

# An Unstructured C-Terminal Region of the Hsp90 Co-chaperone p23 is Important for its Chaperone Function

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p23 is a co-chaperone of the heat shock protein Hsp90. p23 binds to Hsp90 in its ATP-bound state and, on its own, interacts specifically with non-native proteins. In our attempt to correlate these functions to specific regions of p23 we have identified an unstructured region in p23 that maps to the C-terminal part of the protein sequence. This unstructured region is dispensable for interaction of p23 with Hsp90, since truncated p23 can still form complexes with Hsp90. In contrast, however, truncation of the C-terminal 30 amino acid residues of p23 affects the ability of p23 to bind non-native proteins and to prevent their non-specific aggregation. The isolated C-terminal region itself is not able to act as a chaperone nor is it possible to complement truncated p23 by addition of this peptide. These results imply that the binding site for Hsp90 is contained in the folded domain of p23 and that for efficient interaction of p23 with non-native proteins both the folded domain and the C-terminal unstructured region are required.

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## Introduction

The molecular chaperone Hsp90 is one of the most abundant heat shock proteins in the cytosol of eukaryotic cells. Its level of expression is increased several fold when cells are exposed to environmental stress, and Hsp90 is hence thought to adopt a general protective function for cytosolic proteins under stress conditions. However, also under physiological conditions specific client proteins depend in their folding on the association with Hsp90 (for a review, see Buchner, 1999). These client proteins include steroid receptors (Pratt & Toft, 1997), tyrosine (Xu & Lindquist, 1993) and serine/threonine kinases (Schulte *et al.*, 1995) and reverse transcriptase (Hu & Seeger, 1996) among others. To assist the maturation process of these substrate proteins, the chaperone function of Hsp90 requires the cooperation with several partner proteins. Interestingly, some of these partner proteins are molecular chaperones themselves (Bose *et al.*, 1996; Freeman *et al.*, 1996) thus contributing to a "multi-chaperone complex".

As established for steroid hormone receptors, the most thoroughly investigated Hsp90 client proteins, the substrate sequentially moves through at least three defined chaperone complexes to reach the hormone binding state (Pratt & Toft, 1997).

p23 is one of the Hsp90 co-chaperones present in these substrate-chaperone heterocomplexes. It was first identified as a component of the mature progesterone receptor complex (Johnson *et al.*, 1994). p23 binds directly to Hsp90 even in the absence of substrate (Johnson & Toft, 1994) and this interaction is ATP-dependent (Johnson & Toft, 1995). The complex can be disrupted by geldanamycin (Johnson & Toft, 1995), an ansamycin antibiotic that had been described as an inhibitor of specific Hsp90 functions (Whitesell *et al.*, 1994; Whitesell & Cook, 1996; for a review, see Scheibel & Buchner, 1998).

Besides steroid hormone receptors, several other Hsp90 substrates including reverse transcriptase and, most recently, telomerase (Hu *et al.*, 1997; Holt *et al.*, 1999) have subsequently been described which seem to require the presence of p23 in the Hsp90 complex for folding. The function of p23 in this context is not understood yet, but *in vitro* assembly studies of glucocorticoid aporeceptor complexes suggest a stabilization of the hormone

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binding form of the steroid hormone receptor by p23 (Dittmar *et al.*, 1997). This observation is supported by more recent experiments in *Saccharomyces cerevisiae*. Deletion of *sba1*, the yeast p23 homologue, results in an increased sensitivity of Hsp90-dependent folding processes towards treatment with ansamycin antibiotics (Bohen, 1998), implying a destabilization of the Hsp90-substrate protein complex in the absence of p23.

*In vitro*, p23 has been attributed chaperone-like properties. It was shown specifically to bind partially folded proteins thus preventing their aggregation and maintaining them in a folding-competent state (Bose *et al.*, 1996; Freeman *et al.*, 1996). These findings suggest that in the Hsp90 complex, p23 contacts both the non-native protein and Hsp90 directly.

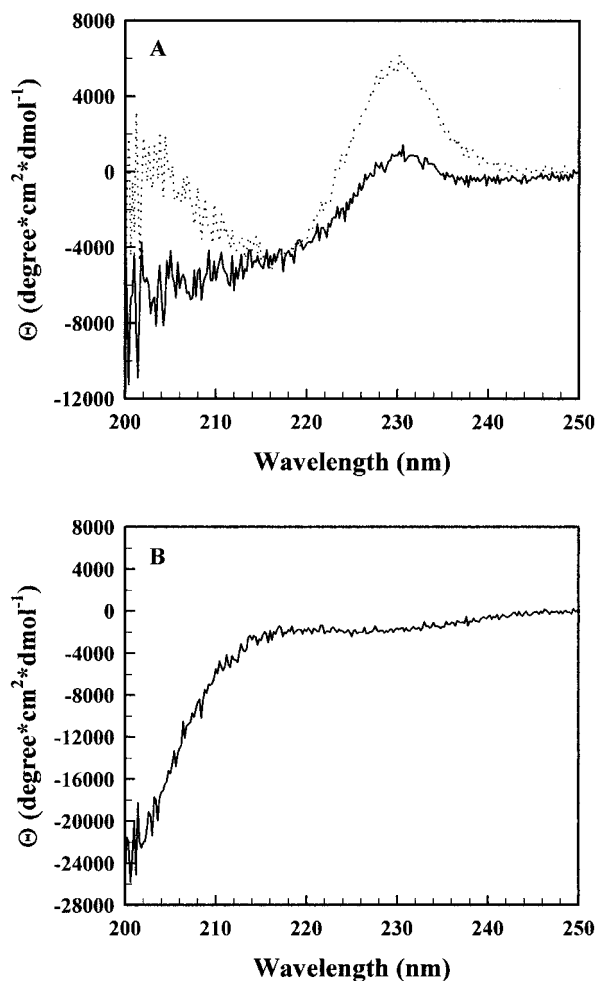
In an attempt to define the contribution of p23 to the chaperone activity of the Hsp90 complex we embarked on a structure-function analysis of p23. We found that p23 contains an unstructured C-terminal tail, which is required for its chaperone activity. Removal of this unstructured region leads to a complete loss of p23's chaperone activity. In contrast, the truncation does not inhibit the ATP-dependent association of p23 with Hsp90.

## Results

### p23 is a $\beta$ -sheet protein containing an unstructured C-terminal part

To gain insight into the secondary structure of human p23, a far-UV CD spectrum from 250 nm to 200 nm was recorded (Figure 1(a)). The spectrum exhibits an unusual peak at 230 nm which can be attributed to the high content of aromatic residues in the protein. The local minimum around 218 nm together with the low ellipticity suggest a  $\beta$ -sheet structure for p23. However, the negative ellipticity around 200 nm is uncharacteristic for natively folded proteins and led us to speculate that p23 may contain an unstructured region.

To test this hypothesis, we performed limited proteolysis experiments with p23 (data not shown). The incubation of full-length human p23 with proteinase K led to the generation of a stable fragment which is approximately 3 kDa smaller than the wild-type protein as judged from SDS-PAGE gels. This fragment is stable even after prolonged incubation or increased protease concentrations (data not shown). Similar results were obtained when trypsin instead of proteinase K was used (data not shown). The size of the proteolytic product was analysed by mass spectrometry. The fragment was determined to have a mass of 15,400 Da. Using N-terminal sequencing, the first amino acids of the fragment were identified as MQPA-SAK which corresponds to the N-terminal sequence of the wild-type protein. The results of these experiments, combined with the information on possible cleavage sites for proteinase K in human p23, unambiguously identified the



**Figure 1.** Secondary structure of human p23,  $\Delta$ p23 and PEP1 (a) Far-UV CD spectra of 0.2 mg/ml human p23 (—) and 0.2 mg/ml  $\Delta$ p23 (· · · · ·) in 40 mM potassium phosphate (pH 6.8) at 20°C. (b) Far-U-V CD spectrum of 0.2 mg/ml PEP1 in 40 mM potassium phosphate (pH 6.8) at 20°C.

fragment generated by proteolytic cleavage. It comprises amino acids 1 to 130 of p23 and lacks the 30 C-terminal amino acid residues. Taken together, these data tempted us to speculate, that human p23 has a poorly folded C-terminal tail of approximately 30 amino acid residues which is accessible to proteases.

### $\Delta$ p23 is a stable fragment with a well-defined secondary structure

To compare the structural features of both wild-type human p23 and the fragment identified by limited proteolysis, we cloned the fragment  $\Delta$ p23 (lacking the 30 C-terminal amino acid residues) into a bacterial vector and purified the recombinantly expressed protein.

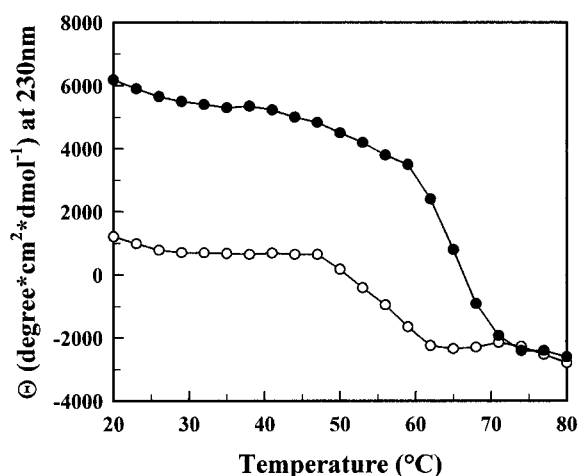
The far-UV CD spectrum of  $\Delta$ p23 is typical for a  $\beta$ -sheet protein with a local minimum of  $-5000$

degrees  $\text{cm}^2 \text{dmol}^{-1}$  around 218 nm (Figure 1(a)). The peak at 230 nm is even more prominent in  $\Delta\text{p}23$  than in the wild-type protein. As expected, the far-UV CD signal of  $\Delta\text{p}23$  increases between 210 and 200 nm, indicating a folded structure. In addition, we recorded a far-UV CD spectrum of PEP1, a chemically synthesized peptide corresponding to the last 37 amino acid residues of full-length human p23 (Figure 1(b)). In agreement with our hypothesis, the CD spectrum of this peptide does not indicate any elements of secondary structure.

Next, we compared the stability of  $\Delta\text{p}23$  to that of human p23 by monitoring the CD signal of both human p23 and  $\Delta\text{p}23$  at 230 nm during constant heating (Figure 2). Whereas in the case of p23 the CD signal starts to decrease at approximately 45°C, for  $\Delta\text{p}23$  it remains constant up to 60°C. Above 60°C, a sharp decrease in ellipticity was observed, indicative of a cooperative loss of secondary structure due to thermal unfolding. For both proteins, the unfolding process is completed at approximately 70°C and thermal unfolding is irreversible (data not shown). Based on these experiments we conclude that removal of the unfolded C-terminal tail does not decrease the thermal stability of p23, but rather leads to a more cooperative thermal unfolding transition.

### The unfolded C-terminal tail of p23 is not necessary for Hsp90 binding

To analyse the functional properties of  $\Delta\text{p}23$  we tested its interaction with Hsp90. Conditions which allow complex formation *in vitro* have been established by Toft and co-workers (Sullivan *et al.*, 1997; Grenert *et al.*, 1999). Using these conditions, we



**Figure 2.** Thermal unfolding of human p23 and  $\Delta\text{p}23$  monitored by CD. The CD signal of both human p23 (○) and  $\Delta\text{p}23$  (●) at 230 nm was monitored during constant heating of the protein solutions at a heating rate of 0.5°C/minute. The final protein concentrations were 0.2 mg/ml in 40 mM potassium phosphate (pH 6.8).

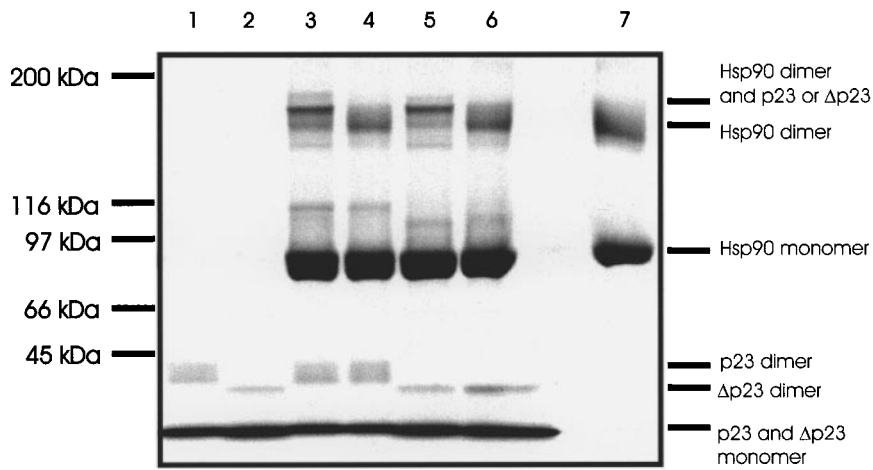
incubated either p23 or  $\Delta\text{p}23$  with Hsp90 in the presence of ATP $\gamma$ S. In some of the experiments geldanamycin was added as a control. Geldanamycin efficiently prevents complex formation by binding to the ATP pocket of Hsp90 (Prodromou *et al.*, 1997; Stebbins *et al.*, 1997). After chemical cross-linking, the complexes were analysed on SDS-PAGE. As shown in Figure 3, both p23 and  $\Delta\text{p}23$  are equally able to bind to Hsp90 (Figure 3, compare lanes 3 and 5), and in both cases the complex can be specifically disrupted by the addition of geldanamycin (Figure 3, lanes 4 and 6). Thus,  $\Delta\text{p}23$  exhibits the same properties as p23 as far as its association with Hsp90 is concerned. We therefore propose that the unfolded C-terminal part of p23 is dispensable for the association between p23 and Hsp90.

### Removal of the C-terminal tail impairs chaperone function of p23

p23 binds specifically to non-native proteins, thus displaying the properties of a molecular chaperone (Bose *et al.*, 1996; Freeman *et al.*, 1996). To test whether deletion of the C-terminal 30 amino acid residues alters the behaviour of p23 towards non-native proteins, we performed the well-established citrate synthase (CS) aggregation and inactivation assays (Buchner *et al.*, 1998b). Dimeric CS loses its activity and rapidly aggregates when incubated at 43°C. This aggregation can be visualized by monitoring the turbidity of the protein solution (Figure 4(a)). The presence of a molar excess of human p23 effectively suppressed CS aggregation. Over the time course of the experiment, a slight increase in CS aggregation could be detected (Figure 4(a)).

In contrast, the presence of  $\Delta\text{p}23$  did not have any influence on the aggregation behaviour of CS at concentrations where full-length p23 proved to be very effective (Figure 4(a)). This suggests that the C-terminal region is required for the efficient interaction of p23 with non-native proteins. Interestingly, isolated PEP1, the peptide corresponding to this region, has no influence on the spontaneous aggregation reaction of CS (data not shown). Next, we asked whether PEP1 could act *in trans* and complement the function of  $\Delta\text{p}23$  when both were added together to the aggregation reaction. However, even at a tenfold molar excess of  $\Delta\text{p}23$  and a 20-fold molar excess of PEP1 over CS, we still were not able to achieve significant suppression of CS aggregation (data not shown).

Similar results were obtained when the influence of p23 and  $\Delta\text{p}23$  on the inactivation kinetics of CS was assessed (Figure 4(b)). Wild-type p23 slowed down the inactivation process of CS efficiently even at substoichiometric concentrations (Figure 4(b)), which suggests a transient interaction between p23 and CS (Bose *et al.*, 1996). On the other hand, the inactivation rate constants remained unchanged in the presence of excess  $\Delta\text{p}23$  (Figure 4(b)). Again,



**Figure 3.** Complex formation between Hsp90 and p23/ $\Delta$ p23. Proteins were crosslinked under optimum conditions (10 mM  $MgCl_2$ , 2 mM  $ATP\gamma S$ , 10 mM  $Na_2MoO_4$  and 0.01 % Nonidet P-40). Lane 1, human p23; lane 2,  $\Delta$ p23; lane 3, human Hsp90 and human p23; lane 4, human Hsp90, human p23 and geldanamycin; lane 5, human Hsp90 and  $\Delta$ p23; lane 6, human Hsp90,  $\Delta$ p23 and geldanamycin; and lane 7, human hsp90.

the addition of PEP1 to  $\Delta$ p23 did not reconstitute the chaperone properties of  $\Delta$ p23 (data not shown).

From these experiments one can conclude that the C-terminal tail of p23 as an integral part of the molecule plays a crucial role in maintaining its chaperone properties.

## Discussion

We identified a C-terminal region in p23 that is apparently unstructured and can readily be cleaved off by proteases. This unstructured part comprises the 30 C-terminal amino acid residues of the protein. The truncated fragment,  $\Delta$ p23, exhibits the far-UV CD spectrum of a protein with a high  $\beta$ -sheet content. The peak at 230 nm, which is characteristic for proteins of the p23 family (T.W. & J.B., unpublished data), is more prominent in  $\Delta$ p23 than in the wild-type protein. This observation can be explained by the fact that all the aromatic residues that contribute to this signal in human p23 are located within the shorter  $\Delta$ p23 sequence. In contrast, the 30 C-terminal amino acid residues that are not present in  $\Delta$ p23 contain many acidic amino acids. Thus, truncation of the protein also leads to a significant increase of its pI. The suggestion that the C-terminal tail of p23 is unstructured is further supported by the far-UV CD spectrum of PEP1, a peptide comprising the last 37 amino acid residues of p23. Here, no ordered secondary structure elements could be detected. Based on this information and the stability measurements we conclude that p23 consists of two parts: (i) a stably folded and highly cooperative  $\beta$ -sheet domain; and (ii) a mainly unstructured highly acidic C-terminal tail.

Functional characterizations of p23 have focused on either the binding of p23 to Hsp90 or the *in vitro* chaperone properties of isolated p23. Complex formation between p23 and Hsp90 is strictly ATP-dependent and the complex can therefore be disrupted by the addition of the ansamycin antibiotic geldanamycin which is known to bind the nucleo-

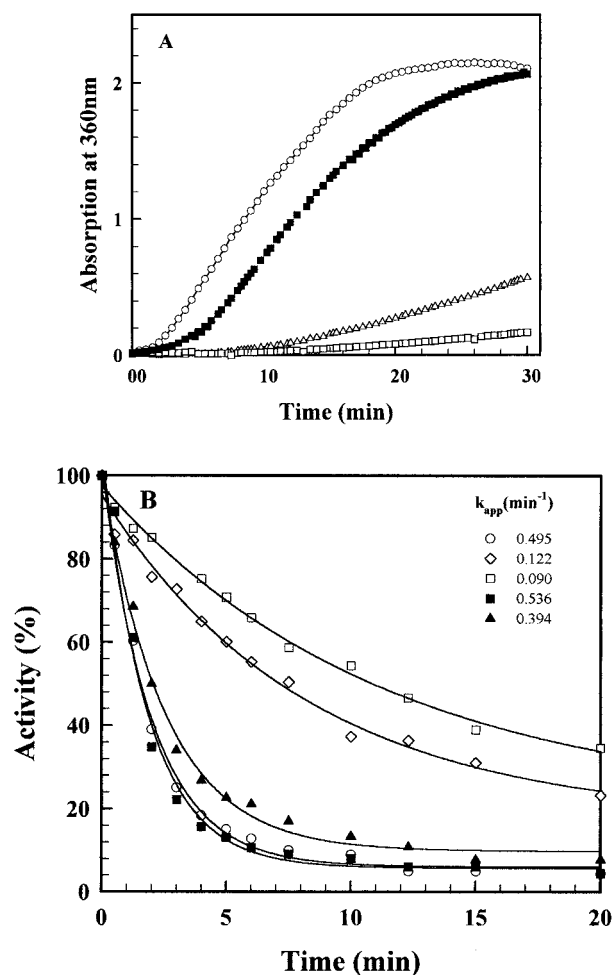
tide pocket of Hsp90 (Stebbins *et al.*, 1997; Prodromou *et al.*, 1997). The use of non-hydrolyzable ATP analogues instead of ATP promotes the formation of the complex (Sullivan *et al.*, 1997), whereas ATP hydrolysis seems to have a negative influence as has been demonstrated with ATP hydrolysis mutants of Hsp90 (Grenert *et al.*, 1999). Moreover the complex is stabilized by molybdate and the anionic detergent Nonidet P-40 (Sullivan *et al.*, 1997). It has been speculated that molybdate enhances complex formation by binding in the Hsp90 nucleotide pocket upon hydrolysis of ATP, keeping Hsp90 in its ATP-bound conformation (Grenert *et al.*, 1999).

This tight regulation of association mediated by the nucleotide-bound state of Hsp90 is especially interesting, since it became evident that adenosine nucleotides also control the affinity of the N-terminal chaperone site of Hsp90 for substrate proteins (Scheibel *et al.*, 1998). The N-terminal region of Hsp90 which contains the ATPase domain is believed to mediate at least partially the interaction with p23 (Chen *et al.*, 1998; Fang *et al.*, 1998). However, it is not possible to crosslink p23 to the isolated N-terminal domain of Hsp90 (Scheibel *et al.*, 1999). Therefore, one has to assume that other parts of Hsp90 are also important for the association with p23.

Whereas the binding between p23 and Hsp90 has been studied previously by immunoprecipitation experiments (Sullivan *et al.*, 1997), we assessed complex formation here by chemical crosslinking. For both methods, complex formation requires the same components (i.e. molybdate, Nonidet P-40 and  $ATP\gamma S$ ). However, only a certain fraction of the total protein was crosslinked in the complex with our method as can be seen from the percentage of unbound protein on the SDS gel (Figure 3).

Using this method we could show that  $\Delta$ p23 has the same affinity for the heat shock protein as full-length p23 and it is also released upon treatment of the complex with geldanamycin. These results strongly suggest that the Hsp90 binding site is contained within the  $\beta$ -sheet domain of p23.





**Figure 4.** Interaction of p23 and  $\Delta$ p23 with non-native CS. (a) Influence of human p23 and  $\Delta$ p23 on aggregation of thermally denatured CS. A 4  $\mu\text{M}$  CS (monomer) sample was incubated at 43°C in 40 mM Hepes-KOH (pH 7.5). CS aggregation was monitored by measuring the absorption at 360 nm in the absence of additional components (○) or in the presence of a fivefold molar excess of hp23 ( $\Delta$ ), a tenfold molar excess of hp23 (□) or a tenfold molar excess of  $\Delta$ p23 (■). (b) Influence of human p23 and  $\Delta$ p23 on thermal inactivation of CS. Inactivation kinetics of citrate synthase (0.15  $\mu\text{M}$  monomer) following incubation at 43°C (○). The effect of human p23 on inactivation kinetics was measured with equimolar amounts of hp23 (□) or a twofold excess of CS over hp23 (◇). The effect of  $\Delta$ p23 was determined in the presence of equimolar amounts of  $\Delta$ p23 (■) or a fivefold molar excess of  $\Delta$ p23 (▲). The continuous lines represent single exponential functions with apparent rate constants as shown in the inset.

Importantly, removal of the C-terminal amino acid residues of p23 drastically alters the interaction with unfolding proteins. Intact p23 efficiently suppresses the aggregation of thermally denatured CS. The slight increase in light scattering over time argues in favour of a model where p23 and non-native CS are in a binding equilibrium with rapid release and rebinding of the substrate

by p23 as opposed to tight and permanent binding. The released protein either rebinds to p23 or aggregates as a consequence of unfolding. p23's ability to interact with non-native proteins is entirely dependent upon the C-terminal region of the protein, since  $\Delta$ p23 is no longer able to suppress CS aggregation or slow down CS inactivation. However, the C-terminal peptide alone does not exhibit any chaperone function nor does it complement the fragment  $\Delta$ p23.

Taken together, we were able to correlate the two functions that have been described for p23 so far (Hsp90 binding and binding to non-native proteins) to distinct structural parts of the protein. How this bipartite structure of p23, in cooperation with Hsp90, generates and maintains an active conformation of their complicated target molecules remains to be elucidated.

## Materials and Methods

### Plasmids, bacterial strains and peptides

For the bacterial expression of both wild-type human p23 and the C-terminally truncated fragment  $\Delta$ p23 we used the *Escherichia coli* strain BL21 (DE3) pLysS. The hp23-pET23 plasmid for bacterial expression of human p23 was a kind gift from Dr David Toft (Mayo Graduate School, Minnesota). The 37 amino acid residue peptide PEP1 comprising amino acids 124 to 160 of human p23 was chemically synthesized by Dr Susanne Modrow at the Institute for Medical Microbiology of the University of Regensburg.

### Cloning of $\Delta$ p23

$\Delta$ p23 was cloned by a 36-cycle PCR amplification (using PWO polymerase and an annealing temperature of 60°C) from the original hp23-pET23 plasmid, using the primers 5'GATCCATATGCAGCCTGCTTCGCA 3' and 5'GATCGAATTCTTATTACATGT TGTTTCATCATC TCAGAGAA 3'. The PCR product was gel purified and cloned into the pET17b vector using the *NedI* and *EcoRI* restriction sites to generate the clone designated  $\Delta$ p23-pET17b.

### Protein purification

Human p23 expressed in *E. coli* was purified as described (Buchner *et al.*, 1998c). The C-terminally truncated fragment  $\Delta$ p23 was purified following the same protocol, with the exception that 2.25 M ammonium sulfate was used instead of 2.5 M for the precipitation of  $\Delta$ p23.  $\Delta$ p23 has a molecular mass of 15,492 Da (human p23, 18,697 Da) and a pI of 5.05 (compared to 4.35 for the wild-type protein).

Human Hsp90 $\beta$  was purified from baculovirus-infected Sf9 cells which were obtained from the Tissue Culture Core Facilities at the University of Colorado Cancer Center. The protein was isolated from the infected cells as described (Buchner *et al.*, 1998a).

The concentrations of wild-type human p23,  $\Delta$ p23 and human Hsp90 $\beta$  were determined using the calculated extinction coefficients of 1.67, 2.02 and 0.65, respectively, for a 1 mg/ml solution in a 1 cm cuvette at 280 nm.

### Proteolytic digest of human p23

Human p23 was digested with Proteinase K in 40 mM Hepes-KOH (pH 7.5), 5 mM EDTA using a 4000-fold molar excess of substrate over protease at 25 °C. At different time points aliquots were withdrawn from the reaction mixture and proteolysis was stopped by addition of Complete<sup>®</sup> protease inhibitors. The aliquots were analysed on 12.5%-20% gradient SDS-PAGE.

### Circular dichroism measurements

Far-UV circular dichroism (CD) spectra of human p23, Δp23 and PEP1 were recorded in a Jasco J715 spectropolarimeter equipped with a thermostatted cell holder. Human p23, Δp23 and PEP1 were dialyzed overnight against 40 mM potassium phosphate (pH 6.8). Far-UV CD spectra were recorded from 250 to 200 nm in thermostatted 0.1 cm quartz cuvettes at 20 °C with a protein concentration of 0.2 mg/ml. All spectra were baseline-corrected.

To determine the thermal stability of human p23 and Δp23, the CD signal of both proteins was monitored at 230 nm from 20 °C to 80 °C with a heating rate of 0.5 °C/minute.

### Chaperone assays

A 4 μM CS (monomer) sample from pig heart (Boehringer Mannheim) was thermally denatured by incubation at 43 °C in 40 mM Hepes-KOH (pH 7.5) for 45 minutes. Aggregation of non-native CS was measured by monitoring the increase of turbidity at 360 nm in a Pharmacia Ultrospec 4060 UV-VIS spectrophotometer equipped with a temperature control unit using microcuvettes (120 μl) with a path length of 1 cm.

Thermal inactivation of pig heart CS was performed as described (Buchner *et al.*, 1998b) with a CS concentration of 0.15 μM.

### Chemical crosslinking

The cleavable crosslinker Dithiobis(succinimidylpropionate) (DSP) was used as the crosslinking reagent. For crosslinking of human p23/Δp23 to human Hsp90, the protein components (final concentration 0.4 mg/ml each) were incubated in 40 mM Hepes-KOH (pH 7.5) containing 10 mM MgCl<sub>2</sub>, 2 mM ATPγS, 10 mM Na<sub>2</sub>MoO<sub>4</sub> and 0.01% Nonidet P-40 for 30 minutes at 30 °C (Sullivan *et al.*, 1997). In geldanamycin-containing control samples, geldanamycin was added in a tenfold molar excess over Hsp90. Proteins were crosslinked afterwards by the addition of 250 μM DSP and further incubation for 30 minutes at room temperature. The crosslinking reaction was stopped by the addition of excess Tris-Cl (pH 8.0). The crosslinked samples were analysed by SDS-PAGE on 4% to 12% gradient gels.

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