Localization of Sites of Interaction between p23 and Hsp90 in Solution*

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The co-chaperone p23 forms a complex with the chaperone Hsp90 that mediates the folding pathway leading to the production of functional steroid receptors. Solution NMR spectroscopy has been used to characterize sites of interaction between Hsp90 and p23. Titration of p23 with Hsp90 results in the selective broadening of certain cross-peaks in the 15N-1H heteronuclear single quantum correlation (HSQC) spectrum. The interaction sites on p23 and Hsp90 have been localized by dissection of Hsp90 into single-domain and two-domain constructs. The N-terminal (N) domain of Hsp90 does not affect the NMR spectrum of p23 either in the presence or absence of the ATP analogue ATPγS. Similarly, the HSQC spectrum of 15N-labeled N domain is unperturbed by the addition of p23. A subset of cross-peaks in the HSQC spectrum of p23 is shifted upon addition of the middle (M) domain of Hsp90, and the same shifts are observed upon the addition of the two-domain construct containing the N and M domains (NM). The addition of the co-chaperone Aha1, which is known to bind to the M domain of Hsp90, displaces p23 from Hsp90. The resonances that shift upon addition of the M and NM Hsp90 constructs correspond to those that were broadened at the lowest ratios of full-length Hsp90 to p23 and define an Hsp90 binding site that includes much of the C-terminal sequence of p23 together with a contiguous β-hairpin from the N terminus. We conclude that p23 forms a specific complex with Hsp90 primarily through binding to its middle domain.

The 90-kDa heat shock protein Hsp90 is a highly conserved molecular chaperone that is present in abundance in eukaryotic cells (~1–2% of cytosolic protein) (1–3). The protein is found as a homodimer; the monomer unit consists of three domains, the N-terminal domain (N), a middle domain (M), and a C-terminal dimerization domain (C) (Fig. 1A). The N domain has been associated with the weak ATPase activity of Hsp90 and may also be involved in co-chaperone and client protein binding. The M domain has been postulated as a major site of client protein binding but also influences the ATPase activity. In eukaryotic Hsp90s, the N and M domains are separated by a charged linker sequence of ~60 residues; this segment is absent in prokaryotes. A popular model for Hsp90 action states that the binding of ATP or inhibitors causes conformational changes that influence the binding of clients and co-chaperones and may (4) or may not (5) promote transient dimerization of the N domain. The C domain may also contain a substrate binding site (6, 7) and has a sequence at the C terminus that is specific for binding of tetratricopeptide repeat proteins.

A number of x-ray crystal structures have been published of domains of Hsp90; there is as yet no crystal structure of the full-length protein. Available structures include that of the N domain (8–13) and of the M domain (14, 15) of eukaryotic Hsp90s. Recently, the structures of the C domain (16) and of a two-domain construct (N+M) of Hsp90, the Escherichia coli analogue of Hsp90 (17), have been reported.

Hsp90 interacts with a number of client proteins, including steroid hormone receptors, kinases, and polymerases (18). Steroid hormone receptors have been found to exist in long-lived complexes with Hsp90 and co-chaperones (particularly p23 and immunophilins) in the absence of steroid ligand (1). A number of co-chaperones are involved in the function of Hsp90, and new co-chaperones are constantly being identified. The literature consensus at present is that the association of co-chaperones with Hsp (Hsc)70 and Hsp90 determines the client specificity of these chaperone systems. Yet, for even the best known and most extensively studied co-chaperones, we are only beginning to understand their specific role in the function of Hsp90 and the structural basis for this interaction. For example, the structure of the complex between the stress-related co-chaperone Aha1 and Hsp90 was recently reported (15), but no such structural studies have been reported on the p23-Hsp90 interaction. In recent years, interest in the Hsp90 system has increased exponentially, as the potential of Hsp90 inhibitors, such as geldanamycin, as anti-cancer agents has been recognized. Nevertheless, there remain a number of unresolved questions about the molecular mechanisms of Hsp90-mediated processes, and contradictory experimental evidence abounds in this field. For example, early in vitro studies implicated Hsp90 in ATP-mediated binding and chaperoning of unfolded proteins (19), but this activity has yet to be demonstrated in vivo, where Hsp90 interacts with specific client proteins after they have apparently attained a degree of tertiary structure (20).

Although the N-terminal domain of Hsp90 is the site of ATP binding (9), the isolated N domain has no detectable ATPase activity (10). The M domain is thought to contribute to the ATPase activity by conformational restriction of the γ-phosphate of ATP, which is disordered in the N domain/ATP structures; the crystal structure of the M domain contains a potential catalytic loop distantly homologous to those found in structurally related proteins (14). Interestingly, this loop shows a number of different conformations in crystallographically independent copies of the M domain (14), an indication that it may be mobile in the absence of the N domain and ATP. The crystal structure of the yeast Hsp90 middle domain complexed with the N-terminal domain of the co-chaperone Aha1 (15) indicates a probable role for Aha1 in stabilizing a conformation of this catalytic loop that facilitates ATP hydrolysis.

There is a complicated relationship between ATP hydrolysis, client protein binding, and co-chaperone interactions. One suggested sequence of events is: (i) binding of client protein to an “open” form of the Hsp90 dimer, (ii) binding of ATP to the N-terminal domain, (iii) tran-

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sient dimerization of the N-terminal domain, clamping the client protein inside, (iv) binding and assistance of co-chaperones, forming a multichaperone complex (reviewed in Ref. 3). Other recent studies focus on the role of bound ATP in increasing the affinity of Hsp90 for client proteins (5) and the effects of bound client protein and p23 on the ATPase activity of Hsp90 (21). The role of p23 remains confusing; it has been suggested that it binds to both the N-and C-terminal domains of Hsp90 and that binding requires the presence of ATP analogues (22). One study concluded that the role of p23 was to stabilize the interaction between Hsp90 and the client protein (23), whereas others appear to implicate p23 in client protein release after ATP hydrolysis (24). Both the N and M domains of Hsp90 have been implicated in the binding of p23 (25, 26). Using NMR, we have examined the interactions of p23 with Hsp90 and its isolated domains in solution. We have found that p23 binds to full-length Hsp90, but does not bind to the isolated N-terminal domain either in the presence or absence of an ATP analogue. Instead, p23 binds via a specific binding site to the M domain either as an isolated domain or in two-domain constructs containing the N and M domains or the M and C domains. In addition, p23 can be displaced from the Hsp90 complex by the addition of the co-chaperone Aha1, which has been shown to bind to the M domain (15).

EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—The p23 constructs p23-(1–160) and p23-(1–119) were amplified by PCR from a human liver cDNA library and cloned into the expression vector pET21a. p23 constructs were expressed as soluble proteins in *E. coli* BL21 DE3 (DNAY) grown in M9 minimal medium with overnight induction at 15 °C. Cell pellets from p23 cultures were lysed in 10 mM Tris, pH 8, 20 mM DTT, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and the soluble protein was purified on HiTrapQ ion exchange columns (GE Healthcare) equilibrated with Buffer A (10 mM Tris, pH 8, 200 mM NaCl, 0.5 mM EDTA, 1 mM DTT). The purity of the proteins was assessed by SDS-PAGE. The proteins were refolded by diluting the purified samples into Buffer B (100 mM Tris, pH 8, 150 mM NaCl, 2 mM DTT, 20% glycerol) and allowed to equilibrate at 4 °C overnight. The refolded proteins were concentrated and dialyzed against Buffer C (20 mM Tris, pH 8, 200 mM NaCl, 0.5 mM EDTA, 1 mM DTT) before being stored at −20 °C.

2 The abbreviations used are: DTT, dithiothreitol; HSQC, heteronuclear single quantum correlation; ATP-γ-S, adenosine 5'-3-O-(thio)triphosphate; AMP-PNP, 5'-adenylylimidodiphosphate; CD, circular dichroism.

![FIGURE 1](image1.png)

**FIGURE 1.** A, schematic diagram of the domain organization of human Hsp90. The C-terminal MEEVD sequence is the binding site for tetratricopeptide repeat proteins. The N-terminal domain is the site of ATPase activity. B, alignment of the amino acid sequences of p23 from a variety of species and related proteins SBA1 from yeast and human SGT1. Conserved amino acids are colored according to type: hydrophobic residues (A, V, I, L, M) are shown in various shades of green, positively charged residues (R, K) in blue, negatively charged residues (D, E) in red, cysteine in yellow, small hydrophilic amino acids (T, S) in light yellow, N, Q, P, G in pink, and aromatic residues (H, F, Y, W) in purple.

![FIGURE 2](image2.png)

**FIGURE 2.** Far-UV circular dichroism spectra. A, p23-(1–119) (squares) and p23-(1–160) (triangles). B, Hsp90 single-domain constructs N (squares), M (triangles), C, Hsp90 two-domain constructs NM (triangles) and MC (squares).
ibrated with 10 mM Tris, pH 8, 2 mM EDTA, 1 mM DTT. Bound p23-(1–160) was eluted from the Q column with a linear gradient to 1 M NaCl. Fractions containing p23-(1–160) were pooled and further purified by gel filtration chromatography on a Sephacryl S100 HR XK16 column in 10 mM Tris, pH 7, 100 mM NaCl. Protein yields were ~50 mg/liter for p23-(1–160) and ~17 mg/liter for p23-(1–119).

Full-length Hsp90 was prepared by expression in E. coli. Clones for human and chicken Hsp90 were obtained from Dr. David Toft (Mayo Clinic, Rochester, MN). In our hands, the chicken protein showed a slightly greater expression level, and it was therefore chosen for further experiments. Chicken Hsp90 is 91% identical to human Hsp90α over the entire protein sequence. Full-length chicken Hsp90 was prepared by a modification of the published method (27). The protein was expressed in minimal medium in the host BL21 DE3 pLysS with induction at 15 °C for 12–20 h. Cells were lysed in 25 mM Tris, pH 8, 40 mM KCl, 4 mM EDTA, 10 mM DTT by sonication and the soluble fraction applied to a 60-ml Sepharose Q FF column equilibrated with 25 mM Tris, pH 7.5, 40 mM KCl, 4 mM EDTA, and 2 mM DTT. Full-length Hsp90 was eluted with a linear gradient to 1 M KCl and dialyzed against 25 mM Tris, pH 7.5, 50 mM KCl, 4 mM EDTA, and 1 mM DTT. Hsp90 was applied to a 40-ml heparin-Sepharose CL-6B column equilibrated with the same buffer and eluted with a linear gradient to 0.5 M KCl. The protein was concentrated in a Centriprep10 centrifugal filter device (Amicon) and purified by gel filtration chromatography on an 180-ml Sephacryl S200HR XK16 column in 20 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 200 mM KCl. Hsp90 (yield ~5 mg/l minimal medium) was concentrated and exchanged by dialysis into 10 mM Tris, pH 6.9, 50 mM KCl, 8 mM MgCl2, and 2 mM DTT for NMR.

Constructs for the N-terminal domain (N domain, residues 1–234), middle domain (M domain, residues 290–550), N terminus plus middle domains (NM construct, residues 1–550), and middle plus C-terminal domains (MC construct, residues 290–728) of chicken Hsp90 and the N-terminal region of Aha1 (human residues 1–162) were cloned into the T7 expression vector pET15b (Novagen), which introduces an N-terminal His tag and thrombin cleavage site into the expressed protein. All truncated Hsp90 constructs and Aha1-(1–162) were expressed in BL21 DE3 (DNAY) with induction at 15 °C for 12–20 h. Cells were lysed by sonication in 20 mM Tris, pH 8, 200 mM KCl, and 20 mM imidazole, and the soluble fraction of the cell lysate was purified by metal affinity chromatography on nickel-nitrilotriacetic acid (Qiagen). His-tagged proteins were eluted with 180 mM imidazole and then purified by anion exchange chromatography on a HiTrap Q-Sepharose HP column (GE Healthcare) in 25 mM Tris, pH 8, 50 mM KCl, 2 mM DTT, and 0.5 mM EDTA using a linear gradient to 1 M KCl. The protein constructs were further purified by gel filtration on a Sephacryl S100HR column (2.6 × 65 cm) in 25 mM Tris, pH 7.5, 150 mM KCl, 0.5 mM

FIGURE 3. Overlaid 15N-1H TROSY-HSQC spectra of p23-(1–160) (black) and p23-(1–119) (red). Inset, central portion of the p23-(1–160) spectrum is shown in black. Cross-peak assignments are indicated in red for residues common to the two proteins and black for those present only in the C-terminal tail of p23., ppm, parts/million.
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EDTA, and 2 mM DTT. All His-tagged Hsp90 constructs were expressed exclusively as soluble proteins. The N, M, and NM constructs appear to be monomeric by gel filtration chromatography, whereas the MC and full-length constructs are dimeric.

**NMR Spectroscopy**—NMR sample concentration for p23 was 0.3 mM in 0.5 ml of NMR buffer at pH 6.8 (p23-(1–119)) or 7.0 (p23-(1–160)) containing 95% H$_2$O/5% D$_2$O or 99.99% D$_2$O. NMR spectra were acquired at 20°C on Bruker DRX600, AVANCE 800, and AVANCE 900 spectrometers. Spectra were processed with NMRPipe (28) and analyzed with NMRView (29). Backbone resonance assignments were made using HNCA, HNCO, CBCA(CO)NH, HNCACB and HCA(CO)CANH spectra. Side chain assignments were made using three-dimensional $^{15}$N total correlation spectroscopy and nuclear Overhauser effect spectroscopy spectra and three-dimensional HCCH-COSY. Resonance assignments for p23 have been deposited in the Biomagnetic Resonance data bank (accession codes 6973 and 6974). For NMR binding studies, p23, Aha, and Hsp90 constructs were concentrated and dialyzed into 10 mM Tris, pH 6.9, 50 mM KCl, 8 mM MgCl$_2$, and 2 mM DTT.

**CD Spectroscopy**—CD spectra were collected with 5–10 μM protein in 3 mM Tris, pH 8, at 25°C in a 0.2-cm cell using an Aviv Model 202 CD spectrometer.

**RESULTS**

**Design and Characterization of p23 Constructs**—To explore the conformational preference of the C-terminal tail of p23, as well as to confirm the structure of the N-terminal domain as delineated in the x-ray crystal structure (30), two p23 constructs of different lengths were prepared, p23-(1–119) (encompassing the folded domain) and p23-(1–160) (the complete protein). The amino acid sequence of human p23 is shown in Fig. 1 aligned with a number of p23 sequences from eukaryotic organisms, including yeast and fungi. Only 10 residues are identical among all of these organisms (W8, R11, P61, K79, W86, L89, K95, F103, W106, and D108), although many others form highly homologous sets. In particular, the p23 sequences of the higher eukaryotes exhibit nearly total identity, even in the C-terminal sequence (Fig. 1B).

The CD spectra of human p23-(1–119) and p23-(1–160) are unusual (Fig. 2A). The CD spectra, which are similar to published spectra (31), are consistent with β-structure in the N-terminal domain together with an unstructured region at the C terminus (shown by the increase in the negative ellipticity at 202 nm for p23-(1–160). The positive ellipticity at 178° nm is due to the high proportion of tryptophan residues (a total of 5) in the protein.

A comparison of the $^1$H-$^15$N HSQC spectra of $^15$N-labeled p23-(1–119) and p23-(1–160) (Fig. 3) confirms that the N-terminal domain is folded and the C-terminal acidic tail is unfolded. There are very few cross-peaks that differ significantly between the two constructs. These differences mostly arise for histidines and for those residues, such as Cys-75, that are in close proximity to histidine side chains in the folded N-terminal domain and are thus consistent with the slight pH difference between the solutions used for the two spectra. These results confirm that the presence of the C-terminal sequence neither influences the structure of the N-terminal domain nor makes specific contacts with it.

An x-ray structure has been published for the N-terminal domain (residues 1–110) of p23 (30). The structure consists of a β-sandwich, formed of 5-strand and 3-strand β-sheets. Examination of the three-dimensional $^1$H-$^15$N nuclear Overhauser effect spectrum of p23-(1–119) reveals that the β-sheet structure of p23 is the same in solution as it is in the crystal. A map of the cross-strand NOEs observed in the nuclear Overhauser effect spectrum confirms the presence of the two β-sheets of the β-sandwich.

**Interaction of p23 with Hsp90**—The addition of full-length Hsp90 to p23 in the presence or absence of ATPγS results in broadening of the NMR signals in the HSQC spectrum, consistent with the binding of a large dimeric partner (Fig. 5A). As Hsp90 is titrated into the solution of $^15$N-labeled p23-(1–160) or p23-(1–119), certain resonances, such as Gly-83 and Lys-107, are found to broaden even at the lowest Hsp90 concentration, whereas others, including those assigned to the C-terminal tail, remain visible even at the highest Hsp90 concentration. The resonances of several of the tryptophan side chains show small shifts at low concentrations of Hsp90 before being broadened beyond detection at higher Hsp90 concentrations. This is also true of some of the backbone amides, such as those of Asp-111 and Val-15.
Interaction of p23 with Domains of Hsp90—Complex formation between p23 and Hsp90 was shown to depend on the presence of ATP (33); the binding site for p23 has therefore been assumed to be to the N-terminal domain of Hsp90, which contains the major ATP binding site. However, there is no published structural evidence that this is so. To define more precisely the sites of interaction of p23 and Hsp90, the one- and two-domain constructs of Hsp90 were added to labeled samples of p23, and unlabeled samples of p23 were added to 15N-labeled samples of the Hsp90 N and M domains and the NM construct. No effect was observed on the spectrum of 15N-labeled p23 upon the addition of the N domain construct (Fig. 6B). Similarly, no effect was observed on the spectrum of 15N-labeled N domain upon the addition of p23 (data not shown). However, specific concentration-dependent changes in the spectrum of p23 were observed upon the addition of the M domain (Fig. 5B).

The NMR spectrum of p23 is affected in almost identical ways by the addition of the NM construct (Fig. 6A), and these resonances correspond to those that are preferentially broadened and/or shifted upon the addition of full-length Hsp90 (Fig. 5A). The magnitudes of the chemical shift changes and resonance broadening observed in the presence of the NM construct are the same with and without the ATP homologue ATPγS (Fig. 6A). A comparison of the changes caused by addition of the N, M, and NM constructs in a portion of the HSQC spectrum of p23 is shown in Fig. 6B.

Competition between p23 and Aha1 for Binding to the Hsp90 Middle Domain—Aha1, a stress-related co-chaperone of Hsp90, has been rel-
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Localization of the Binding Site of Hsp90 on p23—The $^1$H-$^1$N HSQC spectrum of p23 shows differential broadening and shift of some cross-peaks upon the addition of full-length Hsp90. The residues that show changes are mapped onto the structure of p23 (30) in Fig. 7A. The changes in the $^1$N HSQC spectrum of p23 with the addition of the one- and two-domain parts of Hsp90 are consistent with specific binding to a well-defined region at the tip of the p23 structure (Fig. 7B) that is completely consistent with the region affected by full-length Hsp90 (Fig. 7A). The binding site is shown in Fig. 7B to encompass a face of the folded portion of p23 that includes several of the conserved tryptophan side chains. This is reflected, for example, in the shifts of the indole NH resonances of these side chains (Figs. 5 and 6). Much of the sequence between residues 100–112 appears to be affected by Hsp90 binding, and some of the most significant effects are observed on Asp-111, which was undefined in the x-ray coordinates. The surface of p23 that interacts with Hsp90 has no unusual charge or hydrophobicity characteristics. The surface contains a number of charged side chains, including negative charges at positions 13, 81, 108, and 110 and positive charges at positions 11, 88, and 107. These charged side chains form a cap over the tryptophan side chains of the interaction site (Trp-8, -86, -106, and -109). Some of these residues are conserved, not only within the eukaryotic p23 sequences, but even to quite distantly related sequences such as yeast Sba1 and human Sgt1 (Fig. 1). The tryptophans show the most consistent sequence identity, with Trp-8 and -86 completely conserved throughout the entire set shown in Fig. 1. Trp-106 is conserved in p23 and Sba1, and Trp-109 is conserved only in higher eukaryotes. A conservation of negatively charged residues in the region 108–112 (human numbering) is common to all sequences except Sgt1, and the prominent presence of this region of the sequence in the Hsp90 binding site identified by NMR is an indication that a negative charge is likely required for binding by p23 (although not apparently for Sgt1). The fact that the crystal structure (30) is undefined for residues after 110 may be an indication that this region is not well structured in the free p23 but becomes less mobile in the Hsp90 complex.

Localization of the p23 Binding Site on Hsp90—It was previously thought that p23 interacted exclusively with the nucleotide-bound state of Hsp90 (22). However, recent results show that human p23 binds, albeit ∼10-fold more weakly, to nucleotide-free Hsp90, as well as to the Hsp90-AMP-PNP complex, as measured by calorimetry or surface plasmon resonance (26). Conformational changes resulting from both binding of nucleotide and N-terminal domain dimerization are thought to enhance the affinity of p23 for Hsp90 (26, 35). As a complement to these studies, we present the first structural characterization of the interaction site between p23 and Hsp90. The spectral changes that result from the addition of p23 to the Hsp90 N and M domain constructs show unequivocally that the primary binding site for p23 is the middle domain of Hsp90. This result is consistent with previous mapping and mutagenesis studies indicating that the Hsp90 middle domain is required for binding of p23 (25, 35). Our NMR studies indicate no requirement for the presence of ATPγS, as evidenced by lack of change in line width or chemical shift, for any of the interactions of p23 with Hsp90, either full-length or its fragments. However, an enhancement in affinity due to nucleotide binding

**FIGURE 6.** A, overlay of portions of the 800 MHz $^1$H-$^1$N HSQC spectra at 30 °C of p23-(1–119) (black), after the addition of 0.2× molar ratio (red) and 0.4× molar ratio (yellow) of the Hsp90 NM construct and 0.4× molar ratio Hsp90 NM + ATPγS (green outline). B, portion of an 800 MHz HSQC spectrum at 30 °C of p23-(1–119) (black) in the presence of 3.8× molar ratio of Hsp90 N domain (red outline) compared with the spectra obtained with 1.0× molar ratio of the NM construct (yellow) and a 1.0× molar ratio of Hsp90 M domain (green outline). C, portion of an 800 MHz TROSY spectrum at 30 °C of p23-(1–119) (black) in the presence of a 0.5 molar ratio of the Hsp90 M domain (green) and upon further addition of a 0.5× molar ratio of Aha1-(1–162) (red outline). The black and red cross-peaks are coincident, indicating that the spectrum of p23, shifted by the addition of the M domain protein (to the green spectrum), has reverted to the same spectrum as the free p23 upon the addition of Aha1. ppm, parts/million.

At first glance, it appears that Hsp90 is well characterized in its interactions with Hsp90 (15, 34). In particular, the N-terminal domain of Aha1 has been shown to bind to the middle domain of Hsp90, and the interaction has been structurally characterized (15). The co-chaperones Aha1 and p23 bind Hsp90 with similar affinities; the dissociation constant ($K_d$) for the binding of Aha1 to yeast Hsp90 in the presence of the ATP analogue AMP-PNP has been reported as 0.7 μM (34), whereas the $K_d$ values for p23 binding to human Hsp90 are 0.8× molar ratio of Hsp90 AMP-PNP and the yeast p23 analogue Sba1 to yeast Hsp90-AMP-PNP are 1.5 and 1.75 μM, respectively (26, 35). As shown above (Fig. 5B), certain resonances in the $^1$H-$^1$N HSQC spectrum of p23 are shifted by the addition of the unlabeled M domain. Addition of unlabeled Aha1 (1–162) to this mixture (Fig. 6C) causes the p23 cross-peaks to return to chemical shifts coincident with the unlabeled state. The same result was obtained upon the addition of Aha1 to mixtures of $^1$N-labeled p23 with full-length Hsp90 or the two-domain constructs NM and MC, indicating that the p23 binding site in all Hsp90 constructs overlaps with that of Aha1. This result is consistent with previous gel filtration analyses of yeast Hsp90, Aha1, and Sba1 mixtures (36).
would be difficult to measure by this method, especially for the larger complexes, because of resonance broadening.

**Effect of p23 on Hsp90 ATPase Activity**—The co-chaperones Aha1 and p23 have opposite effects on the ATPase activity of Hsp90. Although Aha1 enhances the ATPase activity ~12-fold, p23 inhibits Hsp90 ATPase activity (34, 35, 37). It has been proposed that p23 "locks" Hsp90 in a nucleotide-bound state that has high affinity for client proteins (26). Our results give insight into this suggestion. The crystal structure of the complex between Aha1 and the middle domain of yeast Hsp90 shows an extensive contact surface between the two proteins (15). Our results indicate that p23 uses a smaller surface to bind Hsp90. Under solution NMR conditions, Aha1-(1–162) displaces p23 from complexes with full-length or truncated Hsp90 constructs. Previous mapping studies have implicated the N-terminal α-β-α and part of the α-coil regions of the Hsp90 middle domain as necessary for p23 binding (25). This region of the Hsp90 middle domain contains residues that contact the N-terminal domain as well as a conformationally flexible loop (residues 370–390) implicated in ATPase activity. Residues of Aha1 that contact this loop in the Hsp90 middle domain are required for enhancement of the ATPase activity of Hsp90 (15). A possible mechanism for p23 inhibition of Hsp90 ATPase activity consistent with these observations is that p23 binds the Hsp90 middle domain in the region of the catalytic loop, perhaps locking the flexible loop in a conformation where it is unavailable to assist in orienting the phosphate group for hydrolysis.

**Role of the C-terminal Tail**—The role of the C-terminal ~45 residues of p23 remains obscure. It remains highly flexible and unstructured according to the NMR spectra under all conditions tested. We have been unable to demonstrate any detectable influence of peptides, unfolded protein sequences or folded proteins, including Hsp90, on the spectrum of this region of the protein. In terms of the interaction of Hsp90 with p23, it is clear that the C-terminal tail has no influence at all, because the spectral changes that accompany the addition of Hsp90 or its fragments are identical for p23-(1–160) and p23-(1–119). Sequence conservation of this region is lower than for other regions of the protein (Fig. 1B), although it retains a high proportion of sequence identity between higher eukaryotes. This region of the protein is highly negatively charged and also contains in all species many of the sequence markers for natively unfolded proteins (38). It appears likely that the C-terminal tail has a function related to its flexible and negatively charged nature, perhaps as a "fly casting" (39) lure for partner proteins or possibly in a nonspecific but charge-facilitated interaction with other chaperones, co-chaperones, or client proteins. A recent report implicates the C-terminal tail of p23 in cell-death response in the endoplasmic reticulum (40).

**CONCLUSION**

Examination of the interaction of Hsp90 with the co-chaperone p23 by NMR titrations of full-length and one- and two-domain constructs of Hsp90 reveal that p23 interacts with Hsp90 via a well defined site that encompasses much of the C terminus, plus an N-terminal hairpin loop. The resonances that are changed in the NMR spectrum of p23 upon addition of the N, M, NM, and full-length Hsp90 proteins are very similar, indicating that the more specific results obtained with the smaller Hsp90 constructs can be applied to infer that the location of the p23 binding site on Hsp90 is primarily via the middle (M) domain and that, under NMR conditions, the addition of the ATP analogue ATPγS has no influence on the p23-Hsp90 interaction. These results will form the basis for further work to characterize the Hsp90-p23 interaction in solution.

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