Genetic Dissection of p23, an Hsp90 Cochaperone, Reveals a Distinct Surface Involved in Estrogen Receptor Signaling*

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p23 is an Hsp90-associated protein that regulates signal transduction by the estrogen receptor α (ER); however, the mechanism through which p23 governs ER function remains enigmatic. To obtain a collection of p23 molecules with distinct effects on ER signaling, we screened in yeast a series of random mutations as well as specific sequence alterations based on the p23 crystal structure and further analyzed these mutations for their effect on p23-Hsp90 association in vitro and in vivo. We found that the ability of the p23 mutants to decrease or increase ER signal transduction correlated with their association with Hsp90. We also identified a mutation in the C-terminal tail of p23, which displayed a dominant inhibitory effect on ER transcriptional activation and associates more avidly with Hsp90 relative to the wild type p23. Interestingly, this mutant interacts with Hsp90 in its non-ATP-bound state, whereas the wild type p23 protein interacts exclusively with the ATP-bound form of Hsp90, which may account for its dominant phenotype. In addition, we have uncovered a novel activity of p23 that antagonizes Hsp90 action during times of cellular stress. Using molecular modeling and the p23 crystal structure, we found that the p23 mutations affecting ER signaling identified in the screen localized to one face of the molecule, whereas those that had no effect mapped to other parts of the protein. Thus, our structure/function analysis has identified an important regulatory surface on p23 involved in ER signaling and p23 binding to Hsp90.

The estrogen receptor α (ER)† is a member of the intracellular receptor family of transcriptional regulatory proteins that transduces the signaling information conveyed by estrogen (1). Activation of ER plays a critical role in the initiation and progression of breast cancer by regulating genes and signaling pathways involved in cellular proliferation (2). In the absence of estradiol, ER is found predominantly in the nucleus and is maintained in a high affinity hormone binding conformation by the Hsp90 molecular chaperone complex (3, 4). Upon binding to estradiol, ER associates with specific DNA sequences, termed estrogen response elements (EREs), and modulates transcription initiation from nearby promoters (5).

Previous work from our laboratory and others has demonstrated a role for p23 in ER signal transduction (6–9). p23, a ubiquitous and evolutionarily conserved Hsp90-binding protein, appears to regulate ER sensitivity to the steroid hormone under physiological low ligand and receptor concentrations (8). Ligand binding and transcriptional activation by ER under these conditions directly correlates with the concentration of p23 in the cell. Overexpression of p23 increases ER transcriptional activity by increasing the number of receptors capable of signaling; conversely, a lack of p23 decreases ER transcriptional activation by reducing the number of ER molecules capable of ligand binding (8). The ability of p23 to increase ER signaling is mediated through Hsp90 binding and stabilization of aporeceptor complexes. In addition, findings from the Yamamoto laboratory have shown that p23 overexpression also influences the transcriptional activity of a number of nuclear receptors independent of ligand binding, including ER, when assayed at high receptor and ligand concentrations (6). However, the precise structural determinants of the p23-ER functional interactions as well as the role of Hsp90 in these processes have yet to be determined.

Hsp90 is an abundant and ubiquitous chaperone with an ability to carry out multiple functions within the cell (10–12). During times of environmental stress, such as heat shock, Hsp90 plays an important role in reactivating damaged proteins, thereby preserving cell viability (13). Under normal conditions, Hsp90 appears to be necessary for the conformational maturation of a growing number of “client” proteins, including steroid receptors (11), the dioxin receptor (14), growth factor receptors (15), kinases, including the double-stranded RNA-dependent protein kinase PKR (16), and enzymes, such as telomerase (17). The ability of Hsp90 to manage the maturation and integrity of such a diverse set of substrates under normal and stressful conditions is due to its ability to associate with distinct accessory proteins, termed cochaperones, that include p23 (18). Changes in the intracellular concentration of cochaperones can potentially affect the equilibrium of the various Hsp90 subcomplexes within the cell, which in turn could affect chaperone-dependent regulatory functions (8, 18). For example, alterations in the intracellular concentration of p23 could affect the ability of Hsp90 to buffer or protect cells during stress. However, whether the expression of p23 varies among mammalian cell types or during pathological processes, such as

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the progression from hormone-dependent to hormone-independent breast cancer, has not been investigated.

Although in vitro p23 binds Hsp90 to stabilize steroid receptor-Hsp90 heterocomplexes, the mechanism of p23 action remains to be elucidated (19). It is important to note that neither p23 nor immunophilins are involved in rendering the receptor competent for hormone binding (11). Newly synthesized receptors initially bind to Hsp70 and Hsp40 and are then transferred to Hsp90 by adapter proteins (20, 21). Biochemical studies indicate that p23 binds exclusively to the ATP-bound form of Hsp90, and when Hsp90 achieves the ATP-bound conformation (22), the adapters are released, and p23 binds along with an immunophilin to produce the final complex (23). In contrast to the stabilizing effect of p23 on steroid receptor-Hsp90 heterocomplexes, other biochemical studies suggest that p23 enhances the release of substrates from Hsp90 without altering its rate of ATP hydrolysis (24, 25). Geldanamycin, a specific inhibitor of Hsp90 that competes with ATP for binding, can disrupt the Hsp90-p23 complex (26). In addition, several activities of p23 appear independent of Hsp90. For example, p23 exhibits a passive chaperone activity, in that it can prevent the aggregation of denatured proteins in vitro (27). Removal of the extreme C-terminal tail of p23 compromises this activity but not heterocomplex formation with Hsp90. p23 has also been shown to aid in the disassembly of transcription factors at cognate response elements, perhaps independent of Hsp90 (28, 29). The recently determined structure of a human p23 derivative lacking the final 35 carboxyl terminal residues reveals a compact globular protein with a shallow cavity formed by a set of residues held in common among p23s from distinct species (30). This “cleft” has been proposed to be a surface for Hsp90 or substrate binding, although this has yet to be examined. Thus, p23 cooperates with Hsp90 to facilitate, for example, ligand binding by ER, but it may also function to modulate protein-protein or protein-DNA interactions in an Hsp90-independent manner.

In light of the apparent complexity of p23 action, we sought to develop a genetic approach for dissecting p23 function that would capitalize on its capacity to increase ER hormone-dependent transcriptional activity in Saccharomyces cerevisiae. We have assayed the yeast homologue of human p23/SBA1 (hereafter referred to as Yhp23) deletions and random as well as targeted point mutations based on the p23 crystal structure for effects on ER signal transduction and further characterized these mutants for their ability to associate with Hsp90. Our findings suggest that Yhp23 mutations affecting ER signal transduction correlate with their association with Hsp90.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture Conditions**—The yeast strains used were W303a derivatives and included YF240, an sba1 (yhp23) deletion strain (9), and CLD82A, a has2 deletion strain (13). Yeast cultures were grown at 30 °C in selective minimal medium with 2% glucose or 2% galactose plus 1% raffinose, supplemented with amino acids. Plasmid Constructs—The pRS314GPD (HA-yhp23-GFP) vector used to express HA-Yhp23-GFP fusion protein was described by Knooblauch and Garabedian (8). The pRS314GPD vector contains a glyceraldehyde dehydrogenase promoter and the TRP1 gene as a selectable marker. To facilitate cloning of the yhp23 mutated genes, the pRS314GPD vector was modified by removing two HindIII sites, one within the linker between yhp23 and GFP and the other in the TRP1 gene, by site-directed mutagenesis (Stratagene). The HindIII site located within the linker was mutated with the following primers: 5′-ATGAAGGAAGCAAAATAGAGCCGGAGACG-3′ and 5′-CTACCTCTCTGTCATTTTCCCTCTC-3′. ER A (G400V) was expressed from the p2H-GAL vector that contains the Gal1-10 promoter, the HIS3 gene as a selectable marker, and the 2 μ replication origin (p2H-Gal-ERGALV). The reporter plasmid ERE-CYC1-LacZ contains a single ERE upstream of a truncated CYC1 promoter linked to the β-galactosidase gene and contains a URA3 gene as a selectable marker and the yeast 2 μ replication origin. Preparation of Yeast Extracts and Immunoblotting—For immunoblotting, yeast extracts were prepared from a 2 ml culture. Cells were grown to exponential phase in synthetic minimal medium complemented with 2% glucose or 2% galactose plus 1% raffinose at either 30 or 37 °C. Cells were harvested by centrifugation and washed once in water. The pellet of cells was then resuspended in 200 μl of yeast lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol) supplemented with 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture containing 1 μg/ml leupeptin, pepstatin A, and aprotinin (Sigma). An equal volume of acid-washed beads was added, and the samples were vortexed for 15 min at 4 °C. The extract was transferred to a new tube and centrifuged at 10,000 rpm for 10 min, and the supernatant was placed into a fresh microcentrifuge tube. The protein concentration was determined using a Bio-Rad protein assay and bovine serum albumin as the standard. For immunoblotting, yeast extracts were fractionated on 10% SDS-polyacrylamide gels, transferred to Immobilon membrane (Millipore Corp.) with the following antibodies: HA monoclonal antibody 12CA5 (dilution 1:1000; Roche Applied Science), ER α rabbit polyclonal antibody HC20 (dilution 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Hsp92 rabbit polyclonal antibody (dilution 1:1000; kindly provided by Susan Lindquist, Whitehead Institute for Biomedical Research, Cambridge, MA). A horseradish peroxidase-coupled goat anti-mouse antibody or horseradish peroxidase-protein A was used to label the primary antibodies, and proteins were detected by ECL (Amersham Biosciences).

**Random Mutagenesis of yhp23**—A library of Yhp23 mutants was constructed by error-prone PCR using the HA-tagged yhp23 as template under mutagenic conditions described by Ishiguro et al. (31). To ensure equal mutation rates for each nucleotide, four separate PCRs depleted of a different dNTP were carried out. The resulting products were purified using the Qiagen Qiaquick PCR purification kit, digested with BamHI and HindIII, gel-purified, and subcloned into the pRS314GPD (HA-yhp23-GFP) vector cut with the same enzymes and transformed into DH5α cells. After plating, an estimated 3 × 10^6 transformants were harvested for plasmid preparation.

For screening, the yhp23-deficient strain, YF240, harboring the full-length receptor expression plasmid p2H-Gal-ERGALV and the ERE-CYC1-LacZ reporter plasmid, were transformed with the mutant library and plated on glucose-containing selective medium. The yeast cultures were grown for 2 days, and transformants (~2 × 10^6) were replica-plated onto 2% galactose plus 1% raffinose, X-gal indicator plates containing 1 nm 1β-estradiol, and receptor activity was assessed after 24 h. Colonies displaying decreased receptor activity (light blue color or white) were identified and tested for GFP expression by indirect immunofluorescence or Western blotting using the monoclonal antibody HA, which recognizes an epitope on Yhp23. Plasmids were rescued from isolates displaying GFP expression and were sequenced across yhp23 in its entirety and further characterized. In all cases, the phenotype was confirmed to be plasmid-borne by retransformation and retesting. Individual amino acid changes were introduced by site-directed mutagenesis using the Stratagene site-directed mutagenesis kit and confirmed by sequencing.

**β-Galactosidase Assays**—For liquid β-galactosidase assays, yeast cultures were grown overnight in selective medium containing 2% glucose and then subcultured 1:10 into 2 ml of selective media containing 2% galactose plus 1% raffinose supplemented with 1 μM 1β-estradiol for 12 h at 30 °C. The yeast cultures (typically 200 μl) were transferred to 6-well plates, and the cell number was determined by measuring absorbance at 600 nm using a multiplate reader. 40 μl of permeabilization buffer (5 mM KCl, 0.5 mM MgSO_4·7H_2O, 60 mM Na_2HPO_4·7H_2O, 60 mM NaHPO_4·H_2O, pH 7.0, 5% CHAPS, 40 mM β-mercaptoethanol) was added to wells of a microtiter plate, and 40 μl of yeast culture were added, mixed, and incubated for 15 min at 37 °C. The reaction was initiated by the addition of 50 μl of lactoperoxidase and resorufin to each well, and the resorufin concentration was calculated for 5–15 min. The β-galactosidase activity was determined by measuring A_420. Receptor activity is presented in β-galactosidase units, which are calculated from the following equation: (1000 × A_420/[strain time] × V_{max}(reaction volume) × A_{420}). Plate β-galactosidase assays were performed using the primers 5′-ATACTCGGAGCTCCTTCCTCTC-3′ and 5′-TCATTGGCACTAGATCCTGGC-3′.
performed by replica-plating colonies from glucose plates onto 2% galactose plus 1% raffinose, X-gal indicator plates containing 1 nM 17β-estradiol. Yhp23 and Hsp90 Coimmunoprecipitation—Overnight cultures of strains containing various HA-tagged Yhp23 mutants (5 ml) were subcultured into 50 ml of minimal yeast medium and grown until exponential phase (A600 < 1.0). Cells were pelleted, washed once in deionized water, resuspended in 500 μl of yeast lysis buffer supplemented with 5 mM ATP where indicated, and transferred to a 2-ml microcentrifuge tube. An equal volume of acid-washed beads was added, and the samples were vortexed for 15 min at 4 °C. The beads and cell debris were removed by low speed centrifugation, and the supernatant was transferred to a new tube. The beads were washed once by adding 250 μl of lysis buffer, and the two supernatants were combined. The lysates were centrifuged at 10,000 rpm for 10 min at 4 °C, and protein concentration was determined. Extracts containing 5 mg of protein in 600 μl were used for each immunoprecipitation. An antibody against HA (10 μg; Covance) was added, and the samples were rocked at 4 °C for 2.5 h. A 50-μl slurry of Protein G-agarose equilibrated in lysis buffer was added, and the samples were rotated for an additional 1.5 h. The agarose beads were collected by centrifugation for 2 min at 3,000 rpm and washed five times in 500 μl of lysis buffer. After the last wash, the samples were spun for 4 min at 3,000 rpm, and the residual lysis buffer was removed. The beads were resuspended in 25 μl of 1 × SDS-sample buffer, boiled for 10 min, and pelleted, and a portion of the supernatant was loaded onto a 10% SDS-polyacrylamide gel.

Serial Dilution Drop Test—Yeast strains were grown in minimal yeast medium at 30 °C until exponential phase. The yeast cultures were diluted with deionized water to 107, 106, 105, 104, and 103 cells/ml, and 10 μl were carefully placed as drops onto plates and incubated for 24 h at the indicated temperatures.

RESULTS

Effects of Yhp23 C-terminal Truncations on Estrogen Receptor Signaling in Yeast—To begin to assess the region(s) of Yhp23 that affect ER function, we constructed a series of Yhp23 C-terminal truncations (Fig. 1A), which lack 38 (Yhp23-(1–178)), 71 (Yhp23-(1–145)), or 93 residues (Yhp23-(1–123)) and assayed their ability to increase ER transcriptional activity in yeast in the presence of 1 nM 17β-estradiol. As seen in Fig. 1B, expression of Yhp23-(1–178) increases ER activity to ~60% of that observed with full-length Yhp23 (Yhp23-(1–216)), whereas overexpression of Yhp23-(1–145) exhibits 30% of wild type activity. No increase in ER activity, however, is observed upon coexpression of the Yhp23-(1–123) derivative. Immunoblotting established that all Yhp23 species and ER are produced at equivalent levels (Fig. 1C). Thus, progressive deletion of the Yhp23 C terminus results in a corresponding reduction of its action on ER, and the minimal effect of Yhp23 on ER transcriptional activity requires residues 123–145. This suggests that the C terminus is required for full Yhp23 activity with respect to ER signal transduction in vivo.

Yhp23 Mutants That Affect ER Signaling—To further characterize Yhp23, we screened a series of random point mutations in yeast for defects in ER signal transduction. To generate a pool of mutants, the yhp23 gene was mutagenized in vitro by
error-prone PCR under conditions that produce, on average, two mutations per \emph{yhp23} molecule. The mutagenized products were reinserted into a yeast GFP-coding expression vector containing an HA epitope to create a library of \emph{yhp23} mutants in the contexts of C-terminal GFP and an N-terminal HA tag. The GFP moiety provides a simple way to eliminate mutations that introduced premature stop codons in the \emph{yhp23} protein, since they would not produce GFP. The mutant \emph{yhp23}-GFP library (of /\text{H}100130,000 clones) was transformed into the \emph{yhp23}-deleted strain containing both an ER expression vector and an ERE-linked \emph{\text{H}}9252-galactosidase reporter plasmid (Fig. 2). Colonies displaying wild-type \emph{yhp23} function are dark blue, whereas colonies with reduced \emph{yhp23} function are light blue or white. In this manner, 90 colonies were selected as possible \emph{yhp23} loss-of-function mutants from a total of 20,000 transformants. Of the 90 colonies, nine expressed full-length \emph{yhp23}-GFP mutants and were isolated and sequenced. From these clones, two single mutants (A13V and E129K) and three double mutants (I117T/K73E, I117N/P44S, and V26D/V112G) representing different regions of \emph{yhp23} were selected for further studies. The remaining mutants had three or more alterations and were not evaluated further. The double mutations were uncoupled by site-directed mutagenesis to enable the identification of the alteration responsible for the loss of \emph{yhp23} function. Additionally, a frameshift mutation was fortuitously identified at residue 189 (189-shift) that results in a change of 20 amino acids at the C terminus of \emph{yhp23} (Fig. 3A). The original frameshift mutation resulted in a stop codon at position 209 and no longer expressed GFP but was engineered to place the GFP moiety back in frame. The GFP fusion form of 189-shift was used for the subsequent experiments and behaves as the original mutant in all of the assays.

The selected \emph{yhp23} mutants were analyzed for their ability to increase ER transcriptional activation in yeast as compared with wild type \emph{yhp23} in the presence of 1 nM 17\beta-estradiol (Fig. 3B). Various mutant phenotypes were observed, ranging from subtle alterations to complete loss of \emph{yhp23} function in ER signaling. The alterations in two of the double mutants, I117T/K73E and I117N/P44S, when uncoupled, revealed that the K73E and P44S had little effect on ER signal transduction and that the I117T and I117N alterations were responsible for the mutant phenotype. Interestingly, I117N displays a more dramatic decrease in activity than I117T. Uncoupling the double mutant V26D/V112G demonstrated that the V26D alteration is responsible for the mutant phenotype. Single amino acid substitutions A13V and E129K reduce the ability of \emph{yhp23} to enhance ER-dependent transcriptional activation by 30%. Interestingly, the overexpression of mutant 189-shift causes a decrease in ER activity relative to that in yeast transformed with the “empty” expression vector control, suggesting that the 189-shift mutant is behaving in a dominant negative fashion, perhaps by interacting with Hsp90 in a nonproductive fashion. Immunoblot analysis of yeast with an HA antibody revealed that the levels of expression of the \emph{yhp23} mutants were similar to the wild type \emph{yhp23} in every case but two, in which V26D/V112G and V26D mutations appear to destabilize the \emph{yhp23}
protein. The 189-shift alteration migrates slightly faster than the other mutants, most likely as a result of the change in amino acids at the carboxyl-terminal portion of the molecule (Fig. 3C). Thus, the A13V, I117T, I117N, and E129K alterations diminish Yhp23 function, whereas the V26D mutation affects protein stability, and K73E, P44S, and V112G changes appear to not perturb Yhp23 activity when assayed for function in ER signaling.

**Yhp23 Mutants Affecting ER Function**

FIG. 4. Association of Yhp23 with Hsp90 in vitro. A, strain YF240 expressing HA-tagged versions of either the wild type Yhp23 (yhp23), the 189-shift alteration (189-shift), the I117N mutation (I117N), or a vector only (v.o.) control were immunoprecipitated with HA under nondenaturing conditions from whole-cell extracts in the presence of ATP (+ATP), and associated proteins were resolved by SDS-PAGE. Hsp90 associated with Yhp23 was detected by immunoblotting with antibodies against yeast Hsp82 and HA for Yhp23. Note the antibody to Hsp82 reacts with both Hsp82 and Hsc82. The left panel shows the expression of Yhp23 and yeast Hsp90 (Hsp82 and Hsc82) prior to immunoprecipitation (input), and the right panel reveals that Hsp90 was immunoprecipitated with Yhp23 (IP). Two immunoreactive species are observed in the input lanes when probed with the Hsp82 antibody, with the top band representing the products of the two Hsp80 genes in yeast, Hsc82 and Hsp82, whereas the bottom band is a nonspecific cross-reacting species. Only the slower migrating species representing Hsc82 and Hsp82 interact with Yhp23. B, ATP dependence of the Yhp23-Hsp90 interaction. Strains expressing the wild type Yhp23 (yhp23) and the 189-shift alteration (189-shift) were lysed and immunoprecipitated in either the absence (-ATP) or presence of ATP (+ATP). The associated proteins were resolved by SDS-PAGE, and the Hsp90 associated with Yhp23 was detected by immunoblotting.

**Fig. 5. Yhp23 overexpression inhibits yeast growth at 37 °C.** The yhp23 deletion strain (YF240) was transformed with expression plasmids containing yhp23, 189-shift mutant, or the empty vector. These yeast strains were grown in liquid medium overnight and then standardized to A600 = 1. Serial dilutions were spotted onto selective plates and incubated for 24 h at 30 or 37 °C. Growth inhibition was most apparent at 37 °C and occurred in yeast strains expressing the wild type Yhp23 and the 189-shift mutant.
ence of the interaction between Hsp90 and the 189-shift mutant. Since the 189-shift alteration appears to act in a dominant negative fashion to decrease ER signaling, it is conceivable that this mutant is binding inappropriately to the non-ATP-bound form of Hsp90 and thus fails to affect ER-dependent transcriptional activation. As expected, Hsp90 was co-immunoprecipitated with wild type Yhp23 when ATP was present, whereas virtually no Hsp90 was detected in association with the wild type Yhp23 in the absence of ATP (Fig. 4B). In contrast, Hsp90 associates with the 189-shift mutant in the absence of ATP, indicating that this mutant does not have the same strict requirement for the ATP-dependent conformation of Hsp90 as the wild type p23 (Fig. 4B). Together, our results suggest that a loss of interaction of Yhp23 with Hsp90 or Yhp23 binding to the non-ATP-bound form of Hsp90 reduces its ability to promote ER signaling.

Effects of Yhp23 on Cellular Proliferation—In addition to maintaining ER in a conformation competent for ligand binding, Hsp90 also plays an important role in restoring protein function and promoting cell survival during environmental stress such as heat shock at 37 °C. The two yeast Hsp90 proteins, encoded by the hsp82 and hsc82 genes, can complement one another, whereas a double deletion is lethal. In contrast, Yhp23 is not a heat shock protein, and the deletion of the gene does not affect cell survival at elevated temperatures. To determine whether the Yhp23 affects cell viability, we initially analyzed growth as a function of Yhp23 overexpression in a strain containing both Hsp82 and Hsc82 at 30 and 37 °C. Interestingly, we observed a decreased growth rate for the yeast strain overexpressing Yhp23 at 37 but not 30 °C (Fig. 5). Strikingly, this phenotype was more dramatic upon expression of the 189-shift mutant, which associated more strongly with Hsp90 (Fig. 5). These findings suggest that Yhp23 overexpression can reduce cellular proliferation during times of cell stress.
The decreased growth of yeast upon Yhp23 overexpression at 37 °C suggests that Yhp23 may be inhibiting the function of the Hsp90 proteins. We therefore examined the effect of lowering yeast Hsp90 levels on Yhp23-dependent growth inhibition by expressing Yhp23 in an hsc82-deficient strain. Importantly, Yhp23 overexpression now results in growth inhibition at 30 °C in the hsc82 deletion strain, with a more severe phenotype occurring at 37 °C, whereas little difference in growth rates at 30 or 37 °C was observed in strains not overexpressing Yhp23 (Fig. 6A, top two panels). This finding is consistent with the idea that Yhp23 overexpression antagonizes Hsp90 under conditions of stress, when cells experience a greater demand for Hsp90 activity to preserve protein function and cell viability. Thus, Yhp23 sequesters Hsp90 and effectively lowers the buffering capacity of Hsp90 to protect cells against the lethal consequences of heat shock; this may represent a novel assay for Hsp90 and Yhp23 interaction in vivo.

We next examined the effect of the Yhp23 mutants on cellular proliferation in the hsc82 deletion strain at 30 and 37 °C. We would expect that Yhp23 mutants with reduced Hsp90 binding capacity will be less effective at inhibiting growth, whereas Yhp23 alterations that did not effect or increase Hsp90 association will inhibit growth equal to or greater than wild type Yhp23. In general, this prediction holds true. For example, expression of the I117N mutant that lost the ability to activate ER-dependent transcriptional activity did not inhibit growth at either 30 or 37 °C relative to the vector only control, consistent with the lack of interaction between the I117N mutant and Hsp90. Overexpression of Yhp23 mutants with reduced ability to activate ER-dependent transcriptional activity, such as V26D, I117T, A13V, and E129K, displayed intermediate growth inhibition phenotypes at 37 °C (Fig. 6A). Alterations in Yhp23 that retained their ability to activate ER-dependent transcriptional activity (K73E and P44S) inhibit cellular pro-
p23 Mutants Affecting ER Function

**Fig. 8. Response of ER to Yhp23 mutations near the hydrophobic cleft.** A, locations of the point mutations neighboring the hydrophobic pocket on the Yhp23 structure and corresponding to the following colors: green, Ile117; orange, Leu107; purple, Thr108; blue, Tyr114. B, wild type Yhp23 and mutant Yhp23 alleles, I28A, L107A, T108A, and Y114A, were assayed for β-galactosidase activity in yhp23 deletion strain YF240 containing a galactose-inducible ER expression plasmid and an ERE-β-galactosidase reporter plasmid in the presence of 1 μM 17β-estradiol (+E2), with the exception of the first two columns, which correspond to ER activity in the absence of Yhp23 (vector only; v.o.) in the absence (−E2) or presence of hormone (+E2). C, strain YF240 expressing HA-tagged versions of either the wild type Yhp23 (yhp23), Y114A mutation (Y114A), or a vector only (v.o.) control was immunoprecipitated as described in Fig. 4. Yeast Hsp90 (Hsc82/Hsp82) associated with Yhp23 was detected by immunoblotting with antibodies against yeast Hsp82 and HA for Yhp23. The total amount of Yhp23 immunoprecipitated was used to standardize the amount of associated yeast Hsp90 by densitometry, with the wild type Yhp23/Hsp90 ratio arbitrarily set as 1. The Yhp23 Y114A mutant has almost twice as much Hsp90 associated as wild type Yhp23. The average increase in the ratio of Hsp90 associated with the Y114A allele from three independent experiments is 1.94 ± 0.22. Shown is a representative experiment.

DISCUSSION

This report describes the isolation and characterization of Yhp23 mutants. The mutants were recovered on the basis of their ability to affect ER signaling. In addition, we describe a novel assay for Yhp23 function in *vivo*, whereby overexpression of Yhp23 inhibits cellular proliferation at 37 °C in an Hsp90-dependent fashion. The ER signaling phenotypes of the Yhp23 mutants correlate well with their temperature-sensitive growth phenotypes, strongly suggesting that the same function(s) required to promote ER activity are also required to inhibit growth, namely interaction with Hsp90. For example, mutant proteins were identified that fail to promote ER activity (e.g., I117N) and no longer associate with Hsp90 in *vivo* or affect cell growth in *vivo*. A carboxyl-terminal frameshift mutant (189-shift) was also identified, which behaves in a domi-
tant negative fashion, reducing the activity of ER and strongly inhibiting proliferation in vivo. As progressive deletions of the carboxyl domain of Yhp23 retain partial activity toward ER, we would suggest that the 189-shift mutant is a novel gain-of-function alteration that interacts with Hsp90 in its non-ATP bound state, thereby blocking Hsp90 function and the activation of ER. These findings suggest that Yhp23 binding to the ATP-bound form of Hsp90 is crucial for function.

Although the major consequence of Yhp23 on ER appears to be through an Hsp90-dependent effect on ligand binding under our assay conditions, p23 overexpression may also affect the transcriptional activity independent of ligand binding and aid in the disassembly of transcription factors at cognate response elements (6, 28). It will be interesting to examine the Yhp23 mutants for their effects on transcriptional activity and transcription complex disassembly, which will also shed light on the contribution of Hsp90 to these processes. Moreover, by altering the conditions of the assay, a similar genetic strategy could be employed to identify Yhp23 mutations that affect transcriptional activity rather than ligand binding.

Whereas genetic studies alone cannot be used to infer the molecular mechanism of p23 action, the availability of the p23 crystal structure and molecular modeling techniques enabled us to relate functional aspects of p23 to specific structural features. As initially described by Weaver et al. (30), the structure of p23 reveals a hydrophobic cleft that may be a potential Hsp90 binding domain. Consistent with this proposal, the random mutations that affect ER function and Hsp90 interaction cluster near this region, and a targeted mutation adjacent to this domain resulted in Yhp23 with increased ability to stimulate ER transcriptional activity and association with Hsp90, further implicating this region as an Hsp90 binding domain.

Our findings also suggest that Yhp23 can antagonize Hsp90 function during times of cell stress. Overexpression of Yhp23 results in reduced cell viability at 37 °C, as compared with yhp23-deficient cells. Importantly, this phenotype was much more dramatic in a yeast strain lacking hsc82, suggesting that Yhp23 overexpression further exasperates the inherently decreased chaperoning potential of this strain and that Hsp90 activity can be modulated through alterations of Yhp23 expression. Although the relevance of our overexpression experiments to the function of p23 and Hsp90 in mammalian cells remains to be determined, alterations in the intracellular concentration of p23 could affect steroid receptor function. Conceivably, p23 overexpression would have a permissive effect on both ER ligand binding and receptor activity, thereby increasing its transcriptional regulatory properties. Conversely, down-regulation of p23 could decrease ligand binding and lead to the development of steroid resistance.

Alterations in the levels of p23 could have implications broader than its effects on steroid receptor signaling. Recent work has suggested that Hsp90 is involved in masking phenotypic variation on a multicellular level (33, 34). For example, the reduction of an organism’s Hsp90 chaperoning activity results in the expression of a mutant phenotype that was previously hidden. This finding complements previous work that suggests that mutation in certain proteins, such as p53, result in their increased dependence on Hsp90 for normal function (35). Our findings suggest that alterations in the level or activity of p23 can also affect Hsp90 action. We speculate that somatic alterations or amplification of p23 would antagonize Hsp90 activity and render p53 mutations (or other clients) that rely on Hsp90 for activity nonfunctional.

Taken together, our findings suggest that p23 has the potential to affect multiple cellular processes through its ability to associate with and affect Hsp90 activity. By favoring the formation of Hsp90 regulatory complexes, p23 can increase the activity of Hsp90 clients, such as ER, thereby increasing cellular responses to estrogen. p23 also has the potential to compromise the “buffering” capacity of Hsp90, thus revealing mutant phenotypes previously held in check by the action of Hsp90. It would be interesting to examine the effect of p23 overexpression and ablation on ER-dependent cell growth and ER-regulated gene expression as well as to determine whether p23 levels are altered during breast tumor progression.

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