Hsp90 Mutants Disrupt Glucocorticoid Receptor Ligand Binding and Destabilize Aaporeceptor Complexes*

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In order to attain competence to respond to hormone, certain steroid hormone receptors must be assembled into hetero-oligomeric aaporeceptor complexes, containing Hsp90 and other proteins. Members of the Hsp90 gene family are highly conserved, strongly expressed, and required for viability in eukaryotic organisms. To elucidate the role of Hsp90 in the activity of steroid hormone receptors in vivo, four Hsp90 mutants, which cause defects in glucocorticoid receptor (GR) signaling, but support the viability of Saccharomyces cerevisiae, were previously isolated (Bohen, S. P., and Yamamoto, K. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11424-11428). In this study, I characterize the effects of the Hsp90 mutants on GR ligand response, ligand binding activity, and aaporeceptor complex stability. The mutants fall into two classes. Three of the Hsp90 mutants cause defects in GR ligand binding in vivo and form aaporeceptor complexes that are unstable in vitro, relative to those containing wild-type Hsp90. The other mutant affects GR signaling, but aaporeceptor complexes with this mutant are not defective for ligand binding or stability. These findings indicate that the binding of Hsp90 to GR in the aaporeceptor complex is insufficient to induce a high ligand affinity conformation, rather the high ligand affinity of GR requires a specific interaction with Hsp90, which is altered by certain Hsp90 mutants.

The 90-kDa heat shock protein (Hsp90) is a highly conserved stress-induced protein that is abundantly expressed in almost all cells under nonstress conditions and is required for viability in eukaryotes. Biochemical analysis has shown that Hsp90 interacts with various proteins, including signaling molecules (1), other heat shock proteins (2, 3), and cytoskeletal proteins (4, 5), but the function of Hsp90 in these complexes remains unclear. The physical association of Hsp90 with steroid hormone receptors (6–9), the basic-helix-loop-helix dioxin family tyrosine kinases (1, 11), the src protooncogene (12), and the ligand binding activities of the mineralocorticoid (27), progesterone (28), and dioxin receptors (29). Notably, however, the role of Hsp90 in signal transduction is best characterized for steroid hormone receptors where Hsp90 appears to be required for the recognition of and response to ligand. In the absence of bound agonist, some of the steroid receptors, including the glucocorticoid receptor (GR), are components of aaporeceptor complexes composed of certain nonreceptor proteins, minimally a dimer of Hsp90 and a monomer of hsp56/FKBP59 (20, 21). GR activation is a response to an increase in available ligand concentration that drives ligand binding to the receptor, as a component of the aaporeceptor complex. The liganded aaporeceptor then dissociates from the other components of the complex, resulting in receptor “activation” (for review, see Refs. 9 and 22); the activated receptor translocates into the nucleus, binds to specific sites on the chromosome, and modulates the transcriptional activity of target genes. Following ligand withdrawal, unliganded receptors are recycled into cytoplasmic aaporeceptor complexes, reconstituting high ligand affinity, and restoring competence to undergo further rounds of activation (23).

It is the aaporeceptor complex that recognizes and transduces the steroid hormone signal. Several lines of evidence correlate GR-Hsp90 complex formation with competence to respond to ligand (9). For example, the high affinity ligand binding activity of the GR aaporeceptor complex is lost when Hsp90 is dissociated from GR in vitro (24), and reconstitution of the complex in reticulocyte lysates restores high ligand affinity (25). In vivo, expression of decreased levels of Hsp90 in yeast results in a decrease in the sensitivity of receptor to ligand (15). In an analogous situation, GR in rat epididymal cells, which lack Hsp90, displays no ligand binding activity (26). Correlations have also been made in vitro between Hsp90 complex formation and the ligand binding activities of the mineralocorticoid (27), progesterone (28), and dioxin receptors (29). Notably, however, analysis of a series of hsp90 point mutants shows that GR-Hsp90 complex formation alone is not sufficient for normal signal transduction (17).

Four point mutants in HSP82, one of the yeast Hsp90 genes, were previously identified in a yeast genetic screen for decreased sensitivity of GR to ligand (17). These mutants define two phenotypic classes based on their receptor specificity and on their effects on cell growth. One of the mutants, E431K, affects GR signaling, but alters neither the activities of mineralocorticoid, progesterone, and the estrogen receptor nor the growth rate or temperature sensitivity of yeast. In contrast, the
other mutants, G313N, T525I, and A576T/R579K (a double mutant), affect all receptor types tested, result in temperature sensitivity for growth at 37°C, and modestly decrease growth rates at permissive temperature. Interestingly, all of the hsp82 mutants seem to form complexes with GR in vivo, as assessed by communoprecipitation with GR in yeast extracts.

There are several mechanisms whereby hsp90 mutants that remain competent to associate with steroid receptors may produce defects in receptor signaling. (i) Mutant hsp90 may fail to induce a high ligand affinity conformation in the receptor ligand binding domain, resulting in aoreceptor complexes with decreased ability to bind ligand. (ii) Mutants may form aoreceptor complexes with normal ligand binding activity, but mutant hsp90 may not dissociate efficiently from the liganded aoreceptor. (iii) Mutant hsp90 might form aoreceptor complexes that bind ligand normally and efficiently dissociate from the liganded receptor, without facilitating a further conformational change necessary to accomplish transcriptional regulation. In previous experiments, efforts to elucidate the role of Hsp90 in signaling were complicated by the fact that association of Hsp90 with its target proteins was disrupted. Thus, the hsp90 mutants presented a unique opportunity to gain new insight into the role of Hsp90 in steroid receptor signal transduction through biochemical analysis of the GR-hsp90 interaction in defective aoreceptor complexes.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and ligase were from Promega Corp. and New England BioLabs. [3H]Dexamethasone and [3H]dexamethasone (42 or 43 Ci/mmol) were obtained from Sigma and Amersham Corp., respectively. FK506 was kindly provided by Fujisawa U. S. A. CaCl2, and MgCl2-free PBS was from the University of California at San Francisco Cell Culture Facility. AEBFS was from Calbiochem. Aprotinin, leupeptin, and pepstatin A were from Boehringer Mannheim. Immobilon-P membranes were from Millipore Corp. Affi-Prep protein A and alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgG were from Bio-Rad. 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate was from Kirkegaard and Perry. Rabbit anti-yeast Hsp90 antisera was kindly provided by Dr. Susan Lindquist (Howard Hughes Medical Institute, University of Chicago). Other reagents were from Sigma.

Methods

Yeast Strain, Plasmids, and Transformations—Yeast strains are isogenic to UH1-GR2G and are stably transformed with the appropriate hsp82 and rat GR expression plasmids and a β-galactosidase reporter plasmid; strains and plasmids were described previously (17, 30). HSP82 and HSC82 deletions in these strains are complemented by wild-type or mutant hsp82 expressed from a TRP1-marked low copy number plasmid under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. Wild-type and F620S mutant rat GRs are expressed by the glyceraldehyde-3-phosphate dehydrogenase promoter from HIS3-marked high copy number plasmids. The GR expression vectors were constructed by first subcloning the glyceraldehyde-3-phosphate dehydrogenase promoter into a HIS3-marked high copy number plasmid. The GR expression vectors were constructed by first subcloning the glyceraldehyde-3-phosphate dehydrogenase promoter into a HIS3-marked high copy number plasmid. The GR expression vectors (26) were inserted as RI--BamHI fragments.

β-Galactosidase Assays—Ligand response of GR in the context of wild-type and mutant hsp82 was determined by measuring β-galactosidase activity induced in response to varying concentrations of dexamethasone. Appropriate yeast strains were grown to saturation at room temperature in selective medium (SD complete lacking His, Trp, and Ura) (33), and diluted 1:6 into fresh medium containing dexamethasone in ethanol or ethanol only (final concentration of ethanol was 1% in all samples). Cultures were incubated for 30 min at 37°C, and extracts were prepared by sonication. Lysates were centrifuged at 10,000×g for 15 min at 4°C, and samples were assayed for β-galactosidase activity. β-Galactosidase activity was determined as described previously (17).
The number of other steroid receptors are compromised in yeast strains containing mutant hsp82 (17). The isolation and initial characterization of the mutants was conducted using wild-type GR; however, analysis of the ligand binding characteristics of wild-type GR is technically intractable because of the low affinity of wild-type GR for ligand when expressed in yeast. To overcome this technical hurdle and to quantitatively determine the effects of the hsp82 mutants on ligand binding, I expressed F620S mutant GR, which displays increased ligand affinity in yeast (35), in strains expressing wild-type or mutant hsp82.

To test whether F620S GR is an appropriate model for the role of Hsp90 in steroid receptor function, I first assessed the effects of the hsp82 mutants on the F620S GR ligand response (Fig. 1A). As with wild-type GR (Fig. 1B), the different hsp82 mutants produced a spectrum of signal transduction phenotypes; G313N resulted in a very severe decrease in dexamethasone responsiveness, whereas T525I and A576T/R579K caused less severe signaling defects. The E431K mutant produced a substantial but significant decrease in the response of F620S GR. Thus, although F620S GR responds to lower concentrations of dexamethasone than does wild-type GR, signal transduction by wild-type and F620S GR was similarly affected by the hsp82 mutants, and, importantly, the individual mutants followed the same order of severity within the spectrum of mutant phenotypes (Fig. 1, compare A and B). These findings indicate that Hsp90 functions in a similar capacity in signaling by F620S and wild-type GR. Thus, F620S mutant GR provides a useful reagent for testing the effects of hsp82 mutants on the ligand binding activity of steroid receptors.

**GR Displays Decreased Ligand Binding in Cells Expressing hsp82 Mutants—** In light of biochemical evidence that Hsp90 is required for high affinity ligand binding by GR, I hypothesized that mutant hsp82 may produce GR aporeceptor complexes with reduced ligand affinity, leading to the GR signaling defect. To test this hypothesis, I measured the intracellular accumulation of dexamethasone in cells coexpressing wild-type or mutant hsp82 and F620S GR (Fig. 2). There is significantly less dexamethasone binding activity in cells expressing any one of three hsp82 mutants that strongly decrease the GR ligand response; G313N, T525I, and A576T/R579K. These mutants resulted in 3–20-fold less ligand binding activity at 250 nM dexamethasone than in cells expressing wild-type Hsp82. The most severe mutant, G313N, displayed no increase in dexamethasone binding activity at 1 μM dexamethasone. In contrast, the T525I and A576T/R579K mutants showed increased dexamethasone binding at the higher ligand concentration, but binding remained about 3-fold less than in cells expressing wild-type Hsp82. Notably, the severity of the in vivo ligand binding defects of a given mutant correlated with the severity of the ligand response phenotype of that mutant (compare Figs. 1 and 2). These observations support the idea that these hsp82 mutants compromise GR signaling by decreasing ligand binding.

In contrast to the other hsp82 mutants, dexamethasone binding activity in cells coexpressing E431K mutant hsp82 and F620S GR was identical to that in cells expressing the wild-type Hsp82 protein (Fig. 2). Although E431K produced the least severe ligand response defect (Fig. 1), comparison of the ligand response and in vivo dexamethasone binding defects of the other hsp82 mutants suggested that I would have detected a binding defect in the context of E431K if such a defect underlies the E431K ligand response phenotype. The lack of a ligand binding defect suggests that E431K and wild-type Hsp82 are capable of forming GR aporeceptor complexes of equivalent number and affinity. Thus, the E431K phenotype is consistent with the hypothesis that alterations in Hsp90 may affect a step in GR activation downstream of ligand binding; for example, E431K hsp82 might not dissociate efficiently from ligand-bound GR.

**hsp82 Mutants Destabilize Aporreceptor Complexes—** I next examined whether the defects in GR ligand sensitivity and ligand binding correlate with an alteration in the interaction of hsp82 with GR by measuring the stability of isolated aporeceptor complexes containing wild-type or mutant hsp82 (Fig. 3). As wild-type GR is somewhat more sensitive to the effects of E431K mutant hsp82 (Fig. 1) and forms less stable aporeceptor complexes than F620S GR, I reasoned that alterations in

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incubation with \([3H]\) dexamethasone, with or without cold dexamethasone and wild-type or mutant hsp82 were preincubated with FK506 for 2 h. Additionally, the finding that mutant aporeceptor complexes are less stable in yeast extracts is most consistent with signal transduction in vivo; in contrast, E431K caused no ligand binding defect in vivo and no significant decrease in aporeceptor complex stability in vivo. I shall consider the two mutant classes separately.

The GR signaling defect observed in yeast expressing G313N, T525I, or A576T/R579K mutant hsp82 is explained by decreased ligand binding activity. There are several mechanisms whereby these mutants could alter their interaction with GR. The GR could produce the observed phenotypes. Mutant hsp82 could abrogate the formation of aporeceptor complexes, form a normal number of aporeceptor complexes that are defective for ligand binding, result in decreased GR protein levels, or disrupt GR ligand binding in vivo via one of a myriad of potential indirect mechanisms. Several of these models have been tested experimentally. For example, GR protein levels are indistinguishable in strains expressing wild-type and mutant hsp82 (17). Furthermore, the hsp82 mutant proteins coimmunoprecipitate with GR from yeast extracts, suggesting that they assemble into aporeceptor complexes in vivo (Fig. 3A and Ref. 17). (I have demonstrated that the association of hsp82 with GR is specific (17) and that GR-hsp82 complexes form prior to cell lysis (data not shown).) In principle, GR and hsp82 might associate passively and artifactually, for example during chilling before lysis, but pulse-chase experiments have demonstrated that GR-Hsp90 complexes are present in vivo in mammalian cells (38), and aporeceptor complex assembly in reticulocyte lysates requires ATP hydrolysis and moderate temperature (25, 39). Finally, although it is formally possible that the hsp82 mutants might indirectly affect receptor signaling, the finding that mutant aporeceptor complexes are less stable in yeast extracts is most consistent with signal transduction.
duction defects resulting directly from an alteration in the GR-Hsp90 interaction. Thus, although this class of hsp82 mutants is competent to form aporeceptor complexes with GR, the defect in the GR-mutant hsp82 interaction results in a corresponding defect in aporeceptor ligand binding, thereby compromising GR signal transduction and subsequent transcription activation.

In addition to the receptor, Hsp90 and hsp56/FKBp59, other proteins have been identified as components of aporeceptor complexes (40), including hsp70, p60, which is a protein homologous to Sti1, p23 (41), and Ydj1, a yeast DnaJ-like protein (42). It has been demonstrated that p23 (43) and hsp70 (44) are required for aporeceptor complex formation in reticulocyte lysates and that DnaJ plays a role in receptor function in vivo in yeast (42, 45), but how these proteins affect receptor signaling is unknown. Cross-linking studies suggest that Hsp90 is the only protein interacting directly with GR in the aporeceptor complex (20), and characterization of the interaction of Hsp90 with deletion mutants of GR indicates that the Hsp90 binds to GR within the region of the receptor that is responsible for recognizing ligand (46); these observations have led to the hypothesis that the interaction of Hsp90 directly with the GR ligand binding domain induces a high ligand affinity conformation in the receptor. The effects of hsp82 mutants on aporeceptor complex stability and ligand affinity may be the result of alterations in the interaction of Hsp82 with the ligand binding domain of GR directly or with other proteins in the aporeceptor complex that are important for its stability and function.

Given GR ligand binding defects observed in vivo in cells expressing these hsp82 mutants, I attempted to measure the dexamethasone binding affinity of wild-type and mutant aporeceptor complexes in yeast extracts. To my surprise the apparent affinity of receptor for ligand in vitro was not altered by the hsp82 mutants (data not shown). This finding seems to contradict the in vivo binding data; however, several observations compel me to conclude that the problem probably lies in the in vitro binding assay itself. A comparison of total GR levels, as determined by immunoblotting, versus ligand binding suggests that only about 2% of the receptor expressed in yeast binds ligand in vitro (36). Furthermore, reduced expression of Hsp82 to 5% of wild-type levels produces severe defects in GR signaling and ligand binding in vivo, and Hsp82 does not coimmunoprecipitate with GR in these extracts (15), yet no defect in GR ligand binding is observed in these extracts (data not shown). Hence, the residual binding in vitro may represent either a subset of receptors that attains a high affinity conformation without bound Hsp82 or a small fraction of aporeceptor complexes that remains stably associated in the extract. In either case, the in vitro binding assay does not reflect the state of the GR-hsp82 interaction in vivo.

E431K mutant hsp82 affects signaling by F620S and wild-type GR, but no other phenotype of E431K hsp82 has been elicited genetically or biochemically. It is possible that E431K is mechanistically related to the other hsp82 mutants, resulting in aporeceptor complexes with compromised ligand binding activity but that the E431K defect is simply too subtle to be detected in the in vivo ligand binding assay. Alternatively, E431K may be mechanistically distinct and cause a defect in some aspect of GR signal transduction subsequent to ligand binding, such as Hsp90 dissociation or an as yet uncharacterized change in GR conformation that is mediated by Hsp90 and is required for GR to become competent for transcriptional regulation. In this case, E431K would represent a particularly useful reagent for the elucidation of Hsp90 function in receptor signaling subsequent to ligand binding.

The findings presented here yield several interesting insights into the role of Hsp90 in receptor signal transduction. It appears that aporeceptor complex formation alone is not sufficient for efficient ligand binding by steroid receptors; not surprisingly, Hsp90 must assume the proper conformation in these complexes to confer high ligand affinity to receptors. Although alteration of the Hsp90 interaction with target proteins has not been shown to regulate any system, it is interesting that changes in the interaction of Hsp90 with any of a number of target proteins may be used to regulate the activity of diverse signaling pathways. This proposal is given credence by the recent finding that drugs of the benzoquinone ansamycin family, which alter the activities of v-src and steroid receptors in vivo, bind to Hsp90 in vitro (19). Analysis of E431K mutant hsp82 suggests that alterations in Hsp90 conformation may affect steps in signal transduction subsequent to ligand binding. Furthermore, such alterations in Hsp82 may be subtle enough to alter signaling specifically by particular Hsp82 target proteins.

Finally, it is intriguing to consider the origin of the steroid receptor-Hsp90 interaction. Members of the nuclear receptor family display a spectrum of dependences on Hsp90 for signaling (9). For example, in contrast to GR, an interaction of Hsp90 with thyroid and retinoid receptors has not been demonstrated, and these receptors can bind their cognate ligands with high affinity as purified proteins (47); thus, it has been hypothesized that thyroid and retinoid receptors do not require Hsp90 for binding. However, examination of signaling by these receptors in yeast expressing a low level of hsp82 demonstrates that these receptors are dependent on Hsp90 for normal ligand binding activity (48). It seems likely that a transient interaction with Hsp90 is required sometime during or shortly after synthesis of these receptors to achieve a high ligand affinity conformation. GR, mineralocorticoid, and progestrone receptors may represent a subset of receptors whose intrinsic folding demands continuous interaction with Hsp90 to achieve a functional conformation. Such a strong dependence on Hsp90 may simply reflect an unstable conformation or the evolution of an additional potential for the regulation of these receptors. It is interesting to note that a similar interaction has arisen independently in DR (10). DR is a basic-helix-loop-helix protein with no sequence homology to steroid hormone receptors. However, DR is a ligand regulated transcription factor that is dependent on bound Hsp90 to achieve high ligand affinity. Thus, the interaction of steroid receptors with Hsp90 may represent a common form of regulation of signaling systems by linking the proper folding of signal transduction proteins to a stable interaction with Hsp90.

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