and Hop when expressed in HEK29T cells (*22*). Bioinformatics and biochemical approaches have suggested that Harc has a three-domain structure (Figure 1A) in which the middle domain (residues 152−271) mediates Hsp90 binding (*22*). To ascribe possible functions to the C-terminal domain of Harc, a C-terminal truncation mutant of Harc (i.e., FLAG−HarcD12) was expressed in HEK29T cells and its capacity to bind Hop, Hsp70, and Hsp90 was examined via immunoprecipitation assays under different detergent and salt concentrations. As shown in Figure 1B, deletion of the C-terminal domain of Harc abolished its ability to bind Hop as well as markedly reduced its binding of Hsp70 (Figure 1B). Hsp90 binding was not significantly reduced in the presence of 100 mM NaCl; however, increasing the concentration of NaCl from 100 to 500 mM during the immunoprecipitation assays abolished the binding of Hsp90 to FLAG−HarcD12, while Hsp90 binding to FLAG−Harc was partially reduced (Figure 1B). Notably, the binding of Hop to full-length FLAG−Harc was abolished in the presence of 500 mM NaCl (Figure 1B). Together these findings suggest that in addition to being a key determinant of the binding of Hop and Hsp70 to Harc, the C-terminal domain may also influence the affinity of Hsp90 binding.

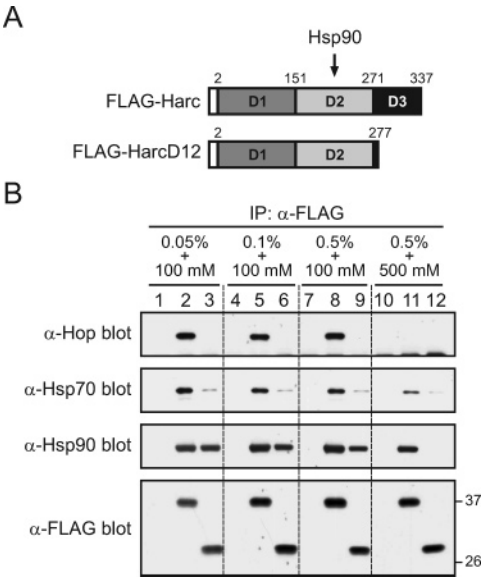

FIGURE 1: Analysis of Hop and Hsp70 binding to Harc. (A) Schematic representation of FLAG-tagged full-length Harc and the C-terminal Harc truncation mutant HarcD12. (B) HEK293T cells expressing FLAG−Harc (lanes 2, 5, 8, and 11) or FLAG−HarcD12 (lanes 3, 6, 9, and 12), or empty vector transfected cells (lanes 1, 4, 7, and 10), were lysed in the presence of 0.05% NP-40. The lysates were adjusted to 0.05%, 0.1%, or 0.5% Nonidet P-40 (NP-40) and either 100 or 500 mM NaCl prior to the immunoprecipitation of FLAG−Harc and FLAG−HarcD12 with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with the indicated antibodies. The positions of the molecular mass markers (kDa) are shown on the right.

Deletion of the C-Terminal Domain Does Not Induce the Gross Destabilization of Harc. Due to the profound effect of deleting the C-terminal domain of Harc on its ability to bind Hop and Hsp70, we assessed the conformational state of FLAG−HarcD12 by subjecting it to in vitro digestion with trypsin. Because the differential binding of Hop, Hsp70, and/or Hsp90 to FLAG−HarcD12 and FLAG−Harc could influence their susceptibility to trypsin digestion, immunoprecipitates of FLAG−HarcD12 and FLAG−Harc were also "salt-stripped" with 1 M NaCl to remove/reduce bound Hop, Hsp70, and/or Hsp90. Little proteolysis of FLAG−HarcD12 was observed at trypsin concentrations of 50−100 ng/mL (Figure 2A). However, significant proteolysis of FLAG−HarcD12 occurred at a trypsin concentration of 200 ng/mL, and it was completely digested when incubated with 500−

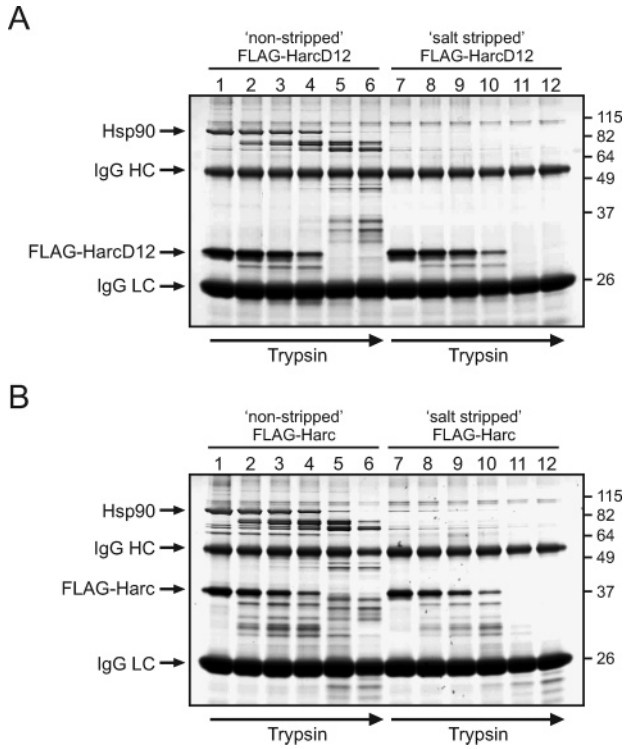

FIGURE 2: Sensitivity of HarcD12 and Harc to in vitro proteolytic digestion by trypsin. (A) FLAG−HarcD12 and (B) FLAG−Harc were immunoprecipitated from lysates of transfected HEK293T cells in the presence of 0.5% NP-40 and 100 mM NaCl ("non-stripped") and 0.5% NP-40 and 1 M NaCl ("salt-stripped"). The immunoprecipitates were then incubated with the following concentrations of trypsin for 30 min at 37 °C, 50 ng/mL (lanes 2 and 8), 100 ng/mL (lanes 3 and 9), 200 ng/mL (lanes 4 and 10), 500 ng/mL (lanes 5 and 11), and 1000 ng/mL (lanes 6 and 12), or with buffer alone (lanes 1 and 7). Aliquots of the digestion reactions were subsequently subjected to SDS−PAGE and the proteins visualized by silver staining. The positions of Hsp90, the heavy and light chains of the anti-FLAG antibodies (IgG HC and IgG LC, respectively), FLAG−HarcD12, and FLAG−Harc are indicated on the left, while the positions of molecular mass markers (kDa) are shown on the right.

1000 ng/mL trypsin (Figure 2A). As shown in Figure 2B, the susceptibility of FLAG−Harc to trypsin digestion was comparable to that of FLAG−HarcD12. The stripping of Hop, Hsp70, and/or Hsp90 from FLAG−HarcD12 and FLAG−Harc appeared to have only modestly increased their susceptibility to in vitro proteolysis by trypsin (Figure 2). The digestion patterns of Hsp90 that was bound to FLAG−HarcD12 and FLAG−Harc appeared comparable (Figure 2).

The C-Terminal Domain of Harc Does Not Bind Hop, but It Can Bind Hsp70. To establish whether the C-terminal domain of Harc represented a bone fide Hop- and/or Hsp70-binding domain, an expression vector encoding a FLAG-tagged form of the C-terminal domain of Harc (i.e., FLAG−HarcD3) was transfected into HEK293T cells. However, FLAG−HarcD3 could not be detected in the lysates of transfected cells (data not shown), the reasons for which are unclear at this stage. Consequently, the C-terminal domain of Harc was expressed as a fusion protein with green fluorescent protein (i.e., FLAG−HarcD3−GFP; see Figure 3A) in HEK293T cells. Although the binding of Hop, Hsp70, and Hsp90 to FLAG−Harc−GFP was comparable to that observed for FLAG−Harc (Figure 3B), FLAG−HarcD3−GFP bound neither Hop nor Hsp90 (Figure 3B). In contrast,

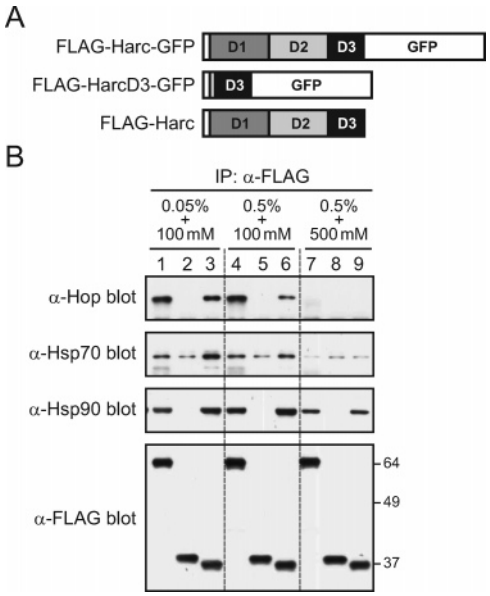

FIGURE 3: Analysis of Hop and Hsp70 binding to the C-terminal domain of Harc. (A) Schematic representation of FLAG−Harc−GFP, FLAG−HarcD3−GFP, and FLAG−Harc. (B) HEK293T cells expressing FLAG−Harc−GFP (lanes 1, 4, and 7), FLAG−HarcD3−GFP (lanes 2, 5, and 8), or FLAG−Harc (lanes 3, 6, and 9) were lysed and the lysates adjusted to 0.05% or 0.5% NP-40 and either 100 or 500 mM NaCl, respectively. The FLAG-tagged proteins were then immunoprecipitated from the cell lysates with anti-FLAG antibodies followed by Western blotting with the indicated antibodies. The positions of molecular mass markers (kDa) are shown on the right.

the binding of Hsp70 to FLAG−HarcD3−GFP was detected (Figure 3B). The ability of bacterially expressed GST fusion proteins of Harc, namely, GST−Harc and GST−HarcD3, to bind Hop, Hsp70, and/or Hsp90 in lysates of nontransfected HEK293T cells was also tested. However, neither fusion protein was capable of binding Hop, Hsp70, or Hsp90 (data not shown).

The above findings suggested that the C-terminal domain of Harc binds to Hsp70 but that it does not bind to Hop. However, it is possible that the middle Hsp90-binding domain of Harc may positively regulate the ability of the C-terminal domain to bind Hop. To test this proposal, we made use of chimeric proteins of Harc and the structurally related Hsp90 cochaperone Cdc37 (Figure 4A). Cdarc consists of the N-terminal domain of Cdc37 and the middle and C-terminal domains of Harc, while Hac37 is composed of the N-terminal domain of Harc and the middle and C-terminal domains of Cdc37. Notably, FLAG−Cdarc did not bind Hop even though the C-terminal domain of Harc was juxtaposed to the middle Hsp90-binding domain of Harc in this chimeric protein (Figure 4B). Conversely, the binding of Hsp70 to FLAG−Cdarc was comparable to that observed for FLAG−Harc (Figure 4B). FLAG−Hac37, which contains the N-terminal domain of Harc and binds Hsp90, did not bind Hop or significant levels of Hsp70 (Figure 4B).

Hop Only Forms a Complex with Harc in the Presence of Hsp90. To further define the nature of the interaction of Hop with Harc, lysates of HEK293T cells expressing FLAG−Harc and FLAG−HarcD12 were subjected to size exclusion chromatography. FLAG−Harc primarily eluted in fractions 61−67, which corresponded to a molecular mass of ∼300−700 kDa (Figure 5A). Notably, the elution profile of FLAG−

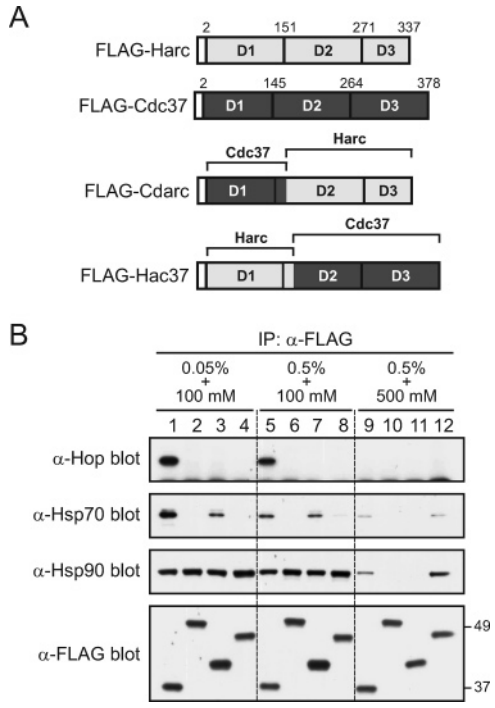

FIGURE 4: Analysis of Hop and Hsp70 binding to Harc−Cdc37 chimeras. (A) Schematic representation of FLAG−Harc, FLAG−Cdc37, FLAG−Cdarc, and FLAG−Hac37. (B) HEK293T cells expressing FLAG−Harc (lanes 1, 5, and 9), FLAG−Cdc37 (lanes 2, 6, and 10), FLAG−Cdarc (lanes 3, 7, and 11), or FLAG−Hac37 (lanes 4, 8, and 12) were lysed in the presence of 0.05% NP-40. The lysates were adjusted to 0.05% or 0.5% NP-40 and either 100 or 500 mM NaCl prior to the immunoprecipitation of FLAG−Harc, FLAG−Cdc37, FLAG−Cdarc, and FLAG−Hac37 with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with the indicated antibodies. The positions of molecular mass markers (kDa) are shown on the right.

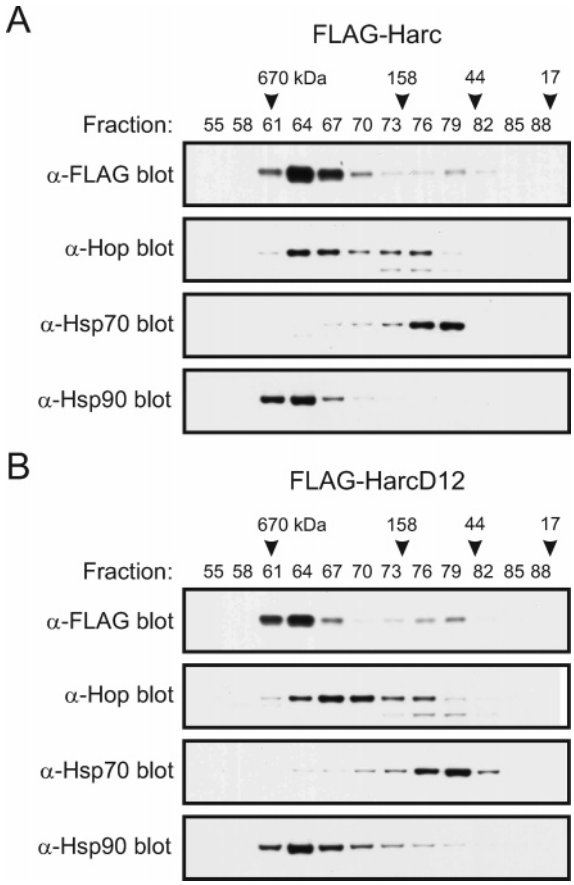

FIGURE 5: Analysis of Harc complexes by size exclusion chromatography. Lysates of HEK293T cells expressing (A) FLAG−Harc or (B) FLAG−HarcD12 were subjected to size exclusion chromatography on a Superose-6 column. Aliquots of the column fractions shown were Western blotted with the indicated antibodies. The elution positions of column calibration standards (kDa) are shown at the top.

HarcD12 was comparable to that of full-length FLAG−Harc (Figure 5B). A significant degree of overlap was observed between the elution profiles of Hop and FLAG−Harc, with the peak fractions of Hop (fractions 64−67) coinciding with the fractions that contained the highest levels of FLAG−Harc (Figure 5A). Although the elution profile of Hop also partially overlapped with that of FLAG−HarcD12, the fractions that contained the highest levels of Hop (fractions 67−70) did not coincide with the peak fractions of FLAG−HarcD12 (Figure 5B). Hsp70 primarily eluted in latter fractions, in particular fractions 76−79, irrespective of whether the cells expressed FLAG−Harc or FLAG−HarcD12 (Figure 5). Hsp90 largely eluted in the same fractions as those that contained FLAG−Harc and FLAG−HarcD12 (Figure 5).

The coimmunoprecipitation of Hop with FLAG−Harc was essentially only detected in pooled fractions 63−65 (Figure 6A) despite the fact that high levels of Hop and FLAG−Harc were also present in pooled fractions 66−68 (Figure 5A). Significantly though, the levels of Hsp90 in pooled fractions 63−65 were higher than those in fractions 66−68 (Figure 5A), suggesting that the interaction of Hop with FLAG−Harc is dependent on the presence of Hsp90. Coimmunoprecipitation of Hsp70 with FLAG−Harc occurred in fractions 60−62 and 63−65 (Figure 6A). However, the levels of Hsp70 coimmunoprecipitated did not correlate with the FLAG−Harc levels; the relative ratio of Hsp70 to FLAG−Harc was greater in fractions 60−62 than in fractions 63−65 (Figure 6A). This suggests that a greater proportion of FLAG−Harc in fractions 60−62 is in complex with Hsp70. Although Hsp70 was detected in fractions 66−68 (Figure 5A), it did not coimmunoprecipitate with FLAG−Harc (Figure 6A). As expected, Hsp90 coimmunoprecipitated with FLAG−Harc in pooled fractions 60−62, 63−65, and 66−68, with the relative levels of Hsp90 correlating with the amount of FLAG−Harc in each immunoprecipitate (Figure 6A). Reciprocal immunoprecipitation assays with anti-Hsp90 antibodies were performed to further establish the composition of the complexes in the column fractions. Hop coimmunoprecipitated with Hsp90 in pooled fractions 60−62, 63−65, 66−68, and 69−71 (Figure 6B), although it did not coimmunoprecipitate with FLAG−Harc in pooled fractions 60−62 and 66−68 (Figure 6A). Thus, FLAG−Harc forms a range of distinct complexes when expressed in HEK293T cells including Harc−Hsp90−Hsp70−Hop and Harc−Hsp90 complexes.

Dimerization of the C-Terminal Domain of Harc Is Necessary for the Binding of Hop. The binding of Hsp90 to Harc is dependent on the dimerization of Harc (*24*). To test whether the same was true for Hop and Hsp70 binding, FLAG−HarcD12 was coexpressed with HA-tagged full-length Harc. As shown in Figure 7, HA−Harc coimmunoprecipitated with FLAG−HarcD12 in anti-FLAG immunoprecipitates, indicating that FLAG−HarcD12 had hetero-

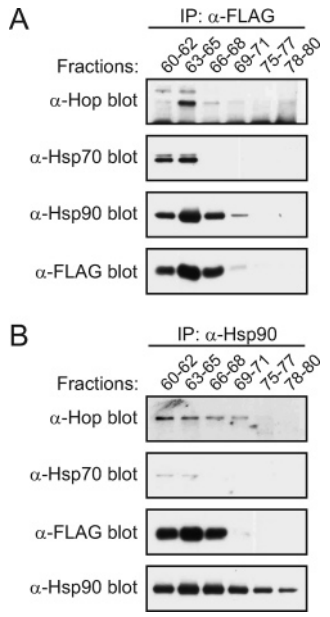

FIGURE 6: Analysis of size exclusion chromatography-fractionated Harc complexes. The indicated column fractions from Figure 5A were pooled and subjected to immunoprecipitation with (A) anti-FLAG and (B) anti-Hsp90 antibodies. The immunoprecipitates were then Western blotted with the indicated antibodies.

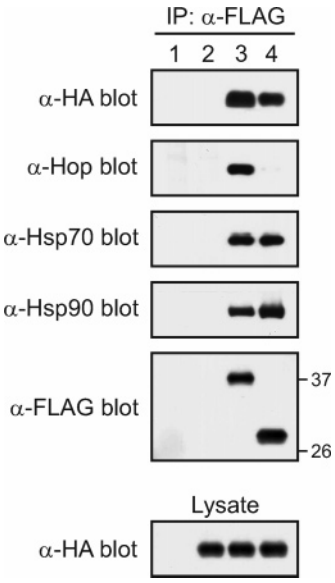

FIGURE 7: Dimerization requirements for the binding of Hop and Hsp70 to Harc. HEK293T cells expressing HA−Harc alone (lane 2) or together with FLAG−Harc (lane 3) and FLAG−HarcD12 (lane 4), or empty vector transfected cells (lane 1), were lysed and FLAG−Harc and FLAG−HarcD12 immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with the indicated antibodies. The cell lysates were blotted with an anti-HA antibody to demonstrate the equal expression of HA−Harc under the different conditions.

dimerized with full-length HA−Harc. However, the coexpression of FLAG−HarcD12 with HA−Harc did not result in the coimmunoprecipitation of Hop with FLAG−HarcD12 (Figure 7). By contrast, the coimmunoprecipitation of Hsp70 with FLAG−HarcD12 was observed when FLAG−HarcD12 was coexpressed with HA−Harc (Figure 7). Western blotting of the cell lysates confirmed that HA−Harc was expressed at comparable levels when coexpressed with FLAG−Harc and FLAG−HarcD12 (Figure 7). Similarly, the expression levels of endogenous Hop and Hsp70 were not affected by the expression of FLAG−Harc and FLAG−HarcD12 (data not shown). Thus, dimerization of the C-terminal domain of Harc is required for complex formation between Hop and Harc but it is not a prerequisite for the binding of Hsp70 to Harc.

Bioinformatics-Based Identification of Alternative mRNA Transcripts Encoding C-Terminally Truncated Forms of Harc. The importance of the C-terminal domain for Harc to form a complex with Hop and Hsp70 prompted us to investigate whether isoforms of Harc that lacked this domain may be expressed under some circumstances. Indeed, searches of the UniProtKB/TrEMBL database identified two potential, alternative human Harc transcripts (accession numbers Q5JVJ2 and A6NFT3) in which exon 7 was replaced with an alternative, but different, exon (Figure 8). Rather than encoding 33 amino acids, each of the alternative exons encode 4 amino acids: Ala-Pro-Arg-Phe and Ile-Pro-Asp-Phe, respectively (Figure 8). The UniProtKB/TrEMBL database also contains two entries for an alternative mouse Harc transcript (accession numbers Q8BP15 and Q3TNC7) in which exon 7 encodes the sequence Ala-Pro-Arg-Phe. On the basis of the data presented above, these shorter isoforms of Harc would be predicted to bind to Hsp90 but not to either Hsp70 or Hop.

Deletion of the C-Terminal Domain Augments the Heat-Shock-Induced Insolubilization of Harc. Because of its ability to form complexes with Hsp90, Hsp70, and Hop, Harc is likely to act in concert with these proteins to protect cells from the deleterious effects of various stress stimuli (e.g., heat stress). However, the markedly different binding properties of the C-terminally truncated Harc mutant suggested that the shorter Harc isoforms identified in the UniProtKB/TrEMBL database may exhibit a different response to stress stimuli than does full-length Harc. Consequently, we first sought to define the response of FLAG−Harc to heat shock, which was then compared to that of Hsp90, Hsp70, Hop, and FLAG−HarcD12. As shown in Figure 9A, FLAG−Harc was detected exclusively in the NP40-soluble fractions of non-heat-shocked HEK293T cells. However, the level of FLAG−Harc in the NP40-soluble fraction was reduced by ∼30−40% after the cells had been subjected to a 90 min heat shock at 43 °C (Figure 9A). Concomitant with the decreased levels of FLAG−Harc in the NP40-soluble fraction of heat-shocked cells was a corresponding increase in FLAG−Harc levels in the NP40-insoluble fraction of the same cells (Figure 9A). To establish whether this was unique to FLAG−Harc, the effect of heat shock on FLAG−Cdc37 was likewise assessed. In contrast to FLAG−Harc, however, heat shock did not result in a notable reduction in the detergent solubility of FLAG−Cdc37 (Figure 9B). The reduction in FLAG−Harc solubility following heat shock was neither augmented nor ameliorated by the prior exposure of the cells to the Hsp90 inhibitor geldanamycin (data not shown). Only low levels of Hop, Hsp70, and Hsp90 were detected in the NP40-insoluble fraction of non-heat-shocked cells (Figure 9C). However, the levels of Hop and Hsp70 in the NP40-insoluble fractions of cells expressing FLAG−Harc (or FLAG−Cdc37) increased significantly in response to heat shock (Figure 9C and data not shown). In the case of Hop, ∼25% of the total Hop was present in the NP40-insoluble fraction after a 90 min heat shock (Figure 9C). Similarly, the level of Hsp70 in the same NP40-insoluble fraction

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Harc_Q9H577  MEQPWPPPGPWSLPRAEGEAEEESDFDVFPSSPRCPQLPGGGAQMYSHGIELACQKQKEFVKSSVACKWNLAEAQQKLGSLALHNSESLDQEHAKAQTAV 100
Harc_Q5JVJ2  MEQPWPPPGPWSLPRAEGEAEEESDFDVFPSSPRCPQLPGGGAQMYSHGIELACQKQKEFVKSSVACKWNLAEAQQKLGSLALHNSESLDQEHAKAQTAV 100
Harc_A6NFT3  MEQPWPPPGPWSLPRAEGEAEEESDFDVFPSSPRCPQLPGGGAQMYSHGIELACQKQKEFVKSSVACKWNLAEAQQKLGSLALHNSESLDQEHAKAQTAV 100

Harc_Q9H577  SELRQREEEWRQKEEALVQREKMCLWSTDAISKDVFNKSFINQDKRKDTEDEDKSESFMQKYEQKIRHFGMLSRWDDSQRFLSDHPYLVCEETAKYLILW 200
Harc_Q5JVJ2  SELRQREEEWRQKEEALVQREKMCLWSTDAISKDVFNKSFINQDKRKDTEDEDKSESFMQKYEQKIRHFGMLSRWDDSQRFLSDHPYLVCEETAKYLILW 200
Harc_A6NFT3  SELRQREEEWRQKEEALVQREKMCLWSTDAISKDVFNKSFINQDKRKDTEDEDKSESFMQKYEQKIRHFGMLSRWDDSQRFLSDHPYLVCEETAKYLILW 200

Harc_Q9H577  CFHLEAEKKGALMEQIAHQAVVMQFIMEMAKNCNVDPRGCFRLFFQKAKAEEEGYFEAFKNELEAFKSRVRLYSQSQSFQPMTVQNHVPHSGVGSIGLLE 300
Harc_Q5JVJ2  CFHLEAEKKGALMEQIAHQAVVMQFIMEMAKNCNVDPRGCFRLFFQKAKAEEEGYFEAFKNELEAFKSRVRLYSQSQSFQPMTVQNHVPHSGVGSIGLLE 300
Harc_A6NFT3  CFHLEAEKKGALMEQIAHQAVVMQFIMEMAKNCNVDPRGCFRLFFQKAKAEEEGYFEAFKNELEAFKSRVRLYSQSQSFQPMTVQNHVPHSGVGSIGLLE 300

Harc_Q9H577  SLPQNPDYLQYSISTALCSLNSVVHKEDDEPKMMDTV 337
Harc_Q5JVJ2  SLPQAPRF---------------------------- 308
Harc_A6NFT3  SLPQIPDF---------------------------- 308
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FIGURE 8: Sequence alignment of putative alternative isoforms of Harc. Amino acid sequences of human Harc from the UniProtKB/TrEMBL database, with the indicated accession numbers, were aligned using the ClustalW program. The C-terminal domain of Harc is boxed, while the amino acids encoded by the last exon of the *Harc* gene are shaded.

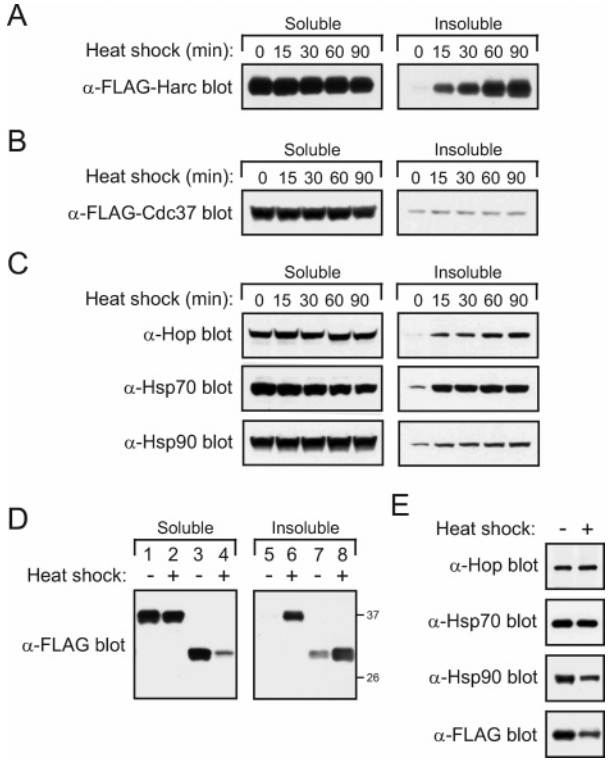

FIGURE 9: Analysis of the effects of heat shock on the solubility of Harc. HEK293T cells expressing (A) FLAG−Harc and (B) FLAG−Cdc37 were subjected to heat shock at 43 °C for the times shown and then lysed. Equal volumes of the NP40-soluble and NP40-insoluble fractions were Western blotted with an anti-FLAG antibody. (C) The NP40-soluble and NP40-insoluble fractions from (A) were also Western blotted with anti-Hop, anti-Hsp70, and anti-Hsp90 antibodies. (D) HEK293T cells expressing FLAG−Harc (lanes 1, 2, 5, and 6) or FLAG−HarcD12 (lanes 3, 4, 7, and 8) were subjected to heat shock at 43 °C for 60 min (lanes 2, 4, 6, and 8) or kept at 37 °C (lanes 1, 3, 5, and 7). The cells were then lysed and equal volumes of the NP40-soluble and NP40-insoluble fractions Western blotted with an anti-FLAG antibody. (E) HEK293T cells expressing FLAG−Harc were subjected to heat shock at 43 °C for 60 min or kept at 37 °C. The cells were lysed, and FLAG−Harc was immunoprecipitated from the NP40-soluble fractions with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with the indicated antibodies.

represented ∼30−40% of the total Hsp70 (Figure 9C). Heat shock resulted in only a relatively small increase (∼2−3-fold) in the levels of Hsp90 in the NP40-insoluble fractions of the cells, irrespective of whether the cells expressed FLAG−Harc or FLAG−Cdc37, and this only accounted for a minor fraction of the total Hsp90 (Figure 9C and data not shown). Notably, deletion of the C-terminal domain of Harc markedly potentiated its heat-shock-induced insolubilization; more than 80% of FLAG−HarcD12 was present in the NP40-insoluble fraction of HEK293T cells that had been subjected to a 60 min heat shock at 43 °C (Figure 9D). The effect of heat shock on the binding of Hsp90, Hsp70, and Hop to Harc was also evaluated. The reduced levels of Hsp90 that coimmunoprecipitated with FLAG−Harc from heat-shocked cells correlated with the reduced levels of FLAG−Harc in the same immunoprecipitates (Figure 9E). However, the levels of Hop and Hsp70 that coimmunoprecipitated with FLAG−Harc from the same heat-shocked cells were not reduced (Figure 9E), suggesting that heat shock increased the interaction of Hop and Hsp70 with Harc.

DISCUSSION

Harc is an Hsp90 cochaperone that is structurally related to Cdc37 but whose role in Hsp90-mediated protein folding has not been defined (*22*). We have previously shown that Harc can form a complex with Hsp90, Hsp70, and Hop in HEK293T cells. Further, we identified the middle domain of Harc as being a bone fide Hsp90-binding domain and established that dimerization of Harc was a prerequisite for its binding to Hsp90 (*22*, *24*). In the present study, we investigated the importance of the C-terminal domain of Harc for its interaction with Hsp90, Hsp70, and Hop as well as determined whether the binding of Hsp70 and Hop was regulated by the dimerization state of Harc. In addition, the response of Harc to cellular stress, namely, to heat shock, was ascertained.

The C-terminal domain of Harc, which represents the region of greatest sequence divergence between Harc and Cdc37, is a key determinant of the interaction of Hop and Hsp70 with Harc. The loss of Hop binding, and the dramatic reduction in the binding of Hsp70, which occurs upon the deletion of the C-terminal domain is unlikely to have been due to a major change in the overall structural integrity of Harc because Hsp90 binding was not significantly reduced. Furthermore, the sensitivity of the Harc C-terminal truncation mutant to in vitro digestion by trypsin was comparable to that of full-length Harc. Interestingly, the "stripping" of Hsp90, Hsp70, and/or Hop from Harc with 1 M NaCl slightly increased the sensitivity of Harc to digestion by trypsin. This suggests that Harc may undergo only a relatively minor conformational change upon the binding of Hsp90.

Our data suggest that the interaction of Hsp70 with Harc is directly mediated by the C-terminal domain of Harc, although other regions of Harc might also contribute to

Hsp70 binding. This conclusion is based on the finding that when fused to a heterologous protein, namely, to green fluorescent protein, the C-terminal domain of Harc bound Hsp70. The C-terminal domain of Harc also bound Hsp70 when in the context of a chimeric protein consisting of the N-terminal domain of Cdc37 and the middle and C-terminal domains of Harc. Thus, the structural organization of Harc is such that it can directly bind Hsp90 and Hsp70 via separate domains.

We have shown previously that dimerization of Harc was necessary for Hsp90 binding (*24*). It is not entirely clear at this stage whether the binding of Hsp70 to Harc is likewise dependent on the dimerization of Harc. If the C-terminal domain of Harc is the primary region involved in the binding of Hsp70, then the coimmunoprecipitation of Hsp70 with FLAG−HarcD12 that was complexed with full-length HA−Harc implies that Hsp70 can bind to monomeric full-length Harc. However, size exclusion chromatography of cell lysates revealed that Hsp70-containing FLAG−Harc complexes were on the order of 400−500 kDa in size. Moreover, the complexes also contained Hsp90, while some contained both Hsp90 and Hop. These findings are therefore more consistent with Hsp70 forming a complex with dimerized Harc, which may also be bound to Hsp90. It is not known whether the binding of Hsp90 and Hsp70 to Harc occurs in a sequential fashion and if so the order of binding. But given that Harc formed a stable complex with Hsp90 in the absence of Hsp70, but not vice versa, it seems likely that the binding of Hsp90 to Harc precedes that by Hsp70.

In contrast to Hsp70, Hop failed to bind to either the HarcD3-GFP fusion protein or Cdarc; it also failed to bind to the N-terminal or middle domain of Harc (data not shown) or to the chimeric protein Hac37. Fractionation of cell lysates by size exclusion chromatography revealed that Hop only formed a complex with Harc in the presence of Hsp90 and Hsp70. Thus, rather than binding directly to Harc, the interaction of Hop with Harc is likely to be mediated via its binding to both Hsp90 and Hsp70. This would also potentially explain why deletion of the C-terminal domain reduced the affinity of Harc for Hsp90; in the absence of the C-terminal domain, Hsp70 and Hop cannot bind to, and thereby stabilize, Harc−Hsp90 complexes. Binding experiments with purified proteins will be necessary to more clearly define the nature of the interaction of Hop with Harc.

In addition to being important for the assembly of Harc−heat shock protein complexes, the C-terminal domain of Harc also appears to be a key determinant of the response of Harc to heat shock. Deletion of the C-terminal domain resulted in the majority (>80%) of Harc rapidly appearing in the detergent-insoluble fraction of heat-shocked cells. Heat shock also resulted in a significant decrease in the detergent solubility of full-length Harc, although not to the same extent as that observed for FLAG−HarcD12. The exposure of cells to heat shock has previously been shown to result in a transient increase in the expression of heat shock genes and in some cases to also trigger a decrease in the solubility of heat shock proteins (*29*−*33*). Furthermore, the subcellular localization of heat shock proteins (e.g., Hsp70) has been found to be altered following heat shock, including the redistribution of significant proportions of the heat shock proteins to the nuclei of cells. Preliminary studies with GFP-tagged Harc have suggested that Harc had become more concentrated in punctuate structures, including in the nuclei of the cells, following heat shock. The biological relevance of the heat shock response of Harc is yet to be established; however, Cdc37 did not exhibit the same response. Thus, the observed effects of heat shock on Harc are unlikely to be a consequence of its ectopic expression but rather reflect its normal response to such a stress stimulus. The fact that Cdc37 did not undergo the same heat-shock-induced detergent insolubilization is also interesting given its central role in regulating the Hsp90-mediated folding of protein kinases (e.g., Cdk4, Raf-1, Akt, and Src kinases) into functional conformations (*19*, *20*, *23*, *25*, *34*). The ability of Cdc37 to remain in a detergent-soluble form in the cytoplasm of heat-shocked cells may allow protein kinases, which regulate cell survival and proliferation, to be protected from heat-induced denaturation.

Bioinformatics analysis indicated that cells may express splice variants of Harc that encode C-terminally truncated isoforms of Harc. These Harc isoforms would lack ∼50% of the C-terminal domain of full-length Harc and hence be predicted to fail to bind Hsp70 and Hop but still be capable of binding Hsp90. However, depending on the relative expression levels of the shorter Harc isoforms and full-length Harc, they may be capable of heterodimerizing with full-length Harc and thereby forming complexes with Hsp90 and Hsp70 but not with Hop. The responses of these shorter Harc isoforms to heat shock is also likely to be significantly different from that of the longer Harc isoform. Although the biochemical and biological consequences of the expression of splice variants of Harc is to be established, it potentially introduces another level of complexity and regulation to the process of Hsp90-mediated protein folding. Our current efforts are directed toward establishing under which conditions these Harc splice variants are expressed and defining their interactions with Hsp90, Hsp70, and Hop.

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REFERENCES

1. Parsell, D. A., and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins, *Annu. Rev. Genet. 27*, 437−496.
2. Morimoto, R. I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators, *Genes Dev. 12*, 3788−3796.
3. Morimoto, R. I. (1993) Cells in stress: transcriptional activation of heat shock genes, *Science 259*, 1409−1410.
4. Lindquist, S. (1986) The heat-shock response, *Annu. Rev. Biochem. 55*, 1151−1191.
5. Jaattela, M. (1999) Heat shock proteins as cellular lifeguards, *Ann. Med. 31*, 261−271.
6. Hohfeld, J., Cyr, D. M., and Patterson, C. (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation, *EMBO Rep. 2*, 885−890.
7. Hayes, S. A., and Dice, J. F. (1996) Roles of molecular chaperones in protein degradation, *J. Cell. Biol. 132*, 255−258.
8. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins, *Nat. Cell Biol. 3*, 93−96.
9. Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F. U. (1996) Pharmacologic

shifting of a balance between protein refolding and degradation mediated by Hsp90, *Proc. Natl. Acad. Sci. U.S.A. 93*, 14536−14541.
10. Hartl, F. U., and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein, *Science 295*, 1852−1858.
11. Pratt, W. B., and Toft, D. O. (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery, *Exp. Biol. Med. (Maywood) 228*, 111−133.
12. Kosano, H., Stensgard, B., Charlesworth, M. C., McMahon, N., and Toft, D. (1998) The assembly of progesterone receptor-hsp90 complexes using purified proteins, *J. Biol. Chem. 273*, 32973−32979.
13. Dittmar, K. D., Banach, M., Galigniana, M. D., and Pratt, W. B. (1998) The role of DnaJ-like proteins in glucocorticoid receptor.hsp90 heterocomplex assembly by the reconstituted hsp90.p60.hsp70 foldosome complex, *J. Biol. Chem. 273*, 7358−7366.
14. Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70, *Mol. Endocrinol. 10*, 682−693.
15. Johnson, J. L., and Toft, D. O. (1995) Binding of p23 and hsp90 during assembly with the progesterone receptor, *Mol. Endocrinol. 9*, 670−678.
16. Hutchison, K. A., Stancato, L. F., Owens-Grillo, J. K., Johnson, J. L., Krishna, P., Toft, D. O., and Pratt, W. B. (1995) The 23-kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp foldosome that is required for assembly of the glucocorticoid receptor into a functional heterocomplex with hsp90, *J. Biol. Chem. 270*, 18841−18847.
17. Owens-Grillo, J. K., Hoffmann, K., Hutchison, K. A., Yem, A. W., Deibel, M. R., Jr., Handschumacher, R. E., and Pratt, W. B. (1995) The cyclosporin A-binding immunophilin CyP-40 and the FK506-binding immunophilin hsp56 bind to a common site on hsp90 and exist in independent cytosolic heterocomplexes with the untransformed glucocorticoid receptor, *J. Biol. Chem. 270*, 20479−20484.
18. Ratajczak, T., and Carrello, A. (1996) Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding, *J. Biol. Chem. 271*, 2961−2965.
19. Kimura, Y., Rutherford, S. L., Miyata, Y., Yahara, I., Freeman, B. C., Yue, L., Morimoto, R. I., and Lindquist, S. (1997) Cdc37 is a molecular chaperone with specific functions in signal transduction, *Genes Dev. 11*, 1775−1785.
20. Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4, *Genes Dev. 10*, 1491−1502.
21. Shao, J., Irwin, A., Hartson, S. D., and Matts, R. L. (2003) Functional dissection of cdc37: characterization of domain structure and amino acid residues critical for protein kinase binding, *Biochemistry 42*, 12577−12588.
22. Scholz, G. M., Cartledge, K., and Hall, N. E. (2001) Identification and characterization of Harc, a novel Hsp90-associating relative of Cdc37, *J. Biol. Chem. 276*, 30971−30979.
23. Scholz, G., Hartson, S. D., Cartledge, K., Hall, N., Shao, J., Dunn, A. R., and Matts, R. L. (2000) p50(Cdc37) can buffer the temperature-sensitive properties of a mutant of Hck, *Mol. Cell. Biol. 20*, 6984−6995.
24. Roiniotis, J., Masendycz, P., Ho, S., and Scholz, G. M. (2005) Domain-mediated dimerization of the Hsp90 cochaperones Harc and Cdc37, *Biochemistry 44*, 6662−6669.
25. Grammatikakis, N., Lin, J. H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function, *Mol. Cell. Biol. 19*, 1661−1672.
26. Roe, S. M., Ali, M. M., Meyer, P., Vaughan, C. K., Panaretou, B., Piper, P. W., Prodromou, C., and Pearl, L. H. (2004) The Mechanism of Hsp90 Regulation by the Protein Kinase-Specific Cochaperone p50(cdc37), *Cell 116*, 87−98.
27. Siligardi, G., Panaretou, B., Meyer, P., Singh, S., Woolfson, D. N., Piper, P. W., Pearl, L. H., and Prodromou, C. (2002) Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37, *J. Biol. Chem. 277*, 20151−20159.
28. Smith, D. B., and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase, *Gene 67*, 31−40.
29. Bensaude, O., Pinto, M., Dubois, M. F., Nguyen, V. T., and Morange, M. (1990) in *Stress Proteins. Induction and Function* (Schlesinger, M. J., Santoro, G., and Garaci, E., Eds.) pp 89−99, Springer-Verlag, Berlin.
30. Arrigo, A. P., Suhan, J. P., and Welch, W. J. (1988) Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein, *Mol. Cell. Biol. 8*, 5059−5071.
31. Collier, N. C., and Schlesinger, M. J. (1986) The dynamic state of heat shock proteins in chicken embryo fibroblasts, *J. Cell. Biol. 103*, 1495−1507.
32. Dubois, M. F., Hovanessian, A. G., and Bensaude, O. (1991) Heat-shock-induced denaturation of proteins. Characterization of the insolubilization of the interferon-induced p68 kinase, *J. Biol. Chem. 266*, 9707−9711.
33. Velazquez, J. M., and Lindquist, S. (1984) hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery, *Cell 36*, 655−662.
34. Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P., and Rosen, N. (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function, *J. Biol. Chem. 277*, 39858−39866.

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