Identification and Characterization of Harc, a Novel Hsp90-associating Relative of Cdc37*

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Although little is known about the precise mechanisms by which the molecular chaperone Hsp90 recognizes its client proteins, Cdc37 has been shown to play a critical role in the targeting of Hsp90 to client protein kinases. Described here is the identification and characterization of a novel 35-kDa human protein that is 31% identical to Cdc37. We have named this novel protein Harc (Hsp90-associating relative of Cdc37). Northern blot analysis revealed the presence of Harc mRNA in several human tissues, including liver, skeletal muscle, and kidney. Biochemical fractionation and immunofluorescent localization of epitope-tagged Harc (i.e. FLAG-Harc) indicated that it is present in the cytoplasm of cells. FLAG-Harc binds Hsp90 but unlike Cdc37 does not bind Src family kinases or Raf-1. Mapping experiments indicate that the central 120 amino acids of both Harc and Cdc37 constitute a Hsp90-binding domain not described previously. FLAG-Harc is basally serine-phosphorylated and hyperphosphorylated when co-expressed with an activated mutant of the Src family kinases. Described here is the identification and characterization of a novel 35-kDa human protein that is 31% identical to Cdc37. We have named this novel protein Harc (Hsp90-associating relative of Cdc37). Northern blot analysis revealed the presence of Harc mRNA in several human tissues, including liver, skeletal muscle, and kidney. Biochemical fractionation and immunofluorescent localization of epitope-tagged Harc (i.e. FLAG-Harc) indicated that it is present in the cytoplasm of cells. FLAG-Harc binds Hsp90 but unlike Cdc37 does not bind Src family kinases or Raf-1. Mapping experiments indicate that the central 120 amino acids of both Harc and Cdc37 constitute a Hsp90-binding domain not described previously. FLAG-Harc is basally serine-phosphorylated and hyperphosphorylated when co-expressed with an activated mutant of the Src family kinases. Notably, FLAG-Harc forms complexes with Hsp90, Hsp70, p60Hop, immunophilins, and an unidentified p22 protein but not with the Hsp90 co-chaperone p23. Thus Harc likely represents a novel participant in Hsp90-mediated protein folding, potentially targeting Hsp90 to Hsp70-client protein heterocomplexes.

The 90-kDa heat shock protein (Hsp90) is an abundant and highly conserved molecular chaperone that is essential for eukaryotic cell survival (1, 2). Hsp90 has a relatively restricted range of client proteins, including steroid receptors (reviewed in Ref. 3), p53 (4), protein kinases (5–8), nitric-oxide synthase (9), and telomerase (10). Prior studies have demonstrated that Hsp90 contains a nucleotide-binding site within its N-terminal domain (11, 12) and intrinsic ATPase activity that is necessary for its in vivo function (13, 14). Accordingly, the chaperoning activity of Hsp90 is likely regulated, at least in part, by cycles of ATP binding and hydrolysis.

Investigation of the mechanism of Hsp90-mediated protein folding, principally on the folding of steroid receptors (e.g. glucocorticoid and progesterone receptors) into ligand-binding competent states has revealed that it acts in concert with Hsp70, co-chaperones, and immunophilins (3). The first step in the paradigm derived from these studies is the association of the ATP-bound conformation of Hsp70 with the steroid receptor, followed by the Hsp40-facilitated conversion of Hsp70 to its ADP-bound conformation (15, 16). p60Hop, which preferentially binds the ADP-bound conformations of Hsp70 and Hsp90 (17), then stabilizes the interaction of Hsp90 with the Hsp70-receptor heterocomplex (15, 16, 18). Further maturation of the complex is accompanied by decreases in the levels of associated Hsp70 and p60Hop and an increase in the level of the Hsp90 co-chaperone p23 (15, 16). Although p23 binds directly to the ATP-bound conformation of Hsp90 (19), its function in the folding process is still unclear. Studies by both Pratt and co-workers (20) and Toft and co-workers (16) suggest that p23 stabilizes the mature receptor in a conformation competent to bind hormone. By contrast, Young and Hartl (21) have recently reported that p23 enhances the release of a peptide substrate encompassing the ligand-binding domain of the glucocorticoid receptor. Furthermore, the activity of exogenous steroid receptors in the yeast Saccharomyces cerevisiae is essentially unaffected by deletion of SBA1, the yeast ortholog of mammalian p23 (22, 23). High molecular mass immunophilins, such as FKBP52 and CyP-40, are additional components of mature Hsp90-receptor heterocomplexes (24, 25). Tetratricopeptide repeat (TPR) domains in immunophilins and a common TPR-acceptor site on Hsp90 facilitate their direct association (25). As a consequence, competition between immunophilins for binding to Hsp90 results in the formation of distinct Hsp90-receptor-immunophilin heterocomplexes (24–26). Although immunophilins possess peptidy1-propyl isomerase activity (27), it is uncertain what function they serve in the Hsp90-receptor heterocomplex. One possibility is that the bound immunophilin regulates the conformational state of the steroid receptor, whereas another, proposed by Pratt and co-workers (28), is that the immunophilin might target steroid receptors to the nucleus.

Despite significant advances in the understanding of the mechanisms of Hsp90-mediated protein folding, relatively little is known about precisely how Hsp90 recognizes its client proteins. Recent studies, however, have revealed a role for Cdc37 in the recognition of client protein kinases by Hsp90. The cdc37 gene was first identified in a mutant strain of S. cerevisiae with a G1 cell cycle arrest phenotype (29). Subsequent analysis revealed that the function of several protein kinases (e.g. Cdc28 and MPS1 kinase) is impaired in yeast cdc37 mutants (30, 31). Mutations in Drosophila melanogaster Cdc37 compromise signaling by the sevenless receptor tyrosine kinase (32). Although yeast and mammalian Cdc37 share only ~20% sequence identity, several studies have shown that overexpression of mammalian Cdc37 is sufficient to stabilize the structure and activ-
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ity of Cdk4 (33), Raf-1 (34), and a temperature-sensitive mutant of the Src family kinase Hck (referred to as tsHck499F) (35). Moreover, these studies demonstrated that Cdc37 facilitated the association of the protein kinases with Hsp90 (34, 35). Its ability to bind both protein kinases and Hsp90 has led to the proposal that Cdc37 acts as a protein kinase-targeting co-chaperone of Hsp90. Notably though, Kimura et al. (36) have shown that yeast Cdc37 possesses in vitro toward some proteins (e.g. β-galactosidase and casein kinase II). Additionally, we have found that a C-terminal truncation mutant of Cdc37 that can bind tsHck499F but not Hsp90 still had a limited ability to stabilize the catalytic activity of the mutant protein kinase (35). These observations argue that rather than acting solely as a passive adapter protein to target Hsp90 to its client protein kinases, Cdc37 may also be a chaperone in its own right.

In this study, we have sought to identify novel Cdc37-related proteins that might serve similar roles to Cdc37. Described here is the identification of a novel 35-kDa human protein that is 31% identical to Cdc37 and 62% identical over a central region of 120 amino acids that represents a Hsp90-binding domain not described previously. We have named this protein Harc (Hsp90-associated relative of Cdc37). Unlike Cdc37 however, Harc does not bind Src family kinases or Raf-1. Harc is a cytoplasmic, phosphoprotein that exists in a complex with Hsp90, Hsp70, p60Hsp, immunophillin, and an unidentified p22 protein but not with the Hsp90 co-chaperone p23.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium and supplements were from Life Technologies, Inc. Fetal calf serum was from CSL, Ltd. (Melbourne, Australia). Pfu DNA polymerase was obtained from Stratagene. The anti-FLAG (DYKDDDDK)-tagged versions of full-length human Harc, whether Cdc37 might represent the founding member of a family of protein kinase targeting co-chaperones of Hsp90, sequence data bases were searched for novel sequences that are related to human Cdc37. One such cDNA sequence, with the accession number AK000646, was identified in the GenBank® data base. The identified cDNA is 1542 nucleotides in length and contains an open reading frame encoding a human protein containing 357 amino acids (which we will subsequently refer to as Harc) and with a predicted molecular mass of 38.7 kDa (Fig. 1). Alignment of the amino acid sequence of human Harc with that of human Cdc37 revealed that the two proteins are 31% identical and that each is comprised of three domains (Fig. 1). Domains 1, 2, and 3 of human Harc are 23, 62, and 9% identical, respectively, with the corresponding domains of human

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Cdc37. Identities between human Harc and murine, chicken, and *D. melanogaster* Cdc37 are 31, 30, and 21%, respectively. Significantly, domain 2 of human Harc (i.e. Glu-152 to Arg-271) is 63, 61, and 43% identical to domain 2 of murine, chicken, and *D. melanogaster* Cdc37. A cDNA sequence encoding murine Harc (accession number AK012312) was recently submitted to GenBank™. Murine and human Harc are 88% identical at both the nucleotide and protein levels, with domain 2 being 97% identical (Fig. 1). Domain 2 of human and murine Harc are 88% identical, whereas domain 3 of both proteins are 72% identical. Expressed sequence tags for bovine, porcine, and rat Harc (accession numbers BE237054, BF444303, and BF552510) have also been identified in the GenBank™ data base. Sequence identities between these expressed sequence tags and human Harc are in the order of 80% or greater. Orthologs of mammalian Harc were not identified in the *D. melanogaster*, *Caenorhabditis elegans*, or *S. cerevisiae* genomes.

Comparison of the protein sequences of human Harc predicted by the cloned cDNA and genomic sequence data at the National Center for Biotechnology Information (accession number XM_005516) and Celera (accession number hCT22486) revealed two amino acid differences. Both sets of genomic data predict glutamic acid rather than glycine (i.e. GAG for GGG) at position 17 and asparagine for serine (i.e. AAU for AGU) at position 305. The predicted protein sequence of murine Harc contains glycine at position 17 and asparagine at position 304 (equivalent to position 305 in human Harc) (Fig. 1). It therefore seems likely that the two amino acid differences in the predicted protein sequences of human Harc arise from polymorphisms in the *HARC* gene. The *HARC* gene resides at position p24.1 on human chromosome 9, whereas the *CDC37* gene resides on chromosome 19 at position p13.13. Two putative *CDC37* pseudogenes were identified on chromosome 16, whereas a third was identified on chromosome 5. The *HARC* and *CDC37* genes are composed of seven and eight exons, respectively. The boundaries of all seven exons in the *HARC* gene and six of seven exon boundaries in the *CDC37* gene are in identical positions in the alignment of their respective amino acid sequences (Fig. 1).

### Tissue Distribution and Subcellular Localization of Harc—

The Harc clone was isolated from a cDNA library constructed using RNA derived from the human signet ring cell gastric carcinoma cell line Kato-III. To determine in which normal human tissues Harc is expressed, Northern blot analysis of poly(A)⁺ RNA from various tissues was performed. Because Harc is encoded by a message indistinguishable in size from that of Cdc37, we eliminated any potential for cross-hybridization by utilizing a Harc-specific probe corresponding to the last 510 base pairs of the Harc cDNA. Harc mRNA was present primarily in liver and skeletal muscle, with considerably lower levels detected in kidney, heart, brain, and placenta (Fig. 2A). *Cdc37* exhibited a markedly less restricted pattern of expression (data not shown), a finding consistent with an earlier report (33). To be able to detect Harc in transfected cells, a mammalian expression vector was constructed such that the FLAG epitope is attached to the N terminus of Harc. Fractionation of transiently transfected 293T cells into S100 (representing the cytosol) and P100 (representing membranes) fractions revealed that FLAG-Harc has a molecular mass of ~35 kDa and is found exclusively in the cytosolic fraction (Fig. 2B). Likewise, FLAG-Cdc37 was also found exclusively within the cytosolic fraction of transfected 293T cells (Fig. 2B). Examination of the subcellular localizations of FLAG-Harc and FLAG-Cdc37 in intact 293T cells by indirect immunofluorescence revealed a somewhat punctate pattern of cytoplasmic staining for both proteins (Fig. 2C).
revealed that FLAG-Harc had eluted primarily in fractions 41–45, representing a molecular mass range of ~220–420 kDa (Fig. 3B). Hsp90 was found to have eluted in essentially the same fractions as had FLAG-Harc (Fig. 3B). Anti-Hsp90 Western blotting of anti-FLAG immunoprecipitates derived from fractions 41, 43, and 45 revealed that FLAG-Harc and Hsp90 present in those fractions were physically associated (Fig. 3C). Taken together, these findings indicate that FLAG-Harc is a Hsp90-binding protein and that when expressed in 293T cells the majority of FLAG-Harc is complexed with endogenous Hsp90.

Harc Contains a Centrally Located Hsp90-binding Domain—To identify the region of Harc that mediates its association with Hsp90, FLAG-tagged full-length Harc and two C-terminal truncation mutants, FLAG-HarcD12 and FLAG-HarcD1 (Fig. 4A), were transiently expressed in 293T cells (Fig. 4B). The FLAG-tagged proteins were then immunoprecipitated from lysates of the transfected cells and Western blotted with an anti-Hsp90 antibody. Deletion of domain 3 had no effect on the ability of FLAG-Harc to associate with Hsp90, whereas deletion of domains 2 and 3 abolished Hsp90 binding (Fig. 4B). Similarly, deletion of domain 3 did not perturb the association of Hsp90 with FLAG-Cdc37, although binding was abolished upon deletion of both domains 2 and 3 (Fig. 4B). The light chain of the anti-FLAG antibody used in the immunoprecipitation reactions obscured the presence of FLAG-Cdc37D1 in the anti-FLAG Western blot shown in Fig. 4B. These findings suggest that amino acids in domain 2 of Harc and Cdc37 are required for their association with Hsp90. To directly test this hypothesis, expression vectors encoding domain 2 of Harc and Cdc37 (Fig. 4C) were transiently expressed in 293T cells (Fig. 4D). Anti-Hsp90 Western blotting of anti-FLAG immunoprecipitates derived from lysates of the transfected cells revealed that domain 2 of both Harc and Cdc37 was capable of binding Hsp90 (Fig. 4D). Because these findings indicated that Harc and Cdc37 use equivalent domains to mediate their association with Hsp90, we next sought to establish whether their binding to Hsp90 was mutually exclusive. Specifically, Hsp90-FLAG-Harc heterocomplexes were immunoprecipitated from lysates of transfected cells with anti-FLAG beads and then Western blotted with an anti-Cdc37 antibody (Fig. 4E). The absence of Cdc37 in Hsp90-FLAG-Harc heterocomplexes was consistent with a mutually exclusive model for the binding of Harc and Cdc37 to Hsp90 (Fig. 4E).

Harc Does Not Bind Src Family Kinases or Raf—We have recently shown that FLAG-Cdc37 is capable of binding the temperature-sensitive Hck mutant tsHck499F and dramatically enhancing its catalytic activity by recruiting the protein kinase to Hsp90 (35). The ability of FLAG-Harc to similarly bind this protein kinase was tested by transiently co-expressing it with tsHck499F in 293T cells. Although tsHck499F co-immunoprecipitated with FLAG-Cdc37, its co-immunoprecipitation with FLAG-Harc was not detected (Fig. 5). Similarly, neither Src527F nor Raf-1 co-immunoprecipitated with FLAG-Harc, although both protein kinases co-immunoprecipitated with FLAG-Cdc37 (data not shown). Additionally, co-expression of FLAG-Harc with tsHck499F did not enhance the cata-
isoform of FLAG-Harc was estimated to be 3-fold (Fig. 6). The increase in phosphorylation of the faster migrating isoform of FLAG-Harc was detected following its co-expression with Hck499F (Fig. 6). However, two phosphorylated isoforms of FLAG-Harc were detected following its co-expression with Hck499F, and molecular mass standards (in kDa) are indicated on the right.

**FIG. 4.** Mapping the Hsp90-binding domain of Harc and Cdc37. A, schematic representation of full-length and truncated versions of FLAG-Harc and FLAG-Cdc37. B, control transfected 293T cells (lane 1) or cells transiently expressing FLAG-Harc (lane 2), FLAG-HarcD12 (lane 3), FLAG-HarcD1 (lane 4), FLAG-Cdc37 (lane 5), or FLAG-Cdc37D12 (lane 6) were lysed and the FLAG-tagged proteins immunoprecipitated (IP) with anti-FLAG beads. The immunoprecipitates were then sequentially Western blotted with anti-Hsp90 and anti-FLAG antibodies. The positions of molecular mass standards (in kDa) and the heavy (Ab H.C.) and light (Ab L.C.) chains of the anti-FLAG antibody are indicated on the right. C, schematic representation of FLAG-HarcD2 and FLAG-Cdc37D2 mutants. D, control transfected 293T cells (lane 1) or cells transiently expressing FLAG-Harc (lane 2), FLAG-HarcD2 (lane 3), FLAG-Cdc37 (lane 4), or FLAG-Cdc37D2 (lane 5) were lysed, and the FLAG-tagged proteins were immunoprecipitated with anti-FLAG beads. The immunoprecipitates were then sequentially Western blotted with anti-Hsp90 and anti-FLAG antibodies. E, control transfected 293T cells (−) or cells expressing FLAG-Harc (+) were lysed, and FLAG-Harc was immunoprecipitated (IP) with anti-FLAG beads. The immunoprecipitates were then sequentially Western blotted with anti-Hsp90, anti-Cdc37, and anti-FLAG antibodies. The whole cell lysates (WCL) were Western blotted with anti-Hsp90 and anti-Cdc37 antibodies.

**FIG. 5.** Analysis of the protein kinase binding activity of FLAG-Harc. 290T cells transiently expressing FLAG-Harc or FLAG-Cdc37 alone or together with tsHck499F were lysed and FLAG-tagged Harc and Cdc37 immunoprecipitated (IP) with anti-FLAG beads. The immunoprecipitates were then sequentially Western blotted with anti-Hck, anti-Hsp90, and anti-FLAG antibodies. The positions of the two isoforms of Hck499F (p59 and p56) and molecular mass standards (in kDa) are indicated on the right. The whole cell lysates (WCL) were blotted with an anti-Hck antibody.

lytic activity of the protein kinase or its association with endogenous Hsp90 (data not shown).

**Harc Is a Phosphoprotein**—Two isoforms of FLAG-Harc were detected following its co-expression with tsHck499F in 293T cells (Fig. 5), suggesting that it had been subjected to post-translational modification (e.g. phosphorylation). To directly test whether FLAG-Harc is phosphorylated when co-expressed with Hck499F, FLAG-Harc was immunoprecipitated from 293T cells that had been metabolically labeled with [32P]orthophosphate. Even in the absence of co-transfected Hck499F, FLAG-Harc was found to be phosphorylated (Fig. 6A). However, two phosphorylated isoforms of FLAG-Harc were detected following its co-expression with Hck499F (Fig. 6A). The increase in phosphorylation of the faster migrating isoform of FLAG-Harc was estimated to be 3-fold (Fig. 6A). A phosphoprotein with a molecular mass of ~90 kDa and that co-migrated with Hsp90 was detected in the immunoprecipitates irrespective of whether FLAG-Harc had been co-expressed with Hck499F (Fig. 6A). Phosphoamino acid analysis of the immunoprecipitated FLAG-Harc isoforms revealed that the faster migrating isoform is basally serine-phosphorylated (Fig. 6B). Surprisingly, both isoforms of FLAG-Harc exhibited increased levels of phosphoserine and only low levels of phosphotyrosine upon co-expression with Hck499F (Fig. 6B). These findings suggested that rather than Hck directly phosphorylating FLAG-Harc, a downstream effector serine kinase of Hck mediated the phosphorylation of FLAG-Harc. Phosphoamino acid analysis of the co-immunoprecipitating the 90-kDa phosphoprotein revealed the presence of only phosphoserine in the protein (data not shown). To demonstrate that Hck-induced phosphorylation of FLAG-Harc accounts for the appearance of the slower migrating isoform, FLAG-Harc was first immunoprecipitated from lysates of transfected cells. One half of the immunoprecipitate was then incubated in the presence of calf intestinal alkaline phosphatase (CIP), whereas the other half was incubated in CIP reaction buffer lacking the phosphatase. Subsequent Western blotting with an anti-FLAG antibody revealed that treating phosphorylated FLAG-Harc with CIP resulted in the disappearance of the slower migrating isoform, FLAG-Harc was first immunoprecipitated from lysates of transfected cells. One half of the immunoprecipitate was then incubated in the presence of calf intestinal alkaline phosphatase (CIP), whereas the other half was incubated in CIP reaction buffer lacking the phosphatase. Subsequent Western blotting with an anti-FLAG antibody revealed that treating phosphorylated FLAG-Harc with CIP resulted in the disappearance of the slower migrating isoform and a concomitant increase in the abundance of the faster migrating FLAG-Harc isoform (Fig. 6C). By contrast, incubation in reaction buffer lacking CIP had no effect on the abundance or electrophoretic mobility of either FLAG-Harc isoform (Fig. 6C). To ascertain whether Hck-induced phosphorylation of FLAG-Harc might influence its association with Hsp90, the ability of the two FLAG-Harc isoforms to co-immunoprecipitate with Hsp90 was determined. As shown in Fig. 6D, both isoforms of FLAG-Harc associated to comparable extents with Hsp90. Similarly, both isoforms of FLAG-Harc co-eluted with Hsp90 when the S100 fraction of 293T cells co-expressing FLAG-Harc and Hck499F was subjected to HPLC size exclusion chromatography (data not shown). No redistribution of FLAG-Harc from the cytosolic to the membrane fraction was observed upon its co-expression with Hck499F (data not shown).

**Complex Formation between FLAG-Harc, Hsp90, Hsp70, p60Hop, and Immunophilins**—To establish whether Harc...
associates with other cellular proteins, FLAG-Harc was immunoprecipitated from 293T cells that had been metabolically labeled with $^{35}$S]methionine. Autoradiography of the immunoprecipitates revealed that in addition to binding Hsp90, FLAG-Harc also bound at least two other $^{35}$S-labeled proteins, namely p60 and p22 (Fig. 7A). However, these two $^{35}$S-labeled proteins were not detected in anti-FLAG immunoprecipitates derived from cells expressing FLAG-Cdc37 (data not shown). Because p60Hop and the Hsp90 co-chaperone p23, which are 60 and 23 kDa in size, respectively, are known to bind Hsp90 (19, 39), they represented likely candidates for the $^{35}$S-labeled p60 and p22 proteins. Western blotting of the immunoprecipitates with anti-p60Hop and anti-p23 antibodies revealed the presence of p60Hop but not p23 in the anti-FLAG immunoprecipitate derived from cells expressing FLAG-Harc (Fig. 7A and data not shown). Authentic p23 exhibited a slightly slower electrophoretic mobility than did $^{35}$S-labeled p22 under the SDS-PAGE conditions used (data not shown). However, because p23 preferentially binds the ATP-bound conformation of Hsp90 (19), we re-examined the possibility that p23 might associate with FLAG-Harc by both lysing the cells and performing the immunoprecipitation reactions in the presence of ATP. Again, p23 was not detected in anti-FLAG immunoprecipitates derived from cells expressing FLAG-Harc (data not shown). Significantly though, only very low levels of p23 were detected in anti-Hsp90 immunoprecipitates (data not shown). However, in reciprocal immunoprecipitation reactions Hsp90, but not FLAG-Harc, was detected in anti-p23 immunoprecipitates (Fig. 7B). By contrast, p60Hop was detected in anti-FLAG, anti-Hsp90, and anti-Hsp70 immunoprecipitates but not in anti-p23 immunoprecipitates (Fig. 7B). Notably, FLAG-Harc was detected in both anti-Hsp90 and anti-Hsp70 immunoprecipitates, whereas Hsp90 and Hsp70 were detected in anti-FLAG immunoprecipitates derived from cells expressing FLAG-Harc but not in those derived from cells that had been transfected with empty vector (Fig. 7B). Association of the immunophilins FKBP52 and Cyp40 with FLAG-Harc was also investigated. As shown in Fig. 7B, both FKBP52 and Cyp40 were detected in anti-FLAG immunoprecipitates derived from cells expressing FLAG-Harc, as well as in anti-p23 immunoprecipitates. However, neither immunophilin was observed in anti-Hsp90 or anti-Hsp70 immunoprecipitates (Fig. 7B). The absence of FKBP52, Cyp40, and p23 in anti-Hsp90 immunoprecipitates suggests that the anti-Hsp90 antibody used in this study (Affinity BioReagents, PA3-013) does not efficiently immunoprecipitate Hsp90 complexed with these proteins, because Hsp90 was detected in reciprocal immunoprecipitation reactions using anti-FKBP52 or anti-p23 antibodies (Fig. 7B and data not shown).

**DISCUSSION**

In the present study, we sought to identify novel proteins that are related to Cdc37, because such proteins might serve an analogous function and act as targeting co-chaperones of Hsp90. We describe here the identification and characterization of Harc, a novel 35-kDa human protein that is 31% identical to human Cdc37. Further searches, including that of the human genome data bases, failed to identify additional Cdc37-related proteins, although three putative CDC37 pseudogenes were identified in the human genome. Alignment of the human Harc protein sequence with that of human Cdc37 suggests that both proteins are comprised of three domains. Additionally, analysis of their genomic sequences suggests that the Harc gene arose from a duplication of the CDC37 gene. The boundaries of all seven exons in the Harc gene and six of seven exon boundaries in the CDC37 gene are in identical positions in the alignment of their respective amino acid sequences. Intriguingly, the Harc cDNA does not contain nucleotides encoding amino acids equivalent to those encoded by the penultimate exon of the CDC37 gene. However, amino acids encoded by the final exon of the Harc gene share identity, albeit limited, with amino acids encoded by the last exon of the CDC37 gene. The absence of sequences encoding amino acids with identity to those encoded by exon 7 of the CDC37 gene is not unique to the human Harc cDNA clone because a murine cDNA clone and expressed sequence tags for bovine, porcine, and rat Harc also lack such sequences. The absence of this sequence in the human Harc cDNA is therefore unlikely to be the consequence of alternative splicing of the Harc mRNA. A more likely explanation is that the exon was deleted or rearranged during or subsequent to the duplication event. Nucleotide diversity between these regions of the Harc and CDC37 genes was too great to allow us to determine whether a nonfunctional exon is present in the human Harc gene. Although orthologs of mammalian Cdc37 can be identified in the D. melanogaster, C. elegans, and S. cerevisiae ge-
homogenization in the presence of 4 mM ATP and 20 mM sodium with control vector (ΔH11002) or pEF-FLAG Harc (ΔH11001) were metabolically labeled with [35S]methionine for 4 h then lysed. Cells transiently transfected with control vector (-) or pEF-FLAG Harc (ΔH11001) were metabolically labeled with [35S]methionine for 4 h then lysed.

This suggests that the duplication of the homologues, no such orthologs of mammalian Harc were identified. Sequencing of the gene that gave rise to the HARC gene occurred during vertebrate evolution. Sequencing of the Danio rerio genome should provide insight as to whether this was a relatively early or late event in vertebrate evolution.

Expression of an epitope (e.g. FLAG)-tagged form of human Harc in 293T cells resulted in its physical association with endogenous Hsp90. Significantly, the association of Hsp90 with FLAG-Harc was not adversely affected by geldanamycin, a specific inhibitor of Hsp90. Geldanamycin inhibits Hsp90 by competitively binding to its nucleotide-binding site, thereby locking Hsp90 into an inactive conformation (11, 12, 40). Thus the lack of an inhibitory effect of geldanamycin on the binding of Hsp90 to FLAG-Harc is consistent with Harc being a co-chaperone of Hsp90 rather than a client protein. The majority of transfected FLAG-Harc was found in a high molecular mass complex with endogenous Hsp90. Harper and co-workers (33) have similarly reported that the majority of Cdc37 is present in a high molecular mass complex with Hsp90 in NIH3T3 fibroblasts. In view of the almost stoichiometric association of FLAG-Harc with Hsp90, it is tempting to speculate that endogenous Harc may almost exclusively exert its biological function in concert with Hsp90.

Domain 2 represents the region of greatest identity between Harc and Cdc37. Significantly, this domain was found to be both necessary and sufficient for Harc and Cdc37 to bind Hsp90, indicating that the central 120 amino acids of these two proteins constitute a novel Hsp90-binding domain. Tertiary structure prediction using the GenTHREADER program (41) was unable to identify the protein fold of Hsp domain 2, although secondary structure analysis, using the PSIpred program (41), suggests the domain is comprised primarily of α-helices. Pattern and sequence profile searches of functionally annotated domain data bases (e.g. InterPro, Ref. 42) were performed in an attempt to ascribe functions to Harc domains 1 and 3. The searches, however, failed to identify any relationship between these two domains and the protein domains described previously.

The binding site for Cdc37 on Hsp90 is thought to lie topologically adjacent to the TPR-acceptor site that mediates the binding of p60Hop and immunophilins to Hsp90 (43). This conclusion was based on the finding that although Cdc37 does not contain a TPR domain, it competed with intact TPR domain-containing proteins, but not a protein fragment containing a TPR domain, for binding to Hsp90 (43). Given that Harc and Cdc37 utilize equivalent Hsp90-binding domains (i.e. domain 2) to facilitate their association with Hsp90, it seems highly likely that Harc binds to the same site on Hsp90 as does Cdc37. This hypothesis predicts that their binding to Hsp90 would be mutually exclusive, resulting in the formation of distinct Hsp90-Harc and Hsp90-Cdc37 heterocomplexes upon their co-expression (e.g. in liver and skeletal muscle). Indeed, endogenous Cdc37 was not detected in Hsp90-FLAG-Harc heterocomplexes. In view of this, Harc might also compete with immunophilins for binding to Hsp90 and hence may govern, to some extent, the composition of Hsp90 heterocomplexes.

In contrast to Cdc37, FLAG-Harc did not associate with Src family kinases (e.g. tsHck499F and Src527F) or Raf-1 in 293T cells, a finding implying that Harc is not a protein kinase targeting co-chaperone of Hsp90. However, because Cdc37 may bind only a subset of protein kinases (e.g. Cdk4 but not Cdc2) (33), we cannot exclude the possibility Harc binds and targets protein kinases other than Src family kinases and Raf-1 to Hsp90. It is also possible that Harc targets another class of client proteins to Hsp90. Owing to its ability to interact with protein kinases but not with several different steroid receptors (e.g. aryl hydrocarbon, estrogen, and glucocorticoid receptors) (44–46), Cdc37 had until recently been thought to function exclusively in Hsp90-mediated folding of client protein kinases. Caplan and co-workers (47), however, have provided evidence that Cdc37 functionally interacts with the anrogen receptor.

It will therefore be of great interest to determine whether Harc can likewise bind to the anrogen receptor or possibly other steroid receptors (e.g. glucocorticoid receptor) and influence its folding into a ligand-binding competent state by Hsp90.

FLAG-Harc is a phosphoprotein that becomes hyperphosphorylated when co-expressed with activated forms of Hck. However, it is not a direct substrate of Hck because co-expression with Hck499F induced primarily serine rather than tyrosine phosphorylation of FLAG-Harc. Therefore, a downstream effector serine kinase of Hck must mediate the phosphorylation of FLAG-Harc, although the identity of this serine kinase is unknown. Preliminary experiments suggest that at least one of the Hck-induced phosphorylation sites resides in the Hsp90-
binding domain of FLAG-Harc.² Notwithstanding this, phosphorylation of FLAG-Harc does not appear to modulate its association with Hsp90. Consequently, the functional significance of both the basal and Hck-induced hyperphosphorylation of FLAG-Harc needs to be established. It is worth noting that the activity of the glucocorticoid and progesterone receptors, at least in vivo, is modulated by the phosphorylation of FLAG-Harc does not appear to modulate its function.

In addition to Hsp90, five other proteins, namely Hsp70, p60Hop, FKBP52, CyP-40, and an unidentified p22 protein, form heterocomplexes with FLAG-Harc in vivo. Notably, the Hsp90 co-chaperone p23 was not detected in these heterocomplexes. Prior studies on the glucocorticoid and progesterone receptors have led to the formulation of a paradigm in which the folding of steroid receptors into active conformations proceeds via the stepwise assembly of chaperone-receptor heterocomplexes (15, 16). In this paradigm, so-called “early” chaperone-receptor heterocomplexes are characterized by the presence of Hsp90, “intermediate” complexes are characterized by the presence of Hsp70, Hsp90, and p60Hop, whereas “late” complexes are characterized by the presence of Hsp90, p23, and immunophilins (e.g., FKBP52 or CyP-40). On the basis of our findings we speculate that Harc might facilitate the formation of intermediate type heterocomplexes by selectively targeting Hsp90 to early Hsp70-client protein complexes (Fig. 8). Notably, FLAG-Harc is also likely to be a component of late chaperone-receptor protein complexes because the immunophilin FKBP52 and to a lesser extent CyP-40 were found in association with FLAG-Harc. Although p23 is a well characterized component of late chaperone-steroid receptor complexes, we failed to detect its presence in Hsp90-FLAG-Harc heterocomplexes. However, we did observe co-immunoprecipitation of a 22-kDa protein with FLAG-Harc from [35S]methionine-labeled cells. Yamamoto and co-workers (48) have shown that the p23-related protein tsp23 can substitute for p23 in stimulating the activity of the glucocorticoid and progesterone receptors, at least in S. cerevisiae. We are currently attempting to establish whether the p22 protein found in Hsp90-FLAG-Harc heterocomplexes is tsp23 or perhaps a p23-related protein not described previously.

It has recently been reported that Cdc37 is present in Hsp90 heterocomplexes containing p23 and the immunophilins FKBP52 and CyP-40, findings that the authors suggested were consistent with a role for Cdc37 in late Hsp90 heterocomplexes (50). Thus although Harc and Cdc37 are at least partially related both structurally and functionally, they likely serve distinct functions in Hsp90-mediated protein folding. Identification of the client protein in Hsp90-Harc heterocomplexes will provide the basis for defining the biochemical and biological function of Harc.

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Identification and Characterization of Harc, a Novel Hsp90-associating Relative of Cdc37
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