Domain-Mediated Dimerization of the Hsp90 Cochaperones Harc and Cdc37†

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ABSTRACT: Hsp90 is a highly conserved molecular chaperone that acts in concert with Hsp70 and a cohort of cochaperones to mediate the folding of client proteins into functional conformations. The novel Hsp90 cochaperone Harc was identified previously on the basis of its amino acid sequence similarity to Cdc37. Although the biochemical role of Harc has not been established, the structural similarities between Harc and Cdc37 suggest that it too may function to regulate the binding of client proteins to Hsp90. We report here that Harc forms dimers in vitro. Functional dissection of Harc revealed that both the N-terminal and middle domains contributed to its dimerization. Notably, dimerization of the middle domain of Harc was required for the binding of Hsp90, suggesting that dimerized Harc binds to Hsp90 dimers. The N-terminal domain of Harc made an important contribution to the dimerization of Harc by facilitating the interaction of Hsp70 with Harc-Hsp90 heterocomplexes. Harc was also found to heterodimerize with Cdc37 in vitro. Titration experiments revealed that Harc homodimerization was favored over heterodimerization with Cdc37 when both cochaperones were at similar levels. However, formation of Harc homodimers and heterodimers of Harc and Cdc37 was comparable when the level of Cdc37 was approximately 10-fold above that of Harc. Furthermore, homo- and heterodimerization of Harc and Cdc37 was a dynamic process. Thus Harc could potentially contribute to the regulation of the Hsp90-mediated folding of Cdc37-dependent protein kinases into functional conformations via dimerization with Cdc37.

The 90 kDa heat shock protein Hsp90[1] is an abundant and highly conserved molecular chaperone that is essential for eukaryotic cell survival (*1*, *2*). Hsp90 acts in concert with other chaperones (e.g. Hsc/Hsp70), cochaperones (e.g. Hop and p23), and immunophilins (e.g. FKBP52) to mediate the folding of client proteins into functional conformations (*3*). Although Hsp90 has a large and expanding number of client proteins, the majority are either transcription factors (e.g. steroid hormone receptors and p53) or protein kinases, such as Src-family kinases and cyclin-dependent protein kinases (*3*).

Extensive mechanistic studies on interactions between Hsp90 and steroid hormone receptors has yielded a generalized paradigm for Hsp90-mediated folding of client proteins into functional conformations (*3*). The first step in this process involves the interaction of Hsp70 and Hsp40 with the client protein to form a so-called "early complex" (*4*, *5*). The Hsp70-Hsp90 organizing protein Hop then stabilizes the interaction of Hsp90 with the Hsp70-client protein, leading to the formation of an "intermediate complex" (*4*−*6*). Further maturation of the complex is accompanied by the loss of Hsp70 and Hop from the complex and interaction of the Hsp90 cochaperone p23 with Hsp90 (*4*, *5*). Immunophilins, such as the FK506-binding protein FKBP52, are additional components of mature Hsp90-client protein heterocomplexes and may in some cases (e.g. steroid hormone receptors) be involved in trafficking client proteins to their final subcellular destinations (*7*−*10*).

A major unresolved question surrounding Hsp90-mediated protein folding is the mechanism underlying the specific recognition of client proteins by Hsp90. For many client protein kinases of Hsp90, the Hsp90 cochaperone Cdc37 likely dictates their recognition by Hsp90. Cdc37 has been shown to bind both protein kinases and Hsp90 (*11*−*14*). Bioinformatic and biochemical approaches have revealed that Cdc37 has a three domain structure in which the N-terminal domain (residues 1−126) is responsible for binding client protein kinases and a middle domain (residues 127−282) that binds Hsp90 (*12*, *13*, *15*, *16*). As yet, the function of the C-terminal domain (residues 283−378) of Cdc37 is not known. The protein kinase binding activity of Cdc37 is regulated at two levels: (i) phosphorylation of serine-13 by the protein kinase CK2 (*17*, *18*) and (ii) conformational switching of bound Hsp90 (*11*−*13*, *19*, *20*). Recently, Prince and Matts have reported the identification of sequence motifs in the catalytic domain of the Src-family kinase Lck that mediates its high affinity interaction with Cdc37 (*21*).

Although Cdc37 was originally thought to simply function as a protein kinase-targeting cochaperone of Hsp90 (*11*−*14*), Siligardi et al. have reported that Cdc37 also inhibits the ATPase activity of Hsp90 (*22*). The binding of ATP in the nucleotide-binding pocket near the N-terminus of Hsp90 induces a conformational change that brings together the N-terminal domains in the Hsp90 dimer and the formation of a "molecular clamp" around the client protein (*23*−*25*).

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[1] Abbreviations: DMSO, dimethyl sulfoxide; GA, geldanamycin; Hsp, heat shock protein.

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Release of the client protein subsequently occurs in response to hydrolysis of bound ATP (*24*). The structural basis for the inhibitory effect of Cdc37 on the ATPase activity of Hsp90 has been elucidated by Roe et al. (*26*) with the solving of the crystal structure of the N-terminal domain of Hsp90 in complex with the C-terminal half of Cdc37. Cdc37 exists as a dimer in the complex and by interacting with the N-terminal domain of Hsp90 prevents closing of the nucleotide-binding pocket, which is a prerequisite for ATP hydrolysis. The presence of Cdc37 dimers in the crystal structure is consistent with an earlier in vitro study by the same group that demonstrated dimerization of Cdc37 in the solution phase (*22*), as well as a recent report by Zhang et al. (*27*).

Harc is a Hsp90-binding protein that was identified on the basis of its high degree of amino acid sequence similarity to Cdc37 (*16*). Like Cdc37, Harc is composed of three domains, with the middle domain mediating its interaction with Hsp90. In contrast to Cdc37, Harc does not bind Src-family kinases or Raf-1, but it does bind Hsp70, Hop, and the immunophilins FKBP52 and Cyp40 (*16*). Although the biochemical role of Harc is still to be established, the structural similarity between Harc and Cdc37 suggests that it too may function to regulate the binding of a specific class of client proteins by Hsp90. Here we have investigated the capacity of Harc to dimerize and interact with Hsp90 and Hsp70. Our findings indicate that Harc has a greater capacity for dimerization than does Cdc37. Moreover, Harc is capable of forming heterodimers with Cdc37 when coexpressed in mammalian cells.

EXPERIMENTAL PROCEDURES

Reagents. Cell culture medium and supplements, fetal calf serum, anti-V5 monoclonal antibody, and precast SDS−PAGE gels were from Invitrogen. The HRP-conjugated and agarose-conjugated anti-FLAG monoclonal (M2) antibodies and geldanamycin were obtained from Sigma. The mouse monoclonal anti-Hsp90 antibody (H38220) was from Transduction Laboratories, whereas the rabbit polyclonal anti-Hsp90 antibody (PA3−013) was from Affinity BioReagents, Inc. The mouse polyclonal anti-Cdc37 and monoclonal anti-Hsp70 (BB70) antibodies were generous gifts from Drs. Steven D. Hartson (Oklahoma State University) and David Toft (Mayo Clinic, Rochester), respectively. Protein G-Sepharose and ECL reagents were from Amersham Biosciences. *Complete* protease inhibitors and FuGENE 6 transfection reagent were from Roche.

Plasmids. Mammalian expression vectors encoding FLAG-tagged versions of human Cdc37, Cdc37D12, Cdc37D1, Cdc37D2, Harc, HarcD12, HarcD1, and HarcD2 were as previously described (*13*, *16*). Expression vectors encoding V5-tagged versions of human Cdc37 and Harc were created by excising the cDNA inserts from pEF-FLAG-Cdc37 and pEF-FLAG-Harc, respectively, with *Mlu*I and subcloning them into the corresponding site in pEF-V5. An expression vector encoding FLAG-Cdarc, a chimeric protein consisting of Val-2 to Met-164 of Cdc37 and Leu-172 to Val-337 of Harc, was generated by a two-step PCR strategy. The primers used to PCR the N-terminal Cdc37 fragment were 5′-CGACGCGT GTG GAC TAC AGC GTG TGG GAC CAC ATT GAG-3 (forward) and 5′-CAT ACC AAA ATG CTT GAT TTG TTT CTC ATA TTT TTC CAC-3 (reverse). The primers used to PCR the C-terminal Harc fragment were 5′-GTG GAA AAA TAT GAG AAA CAA ATC AAG CAT TTT GGT ATG-3 (forward) and 5′-CGACGCGT TTA TAC AGT GTC CAT CAT TTT GGG TTC ATC-3 (reverse). The two products generated were used as templates for a second PCR reaction. The product generated was digested with *Mlu*I and subcloned into the *Mlu*I site in pEF-FLAG. The same strategy was used to create pEF-FLAG-Hac37, which encodes a chimeric protein consisting of Glu-2 to Met-171 of Harc and Leu-165 to Val-378 of Cdc37. The primers used to PCR the N-terminal Harc fragment were 5′-CGACGCGT GAA CAA CCG TGG CCG CCT CCG GGA CCC TGG-3 (forward) and 5′-CAT GCC AAA GTG TCT GAT CTT TTG CTC GTA TTT TTG CAT-3 (reverse). The primers used to PCR the C-terminal Cdc37 fragment were 5′-ATG CAA AAA TAC GAG CAA AAG ATC AGA CAC TTT GGC ATG-3 (forward) and 5′-CGACGCGT TCA CAC ACT GAC ATC CTT CTC ATC GCC CGT-3 (reverse).

Cell Culture and Transfections. Human 293T were maintained in DMEM 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 24−36 h later (*13*, *16*).

Cell Lysis, Western Blotting and Immunoprecipitation. Cells were lysed directly in tissue culture dishes for 30 min with ice-cold NP-40 lysis buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 10 mM NaF, 2 mM EGTA, 1 mM DTT, 0.5% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, and *Complete* protease inhibitors). Lysates were clarified by centrifugation at 13000*g* for 10 min at 4 °C, and then protein concentrations were measured with a Bio-Rad protein assay kit. Western blotting and immunoprecipitation of cell lysates were performed by standard techniques (*13*, *16*).

HPLC Size-Exclusion Chromatography. Transfected 293T cells were lysed by dounce homogenization in lysis buffer containing 0.1% NP-40. Following clarification by centrifugation and filtration through a 0.2 μm syringe filter, a 0.2 mL aliquot of the lysate was applied to a Superose-6 column (HR 10/30, Amersham Biosciences) equilibrated with column buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 10 mM NaF, 2 mM EGTA and 10% glycerol) and elution performed at a flow rate of 0.4 mL/min, with fractions 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).

RESULTS

Dimerization of Harc. Given that purified recombinant Cdc37 is reported to form a dimer in vitro (*22*, *26*, *27*) and Harc is structurally similar to Cdc37 (*16*), we wanted to establish if Harc formed dimers when expressed in mammalian cells. To address this question, epitope tagged versions of human Harc were coexpressed in 293T cells and their interaction was assessed by performing coimmunoprecipitation assays. Specifically, expression plasmids encoding N-terminally FLAG- and V5-tagged versions of Harc (i.e. FLAG-Harc and V5-Harc; see Figure 1A) were transiently

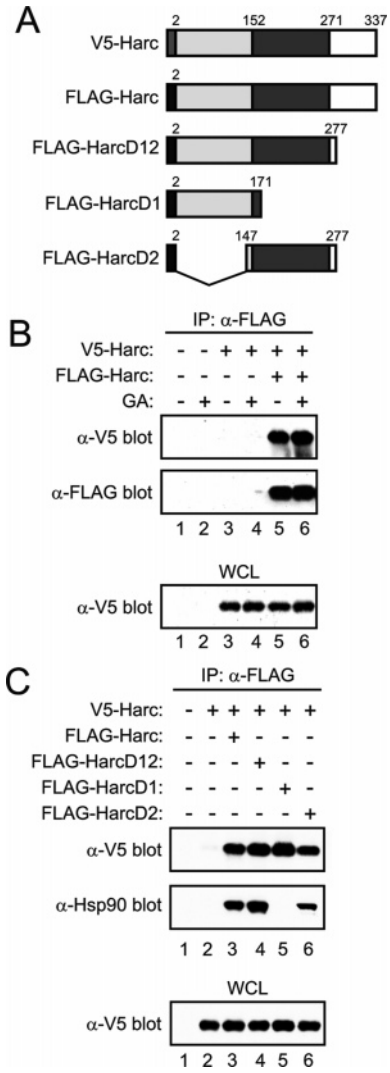

FIGURE 1: Homodimerization of Harc in mammalian cells. (A) Schematic representation of epitope-tagged versions of full-length Harc and derivatives thereof. (B) 293T cells transiently expressing V5-Harc alone (lanes 3 and 4) or together with FLAG-Harc (lanes 5 and 6) were treated with 2 μM geldanamycin (GA) or 0.1% DMSO for 2 h and then lysed. FLAG-Harc was immunoprecipitated from the cell lysates with anti-FLAG antibodies and Western blotted with anti-V5 and anti-FLAG antibodies. The whole cell lysates (WCL) were blotted with an anti-V5 antibody. (C) 293T cells transiently expressing V5-Harc alone (lane 2) or together with FLAG-Harc (lane 3), or derivates thereof (lanes 4−6), were lysed, and FLAG-tagged Harc proteins were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then blotted with anti-V5 and anti-Hsp90 antibodies.

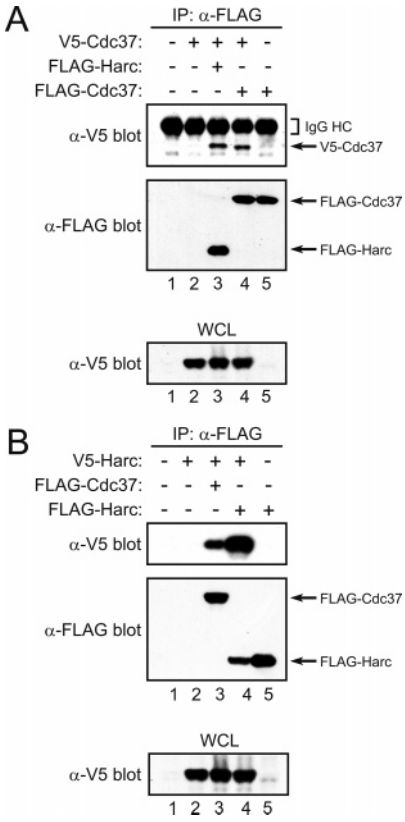

FIGURE 2: Heterodimerization of Harc with Cdc37 in mammalian cells. (A) 293T cells transiently expressing V5-Cdc37 alone (lane 2) or together with FLAG-Harc (lane 3) or FLAG-Cdc37 (lane 4) were lysed, and FLAG-Harc and FLAG-Cdc37 were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with anti-V5 and anti-FLAG antibodies. The whole cell lysates (WCL) were blotted with an anti-V5 antibody. (B) 293T cells transiently expressing V5-Harc alone (lane 2) or together with FLAG-Cdc37 (lane 3) or FLAG-Harc (lane 4) were lysed, and FLAG-Cdc37 and FLAG-Harc were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then blotted with anti-V5 and anti-FLAG antibodies.

cotransfected into 293T cells. Twenty-four hours post-transfection, the cells were treated with the Hsp90 inhibitor geldanamycin (GA) or dimethyl sulfoxide (DMSO) for 2 h. The cells were then lysed, and FLAG-tagged Harc was immunoprecipitated from the cell lysates with anti-FLAG antibodies. As shown in Figure 1B, Western blotting of the immunoprecipitates with anti-V5 antibodies revealed coimmunoprecipitation of V5-Harc with FLAG-Harc. The coimmunoprecipitation of V5-Harc with FLAG-Harc was specific as V5-Harc was not detected in anti-FLAG immunoprecipitates derived from cells that did not express FLAG-Harc (Figure 1B; lane 3 versus lane 5). Inhibition of Hsp90 with GA had no affect on the coimmunoprecipitation of V5-Harc with FLAG-Harc (Figure 1B; lane 5 versus lane 6). Treatment of the cells with GA also did not perturb the coimmunoprecipitation of Hsp90 with FLAG-Harc (data not shown).

We have previously proposed that Harc (and Cdc37) has a three-domain structure in which the middle domain binds Hsp90 (*16*). The function of the N-terminal domain of Cdc37 is to bind client protein kinases (*12*, *13*, *15*), whereas the role of the corresponding domain in Harc remains unknown. The role of the C-terminal domains of Harc and Cdc37 are also unknown. To identify the domain(s) of Harc that mediates its dimerization, various FLAG-tagged versions of Harc (see Figure 1A) were coexpressed with V5-Harc in 293T cells. Deletion of the C-terminal domain of Harc did not affect its ability to dimerize with full-length Harc (Figure 1C; lane 3 versus lane 4). Both the N-terminal and middle domains of Harc (i.e. FLAG-HarcD1 and FLAG-HarcD2, respectively) alone were capable of binding to full-length Harc (Figure 1C; lanes 5 and 6). Notably though, Hsp90 did not coimmunoprecipitate with FLAG-HarcD1 (Figure 1C; lane 5). These findings therefore suggest that both the N-terminal and middle domains contribute to the dimerization of Harc and that binding of Hsp90 to Harc is dependent on dimerization of the middle domain of Harc.

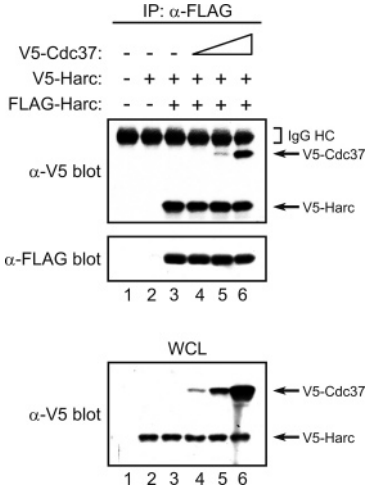

FIGURE 3: Relative dimerization potentials of Harc and Cdc37. 293T cells transiently coexpressing FLAG-Harc, V5-Harc (lanes 3−6), and increasing levels of V5-Cdc37 (lanes 4−6) were lysed, and FLAG-Harc was immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with anti-V5 and anti-FLAG antibodies. The whole cell lysates (WCL) were blotted with an anti-V5 antibody.

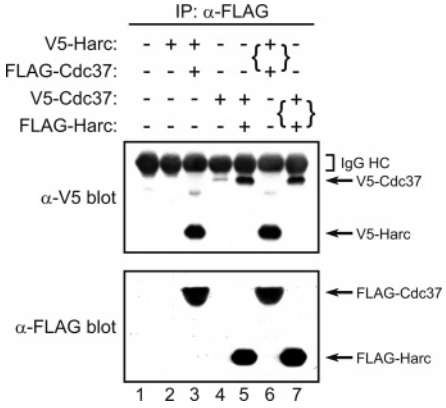

FIGURE 4: Dynamic dimerization of Harc and Cdc37. 293T cells transiently expressing V5-Harc alone (lane 2) or together with FLAG-Cdc37 (lane 3) or V5-Cdc37 alone (lane 4) or together with FLAG-Harc (lane 5) were lysed, and FLAG-Cdc37 and FLAG-Harc were immunoprecipitated with anti-FLAG antibodies. In addition, equivalent amounts of lysates from cells expressing V5-Harc or FLAG-Cdc37 alone (lane 6) and V5-Cdc37 or FLAG-Harc alone (lane 7) were mixed and incubated on ice for 2 h. FLAG-Cdc37 and FLAG-Harc were then immunoprecipitated with anti-FLAG antibodies and Western blotted with anti-V5 and anti-FLAG antibodies.

Heterodimerization of Harc with Cdc37. The ability of Harc to form homodimers promoted the idea that it could potentially heterodimerize with Cdc37 due to the high amino acid sequence similarity between the two Hsp90 cochaperones. Thus V5- and FLAG-tagged versions of Harc and Cdc37 were coexpressed in 293T cells, and their physical interaction was evaluated by performing coimmunoprecipitation assays. These assays revealed that V5-Cdc37 was capable of binding to FLAG-Harc (Figure 2A; lane 3). Again, the coimmunoprecipitation of V5-Cdc37 with FLAG-Harc was specific as V5-Cdc37 was not detected in anti-FLAG immunoprecipitates derived from cells that did not express either FLAG-Harc or FLAG-Cdc37 (Figure 2A; lane 2 versus lanes 3 and 4). The level of V5-Cdc37 that coimmunoprecipitated with FLAG-Harc was comparable to that which coimmunoprecipitated with FLAG-Cdc37 (Figure 2A; lane 3 versus lane 4). V5-Cdc37 was observed to exhibit a marginally faster electrophoretic mobility when isolated as a dimer with FLAG-Cdc37 than when complexed with FLAG-Harc (Figure 2A; lane 3 versus lane 4). This small change in mobility is most likely due to the immunoprecipitated FLAG-Cdc37 protein (which is in excess over V5-Cdc37 in the anti-FLAG immunoprecipitates) distorting the electrophoretic mobility of V5-Cdc37 (data not shown). Notably, V5-Harc was found to coimmunoprecipitate with FLAG-Cdc37 in reciprocal experiments (Figure 2B; lane 3). In this case, however, 4−5-fold more V5-Harc coimmunoprecipitated with FLAG-Harc than with FLAG-Cdc37 (Figure 2B; lane 3 versus lane 4).

Homodimerization of Harc Is Favored over Heterodimerization with Cdc37. To gain insight into the relative ability of Harc to form homodimers and heterodimerize with Cdc37, FLAG-Harc was coexpressed with a constant amount of V5-Harc and increasing levels of V5-Cdc37 in 293T cells (Figure 3). FLAG-Harc was subsequently immunoprecipitated from lysates of the cells and Western blotted with an anti-V5 antibody to detect coimmunoprecipitating V5-Harc and V5-Cdc37. No coimmunoprecipitation of V5-Cdc37 with FLAG-Harc was observed when V5-Cdc37 was expressed at levels approximately 5-fold lower than those of V5-Harc (Figure 3; lane 4). Coimmunoprecipitation of V5-Harc with FLAG-Harc was readily detected under the same conditions (Figure 3; lane 4). However, coimmunoprecipitation of V5-Cdc37 with FLAG-Harc was detected when V5-Cdc37 and V5-Harc were expressed at the same level, although coimmunoprecipitation of V5-Harc was favored over that of V5-Cdc37 (Figure 3; lane 5). Comparable coimmunoprecipitation of V5-Harc and V5-Cdc37 with FLAG-Harc was observed when V5-Cdc37 was expressed at a level approximately 10-fold greater than that of coexpressed V5-Harc (Figure 3; lane 6). Thus while Harc and Cdc37 could heterodimerize, the extent of dimerization was dictated by the relative expression levels of Harc and Cdc37.

Dimerization of Harc Is a Dynamic Process. To establish if dimeric complexes of Harc are static or dynamic, Harc and Cdc37 were either coexpressed in 293T cells or expressed separately and then admixed post cell lysis. Consistent with the data presented in Figure 2 above, Harc and Cdc37 (i.e. V5-Harc and FLAG-Cdc37 or FLAG-Harc and V5-Cdc37) formed a heterodimeric complex when coexpressed in 293T cells (Figure 4; lanes 3 and 5). Notably, though, V5-Harc coimmunoprecipitated with FLAG-Cdc37 when lysates of 293T cells expressing V5-Harc or FLAG-Cdc37 were admixed and then subjected to immunoprecipitation with anti-FLAG antibodies (Figure 4; lane 6). Likewise, V5-Cdc37 and FLAG-Harc formed a complex following their in vitro mixing (Figure 4; lane 7). Interestingly, the extent of complex formation between Harc and Cdc37 was comparable irrespective of whether they had been coexpressed in 293T cells or expressed separately and mixed post cell lysis (Figure 4; lane 3 versus lane 6 and lane 5 versus lane 7). Such findings suggest that the formation of homo- and heterodimeric complexes of Harc and Cdc37 was a highly dynamic process.

Harc and Cdc37 Exhibit Differences in Their Levels of Interaction with Hsp90. The extent to which Harc and Cdc37 interact with Hsp90 might be responsible for determining

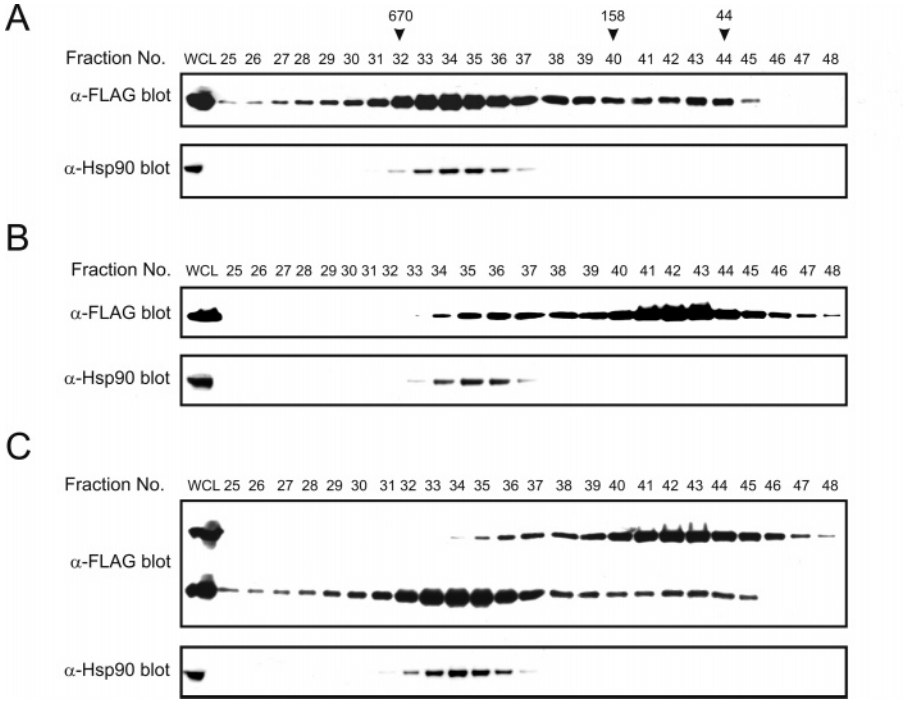

FIGURE 5: Analysis of Harc and Cdc37 by size-exclusion chromatography. 293T cells transiently expressing (A) FLAG-Harc, (B) FLAG-Cdc37, or (C) FLAG-Harc and FLAG-Cdc37 were lysed, and an aliquot (0.2 mL) of the lysates was subjected to size-exclusion chromatography on a Superose-6 column. Samples of the indicated column fractions, as well as the whole cell lysates (WCL), were subjected to Western blotting with anti-FLAG and anti-Hsp90 antibodies. The elution positions of column calibration standards (in kDa) are shown at the top.

their dimerization states. To investigate this possibility, lysates of 293T cells expressing FLAG-Harc and FLAG-Cdc37 were subjected to size-exclusion chromatography. Western blotting of the column fractions revealed that FLAG-Harc primarily (>70%) eluted in fractions 32−36, which corresponded to a molecular mass range of ∼300−600 kDa (Figure 5A). Although FLAG-Cdc37 was detected in fractions 34−36, the majority (>80%) was present in latter fractions, in particular fractions representing a molecular mass range of ∼50−150 kDa (Figure 5B). The elution profiles of FLAG-Harc and FLAG-Cdc37 were essentially unchanged upon their coexpression in 293T cells (Figure 5C). In all three cases Hsp90 principally eluted from the column in fractions 32−36 (Figure 5A−C). Anti-FLAG Western blotting of anti-Hsp90 immunoprecipitates derived from column fractions 34−36 revealed that FLAG-Harc and FLAG-Cdc37 present in these fractions was complexed with Hsp90 (data not shown). Together these findings suggested that the majority of FLAG-Harc present in 293T cells was complexed with Hsp90, whereas only a minor fraction of FLAG-Cdc37 was likewise in complex with Hsp90 under those conditions.

The N-Terminal Domain of Harc Is a Stronger Mediator of Dimerization Than That of Cdc37. In order to explore the relative abilities of the N-terminal domains of Harc and Cdc37 to promote dimerization, chimeric versions of Harc and Cdc37 (i.e. Cdarc and Hac37) were generated (see Figure 6A). Cdarc consisted of the N-terminal domain of Cdc37 and the middle and C-terminal domains of Harc, whereas Hac37 was composed of the N-terminal domain of Harc and the middle and C-terminal domains of Cdc37. We have previously shown that FLAG-Harc binds Hsp90 and Hsp70 when expressed in 293T cells (16). Notably, FLAG-Cdc37 binds Hsp90 but not Hsp70 under the same conditions (Figure 6B; lane 2). In addition to binding Hsp90, the chimeric protein FLAG-Cdarc bound Hsp70, although not to the same extent as did FLAG-Harc (Figure 6B, lane 4 versus lane 3). Significantly, though, FLAG-Hac37 exhibited the same Hsp90- and Hsp70-binding profile as FLAG-Harc (Figure 6B; lane 5). Coimmunoprecipitation experiments revealed that FLAG-Cdc37 and FLAG-Cdarc had comparable abilities to dimerize with V5-Harc when expressed in 293T cells (Figure 6C; lanes 4 and 5). By contrast, the ability of FLAG-Hac37 to dimerize with V5-Harc was significantly greater than that of FLAG-Cdc37 and FLAG-Cdarc (Figure 6C; lane 6 versus lanes 3 and 4). In fact, FLAG-Hac37 and FLAG-Harc exhibited equivalent capacities to bind V5-Harc (Figure 6C; lanes 3 and 6).

The N-Terminus of Harc Has a Greater Capacity To Promote Complex Formation with Hsp90 Than That of Cdc37. If Harc and Cdc37 only interact with Hsp90 in their dimeric states, it would be predicted that Hac37 should interact to a greater extent with Hsp90 than does Cdarc. Indeed, the data presented in Figure 6B revealed greater binding of Hsp90 to FLAG-Hac37 than FLAG-Cdarc. To examine the interaction of FLAG-Cdarc and FLAG-Hac37 with Hsp90 more closely, lysates of 293T cells transiently expressing the two proteins were subjected to size-exclusion chromatography. Western blotting of the column fractions revealed that FLAG-Hac37 eluted rather uniformly across fractions 29−44, an elution profile resembling the combined profiles of FLAG-Cdc37 and FLAG-Harc (Figure 7A versus Figure 5C). By contrast, the elution profile of FLAG-Cdarc resembled that obtained for FLAG-Cdc37 (Figure 7B versus Figure 5B). The major peak of FLAG-Cdarc centered on fractions 40−43 and a smaller peak around fractions 32−34 (Figure 7B). Analysis of the column fractions also revealed that Hsp90 primarily eluted in fractions 30−35, irrespective of whether the cells expressed FLAG-Hac37 or FLAG-Cdarc (Figure 7A and B). Likewise, the elution profile

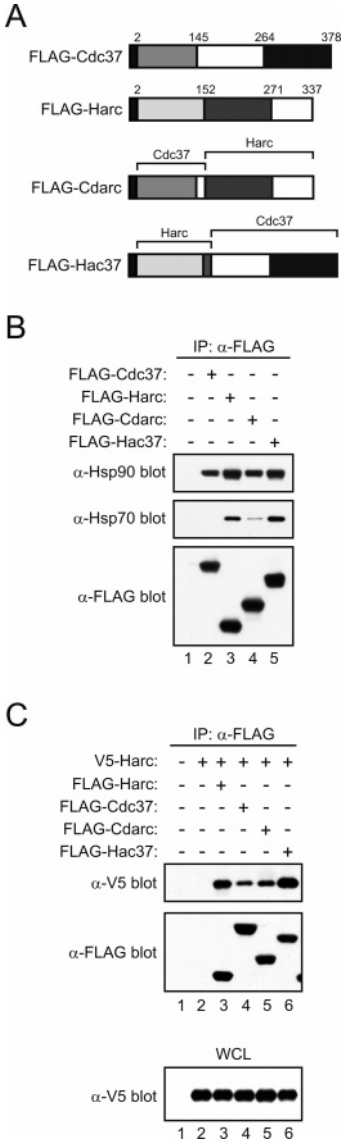

FIGURE 6: Dimerization of chimeric versions of Harc and Cdc37. (A) Schematic representation of FLAG-Cdc37, FLAG-Harc, and chimeric versions of both Cdc37 and Harc. (B) 293T cells transiently expressing FLAG-Cdc37 (lane 2), FLAG-Harc (lane 3), FLAG-Cdarc (lane 4), or FLAG-Hac37 (lane 5) were lysed, and FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with anti-Hsp90, anti-Hsp70, and anti-FLAG antibodies. (C) 293T cells transiently expressing V5-Cdc37 alone (lane 2) or together with FLAG-Harc (lane 3), FLAG-Cdc37 (lane 4), FLAG-Cdarc (lane 5), or FLAG-Hac37 (lane 6) were lysed, and FLAG-tagged proteins were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then Western blotted with anti-V5 and anti-FLAG antibodies. The whole cell lysates (WCL) were blotted with an anti-V5 antibody.

of Hsp70 was similar for lysates derived from cells expressing either FLAG-Hac37 or FLAG-Cdarc (Figure 7A and B). In both cases, Hsp70 was detected relatively uniformly throughout fractions 25−44, with a minor peak centered on fractions 38−42 (Figure 7A and B). Thus a greater proportion of FLAG-Hac37 than FLAG-Cdarc was present in column fractions that contained both Hsp90 and Hsp70.

DISCUSSION

The Hsp90 cochaperone Cdc37 controls the Hsp90-mediated folding of client proteins kinases into active conformations by both recruiting the protein kinase to the chaperone complex and arresting the ATPase cycle of Hsp90, which is necessary for the efficient loading of the protein kinase onto the chaperone (*11*−*14*, *22*, *26*). Recent in vitro studies using purified recombinant Cdc37 have revealed that it transitions between monomeric and dimeric states, with the dimeric form interacting with dimerized Hsp90 (*22*, *26*, *27*). In this report we investigated the capacity of the novel Cdc37-related Hsp90 cochaperone Harc to dimerize with both itself and Cdc37.

The results of our study indicated that Harc primarily existed in a dimeric state when expressed in mammalian cells. Functional dissection of Harc revealed that both the N-terminal and middle Hsp90-binding domains contributed to its dimerization (Figure 1). Significantly though, while the N-terminal domain of Harc was capable of forming a complex with full-length Harc, Hsp90 was not present in the same complex. This indicates that dimerization of the middle domain of Harc is required for Hsp90 binding and hence Harc-Hsp90 heterocomplexes likely consist of dimerized Harc bound to an Hsp90 dimer. Cdc37 is also thought to bind to Hsp90 dimers in a dimeric state, whereby the interaction of the middle domain of Cdc37 with Hsp90 inhibits the ATPase activity of the chaperone (*22*, *26*). Although not experimentally determined here, the middle domain of Harc may likewise regulate the ATPase activity of Hsp90 and in doing so control the loading of client proteins onto Hsp90.

Analogous to Harc, homodimerization of Cdc37 is mediated by both its N-terminal and middle domains (*22*). Siligardi et al. have reported that the affinity of the interaction between purified monomers of the N-terminal domain of human Cdc37 was approximately 6-fold lower ($K_d \sim 970$ μM) than that between monomers of the combined middle and C-terminal domains ($K_d \sim 165$ μM) (*22*). We observed that the N-terminal and middle domains of Harc exhibited similar abilities to homodimerize with full-length Harc when coexpressed in 293T cells. As amino acid sequence alignments of human Harc and Cdc37 revealed 62% identity between their middle domains (*16*), it is likely that these domains possess similar capacities to dimerize. Consequently, our findings imply that the N-terminal domain of Harc has a greater intrinsic potential for dimerization than the N-terminal domain of Cdc37. Indeed, replacement of the N-terminal domain of Harc with the equivalent domain from Cdc37 led to a decrease in Harc dimerization, whereas exchanging the middle and C-terminal domains of Harc with those from Cdc37 had no apparent effect on dimerization (see Figure 6).

Because we examined the ability of Harc to dimerize in total cell lysates, it is possible that other cellular proteins influenced this process, in particular Hsp90. Fractionation of cell lysates by size-exclusion chromatography revealed that the majority of Harc coeluted with Hsp90, whereas only a relatively minor fraction of Cdc37 coeluted with Hsp90 under comparable conditions. Coimmunoprecipitation experiments revealed that Harc and Cdc37 which coeluted with Hsp90 were physically complexed with the chaperone (data not shown). Significantly, we have previously shown that the middle domains of Harc and Cdc37 have similar abilities to bind Hsp90 in 293T cells (*16*). Thus taken together, our present findings indicate that dimerization of Harc "drives"

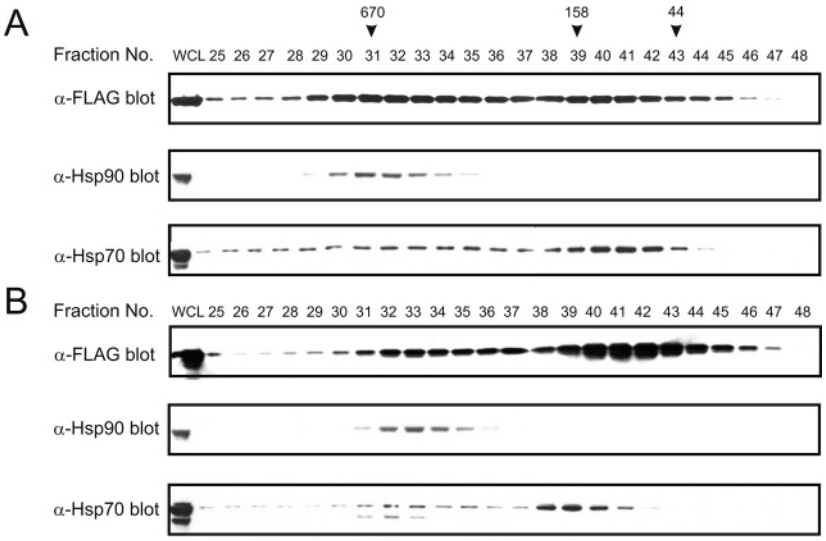

FIGURE 7: Analysis of Hac37 and Cdarc by size-exclusion chromatography. 293T cells transiently expressing (A) FLAG-Hac37 or (B) FLAG-Cdarc were lysed, and an aliquot (0.2 mL) of the lysates was subjected to size-exclusion chromatography on a Superose-6 column. Samples of the indicated column fractions, as well as the whole cell lysates (WCL), were subjected to Western blotting with anti-FLAG, anti-Hsp90, and anti-Hsp70 antibodies. The elution positions of column calibration standards (in kDa) are shown at the top.

its interaction with Hsp90 rather than its dimerization being "driven" by its interaction with Hsp90. Supporting this notion is our finding that a greater proportion of Hac37 (which is composed on the N-terminal domain of Harc and the middle and C-terminal domains of Cdc37; see Figure 6A) coeluted with Hsp90 from the size-exclusion column than did Cdarc (see Figure 7). However, we cannot exclude the possibility that the interaction of dimeric Harc with Hsp90 dimers may in fact stabilize its dimeric state due to more extensive ongoing interactions with Hsp90.

Hsp70 also appears to exert an influence on the dimerization state of Harc. Both Harc and Hac37 were found to efficiently bind Hsp70, whereas Cdc37 and Cdarc either did not bind Hsp70 or only bound low levels of the chaperone, respectively (Figure 6). This indicates that the N-terminal domain of Harc represents an Hsp70-binding domain or that it facilitates the formation of a complex capable of interacting with Hsp70. Our data are therefore consistent with a model in which the N-terminal domain of Harc enhances dimerization by facilitating the interaction of Hsp70 with heterocomplexes of dimerized Harc and Hsp90, which in turn increases the stability of the complex.

It is not yet clear if our observed in vitro heterodimerization of Harc with Cdc37 is biologically relevant. Cdc37 plays a crucial role in controlling the Hsp90-mediated folding of a range of protein kinases (e.g. Cdk4, Raf-1, and Src-family kinases) into functional conformations. As this process is likely to be governed to some extent by the expression level of Cdc37 (*12*, *13*), changes in the expression of either Cdc37 or Harc could alter the cellular levels of homo- and heterodimers of Harc and Cdc37. It is therefore tempting to speculate that Harc could contribute to the regulation of the folding of Cdc37-dependent protein kinases into active conformations via dimerization with Cdc37. Preliminary experiments have indicated that heterodimeric complexes of Harc and Cdc37 do not bind the Src-family kinase Hck (unpublished observation). Thus heterodimerization of Harc with Cdc37 could potentially influence the Hsp90-mediated folding of Cdc37-dependent protein kinases by usurping Cdc37. On the basis of this model, the relative expression levels (and possibly subcellular localizations) of Cdc37 and Harc in cells may dictate the ability of Cdc37 to promote the folding of protein kinases into functional conformations by Hsp90. Future studies should seek to address this potentially important regulatory function of Harc by demonstrating a direct causal relationship between the expression levels of Cdc37-dependent protein kinases and Harc.

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