GCUNC45 Is the First Hsp90 Co-chaperone to Show α/β Isoform Specificity*

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Hsp90 is an essential molecular chaperone required for the normal functioning of many key regulatory proteins in eukaryotic cells. Vertebrates have two closely related isoforms of cytosolic Hsp90 (Hsp90α and Hsp90β). However, specific functions for each isoform are largely unknown, and no Hsp90 co-chaperone has been reported to distinguish between the two isoforms. In this study, we show that the Hsp90 co-chaperone GCUNC45 bound preferentially to the β isoform of Hsp90 in vitro. GCUNC45 efficiently blocked the progression of progesterone receptor chaperoning in an in vitro functional system when Hsp90β was used, but did so with much less efficacy when Hsp90α was used. Knockdown experiments in HeLa cells showed that GCUNC45 is required for the normal cellular distribution of Hsp90β, but not Hsp90α. This is the first example of a co-chaperone with isoform selectivity, and this approach may open novel avenues to understanding the functional differences between Hsp90 isoforms.

The 90-kDa heat shock protein Hsp90 is a ubiquitous and essential molecular chaperone that participates in the final structuring and/or activation of a select group of cellular proteins. Many of these “client” proteins are key cell regulators, including protein kinases and transcription factors (1, 2). In most eukaryotic organisms, there are two cytoplasmic isoforms of Hsp90, termed Hsp90α and Hsp90β (3). In humans, these are closely related proteins (86% identical and 93% similar) with only minor sequence differences that are most evident near their N termini, yet the crystal structures of the two N-terminal domains are very similar (4, 5). The genes for these proteins appear to be differentially regulated during development and in various cell types (6). Hsp90β is distributed throughout the developing mouse embryo during all stages studied, whereas Hsp90α is specifically present in primordial germ line cells (7). A null mutation of mouse Hsp90β was found to be embryonic lethal, where the major phenotype was a lack of placental labyrinth development (8). These studies indicate that there are specific developmental functions of Hsp90β that cannot be supported by Hsp90α. A somewhat converse arrangement may exist in metastatic cancers. Eustace et al. (9) found that Hsp90α, but not Hsp90β, is expressed extracellularly and is necessary for metalloprotease-2 activity and cancer cell invasiveness.

Biochemically, these protein isoforms have often been used interchangeably. Many studies of Hsp90 and co-chaperone requirements for client reconstitution in vitro have used rabbit reticulocyte lysates or purified chaperones of either isoform. Careful analysis of ATP hydrolysis by different forms of Hsp90 showed species variability, but isoform variations were relatively modest (10). Interestingly, when expressed in yeast as the only source of Hsp90, human Hsp90α and Hsp90β differ in their ability to activate some client proteins but not others, and only Hsp90β renders the cells highly sensitive to the Hsp90 inhibitor radicicol (11).

Biochemical approaches using purified systems would seem to be the most straightforward way to study the activities of different Hsp90 isoforms. However, the tools available with which to study isoform-specific functions of Hsp90 are limiting. Most reliable antibodies to mammalian Hsp90 proteins cross-react to different degrees with the other isoforms, and Hsp90 can also form heterodimers (3). Cellular studies are complicated by the fact that Hsp90 is an abundant cellular protein, and thus, it is difficult to knock down one specific isoform for individual experiments. The development of knock-out animals may likely require advanced strategies. One alternative strategy to gain insight into specific functions of Hsp90 isoforms is the identification of co-chaperones that are isoform-specific.

Hsp90 functions with the help of numerous co-chaperone proteins (1, 2). One of these, GCUNC45 (general cell UNC45), is a co-chaperone that has been implicated in the chaperoning of myosins (12–14) and of PR2 (15). In this study, we show that GCUNC45 bound preferentially to the β isoform of Hsp90 in a purified system. Furthermore, GCUNC45 efficiently blocked the progression of PR chaperoning in an in vitro system when Hsp90β was used, but did so with much less efficacy when Hsp90α was used. Knockdown experiments in HeLa cells showed that GCUNC45 is specifically required for the appropriate cellular distribution of Hsp90β, but not Hsp90α. This is the first example of a co-chaperone with isoform selectivity, and the extension of this approach should open novel avenues to understanding the functional differences between these Hsp90 isoforms.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification—Human Hsp90β and Hsp90α were expressed in Sf9 cells and purified as described previously (10). Hsp70, Ydj1, Hop, and p23 were expressed and purified as described previously (16). GCUNC45 was cloned and purified as described previously (15).

*PR Complex Assembly with Purified Proteins—PR expressed in Sf9 cells was adsorbed onto PR22 antibody-protein A-Sepha-

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2 The abbreviations used are: PR, progesterone receptor; siRNA, small interfering RNA; PRB, progesterone receptor B; GR, glucocorticoid receptor; TPR, tetratricopeptide repeat.
rose, stripped of endogenous proteins, and assembled into complexes using purified chaperone proteins as described previously (16). Each reaction contained \( \sim 0.05 \mu M \) PR, \( 1.4 \mu M \) Hsp70, \( 0.8 \mu M \) Hsp90\( \alpha \) or Hsp90\( \beta \) (dimer), \( 0.2 \mu M \) Ydj1, \( 0.08 \mu M \) Hop, and \( 2.6 \mu M \) p23 in reaction buffer (20 mM Tris, \( pH \) 7.5, 5 mM MgCl\(_2\), 2 mM dithiothreitol, 0.01% Nonidet P-40, 50 mM KCl, and 5 mM ATP). Increasing amounts of GCUNC45 were added to the reaction as indicated in the figures. After incubation for 30 min at 30 °C, 0.1 \( \mu M \) [\( ^3H \) ]progesterone (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added, and the incubation was continued on ice for 3 h. The complexes were then washed with reaction buffer. PR resins-bound [\( ^3H \) ]progesterone was quantitated by liquid scintillation counting.

**Protein Binding Assays**—The binding of Hsp90 to GCUNC45 was assayed using GCUNC45 covalently linked to Sepharose 4B as described previously (15). Hsp90 binding to p23 and Hop was determined using antibodies I3 and F5, respectively, bound to protein A-Sepharose. Binding of Hsp90\( \alpha \) or Hsp90\( \beta \) used 15 \( \mu l \) of resin and variable amounts of Hsp90 in a 200-\( \mu l \) final reaction volume in buffer containing 20 mM Tris, 100 mM KCl, 0.02% Nonidet P-40, 2.5% glycerol, 2 mM dithiothreitol, and 5 mM MgCl\(_2\), \( pH \) 7.5. For p23 binding, 5 mM ATP was added to the reactions. Samples were incubated for 30 min at 30 °C and washed four times with 1 ml of the same buffer. Bound proteins were extracted by boiling in SDS sample buffer, resolved by SDS-PAGE, and stained with Coomassie Blue. Arbitrary densitometric units for the appropriate bands of protein were measured and is expressed in cpm as the mean of triplicate samples.

**siRNA Knockdown of GCUNC45**—We used HeLa/PRB cells, which stably express human PRB and a chloramphenicol acetyltransferase reporter gene under the control of progesterone-response elements (17). Cells were cultured in 6-well plates containing glass coverslips. Cells at 30–40% confluence were transfected with 50 \( \mu M \) siRNA duplexes to GCUNC45 using DharmaFECT 1 reagent (Dharmacon) following the manufacturer’s suggestions. The nonspecific control VIII was used to measure any non-target sequence effects. Seventy-two hours after transfection, the coverslips were removed and processed for immunocytochemistry and confocal microscopy. The remaining cells were harvested and lysed, and the extracts were used for Western blotting. Cells on the coverslips were fixed with formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum and 5% glycerol in phosphate-buffered saline. Primary antibodies against Hsp90\( \alpha \) (monoclonal H90.10) and Hsp90\( \alpha \) (rabbit polyclonal RB-119-P1; NeoMarkers) as well as goat anti-mouse and anti-rabbit secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes) as well as goat anti-mouse and anti-rabbit secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes) were used at 1:1000 dilution. All incubations and washes were done at room temperature for 1 h. Coverslips were mounted using ProLong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (Invitrogen). Cells were visualized and recorded using a Zeiss LSM 510 confocal laser scanning microscope.

**RESULTS AND DISCUSSION**

Although there is some biological evidence for distinct roles for the \( \alpha \) and \( \beta \) isoforms of Hsp90, they have been used interchangeably or even as a mixture in most in vitro studies. The results in Fig. 1 show that this is not usually of concern. Using the chaperoning of PR as a functional test, we compared human Hsp90\( \alpha \) and Hsp90\( \beta \) with chicken Hsp90\( \alpha \) for their ability to chaperone PR to its hormone-binding state in the presence of the purified chaperones Hsp70, Hsp40, Hop, and p23. These Hsp90 proteins chaperoned PR with similar efficiencies. The differences among the preparations were small and were representative of experimental variation. The
data in Fig. 1 are consistent with reports showing that both Hsp90α and Hsp90β are in complex with GR in cell lysates (18). Also, when human Hsp90α or Hsp90β is expressed as the sole source of Hsp90 in Saccharomyces cerevisiae, it promotes the transcriptional activity of GR with similar efficiency (11).

We have recently described the Hsp90 co-chaperone GCUNC45 as a significant participant in the chaperoning of PR (15). Although GCUNC45 is a positive factor for PR in the cell, it is inhibitory when added to the in vitro chaperoning system containing Hsp90, Hsp70, Hsp40, Hop, and p23, but the immunophilin FKBP52 can relieve this repression and promote the progression of PR toward the hormone-binding state (15). This suggests that GCUNC45 plays a role in the intermediate steps of the folding cycle, upstream of FKBP52. While investigating the composition of cellular GCUNC45 complexes by pulldown experiments and mass spectrometry analysis, we found a prevalence of Hsp90β in the complexes. This observation prompted us to test the biochemical function of GCUNC45 with different isoforms of Hsp90. As illustrated in Fig. 2, a dose-dependent inhibition of PR activation, previously described using Hsp90β, was not as clearly manifested with Hsp90α.

This unusual behavior of an Hsp90 co-chaperone suggested that GCUNC45 interacts differently with the two isoforms of Hsp90. Therefore, we tested the specificity of GCUNC45 binding to Hsp90 isoforms directly. GCUNC45 was immobilized onto Sepharose beads and incubated with various Hsp90 proteins (Fig. 3). Very little binding was observed using the yeast and chicken proteins, which may reflect species specificity. Although both the α and β forms of human Hsp90 bound GCUNC45, there was a consistent preference for Hsp90β binding to GCUNC45 (Fig. 3A). This selectivity for Hsp90β was more obvious when the two isoforms were tested at various protein concentrations (Fig. 3B). Scatchard analysis showed that Hsp90α and Hsp90β bound GCUNC45 with Kd values of ~50 and 260 nM, respectively, indicating that Hsp90β has a 5-fold higher affinity for GCUNC45. Under similar conditions, both co-chaperones p23 and Hop bound to Hsp90α and Hsp90β with identical affinities (Fig. 3, C and D).

To investigate the biological significance of this selectivity, we took advantage of our ability to knock down GCUNC45 in HeLa cells using siRNA (Fig. 4A). In control cells, Hsp90α was widely distributed throughout the cytoplasm and nucleus (Fig. 4B, upper left panel) as shown previously by others (3, 19). Hsp90β was more abundant in the cytoplasm compared with the nucleus (Fig. 4B, upper right panel). When GCUNC45 expression was disrupted, Hsp90β was found to form clusters in the cytoplasm (Fig. 4B, lower right panel). In contrast, the distribution of Hsp90α was not noticeably affected (Fig. 4B, lower left panel). Overall, cells lacking GCUNC45 also showed marked morphological changes, becoming more rounded and having less cytoplasmic mass. These morphological changes may reflect a specific role for GCUNC45 in recruiting the Hsp90β machine to chaperones and organizing the cytoskeleton myosin (12, 20). Hsp90 has already been shown to interact with actin and tubulin, and Hsp90-GR complexes use microtubules to migrate throughout the cytoplasm (3, 21). Thus, it is tempting to speculate that GCUNC45 may play an important biological role in...
the cytoplasmic trafficking of Hsp90β and, in turn, a subset of Hsp90 clients. These questions will be the focus of future investigations.

In summary, our results show that Hsp90α and Hsp90β have comparable activities in chaperoning PR in vitro (Fig. 1) and that the co-chaperones p23 and Hop (necessary for PR chaperoning) bind equally to these Hsp90 isoforms (Fig. 3). This is not the case with GCUNC45. Its binding and inhibitory activities are most effective in the presence of Hsp90β. GCUNC45 binds through its TPR domain to a site in the N-terminal ATP-binding domain of Hsp90 (15, 22). This is a novel site that is not recognized by the TPR co-chaperone Hop, which also does not manifest isoform specificity. Therefore, the molecular mechanism underlying the preference of GCUNC45 for Hsp90β is apparently related to its distinct mode of interaction with Hsp90. Because the key residues involved in GCUNC45 binding to the N-terminal binding site are conserved in both isoforms (22) and because the structures of the two N-terminal domains are very similar (4, 5), it is likely that the accessibility of this TPR-binding site is regulated by the overall structure and conformational flexibility of full-length Hsp90. The α and β isoforms differ somewhat in structure as indicated by the fact that a significant fraction of Hsp90β, but not Hsp90α, exists in a monomeric state in cell lysates (23).

As isolated proteins, the α and β forms of Hsp90 are very similar in their chaperoning of PR and their interaction with nucleotides and Hsp90 inhibitors (10). However, marked biological differences in the roles of the two isoforms are evident in the cellular context (6, 11). Client proteins and co-chaperones in complex with Hsp90 in vivo are likely to determine these functional differences between isoforms. Our results show one example of a co-chaperone interaction that may selectively facilitate an activity of Hsp90β. GCUNC45 might be involved in the cellular trafficking of the Hsp90β machine with a subset of client proteins. In this way, subtle differences in protein-interacting surfaces may be key determinants of function.

There are at least 20 Hsp90 co-chaperones reported in the literature (1, 2, 24). The biological roles of most of these are unclear, and a thorough comparison may likely reveal additional co-chaperones that would function preferentially with one Hsp90 isoform. Additional demonstrations of isoform-specific co-chaperoning will provide additional insights into the biology of Hsp90.

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