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ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone *in vivo*

Barry Panaretou, Chrisostomos Prodromou, S.Mark Roe, Ronan O'Brien, John E.Ladbury, Peter W.Piper and Laurence H.Pearl[1]

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK

[1]Corresponding author
e-mail: l.pearl@biochem.ucl.ac.uk

B.Panaretou and C.Prodromou contributed equally to this work

Hsp90 is an abundant molecular chaperone essential to the establishment of many cellular regulation and signal transduction systems, but remains one of the least well described chaperones. The biochemical mechanism of protein folding by Hsp90 is poorly understood, and the direct involvement of ATP has been particularly contentious. Here we demonstrate *in vitro* an inherent ATPase activity in both yeast Hsp90 and the *Escherichia coli* homologue HtpG, which is sensitive to inhibition by the Hsp90-specific antibiotic geldanamycin. Mutations of residues implicated in ATP binding and hydrolysis by structural studies abolish this ATPase activity *in vitro* and disrupt Hsp90 function *in vivo*. These results show that Hsp90 is directly ATP dependent *in vivo*, and suggest an ATP-coupled chaperone cycle for Hsp90-mediated protein folding.
Keywords: ATP/chaperone/Hsp90/protein folding

Introduction

Despite their ubiquitous biological distribution and cellular abundance, the Hsp90 family of proteins remain amongst the least well understood of all the molecular chaperones. In eukaryotes, as well as playing a vital role in thermotolerance and stress responses, the cytoplasmic Hsp90s are essential for establishing the function of proteins involved in a wide range of cell regulation and signalling pathways, including steroid hormone receptors (Joab *et al*., 1984), helix–loop–helix transcription factors (Wilhelmsson *et al*., 1990), tyrosine and serine/threonine kinases (Opperman *et al*., 1981; Stancato *et al*., 1993; Aligue *et al*., 1994; Cutforth and Rubin, 1994; Dai *et al*., 1996), and tumour suppressors (Chen *et al*., 1996; Sepehrnia *et al*., 1996), amongst others. *In vitro*, purified Hsp90 binds to denatured protein and displays anti-aggregant properties (Wiech *et al*., 1992). However, *in vivo*, Hsp90-dependent folding and activation of client proteins involves a plethora of accessory factors or co-chaperones which participate in multiprotein complexes with Hsp90. These include the ATP-dependent chaperone Hsp70/DnaK, and its co-chaperones Hsp40/DnaJ (Kimura *et al*., 1995) and p48/Hip (Hohfeld *et al*., 1995) which interact with Hsp90 via p60/Hop/Sti1 (Smith *et al*., 1993). Other accessory factors are associated with particular subclasses of client proteins, so that Hsp90–steroid hormone receptor complexes also contain immunophilins such as cyclophilin 40 or FKBP59/Hsp59 (Owens-Grillo *et al*., 1995), whereas Hsp90–protein kinase complexes contain p50/CDC37 (Hunter and Poon, 1997).

A clear understanding of the biochemistry of Hsp90 has been hampered by a controversy regarding the direct involvement of ATP in its mechanism of action. Thus, cytoplasmic and endoplasmic reticulum Hsp90s have been reported as highly active ATPases (Nadeau *et al*., 1992, 1993) or even GTPases (Nardai *et al*., 1996) that autophosphorylate (Csermely and Kahn, 1991; Csermely *et al*., 1995), undergo conformational changes in the presence of ATP (Csermely *et al*., 1993; Sullivan *et al*., 1997) and require ATP for their interaction with co-chaperones (Johnson and Toft, 1994, 1995) and peptides (Li and Srivastava, 1993). Conversely, Hsp90s purified from different sources lacked ATPase activity (Wiech *et al*., 1993; Scheibel *et al*., 1997), and what ATPase and autophosphorylation activity could be obtained was attributable to contamination with trace amounts of the protein kinases which associate strongly with Hsp90s (Shi *et al*., 1994; Wearsch and Nicchitta, 1996). The question appeared to be settled by a detailed side-by-side comparison of Hsp90 and the known ATP-dependent chaperone Hsp70, in which Hsp90 could not be affinity labelled by azido-ATP, retained on ATP agarose or enhance the fluorescense of an ADP derivative, in contrast to Hsp70 which could do all three (Jakob *et al*., 1996). Subsequently, it has become generally accepted in the chaperone literature that Hsp90 is an ATP-independent chaperone (Buchner, 1996), and observations of ATP dependence in Hsp90-dependent folding processes are attributed entirely to the ATPase activity of associated Hsp70 (e.g. see Schneider *et al*., 1996).

The issue of ATP dependence in the mechanism of Hsp90 has re-emerged as a result of recent structural and biochemical studies (Grenert *et al*., 1997; Prodromou *et al*., 1997b; Scheibel *et al*., 1997) which have demonstrated the presence of an ADP/ATP-binding site in the N-terminal domain of Hsp90. This site is formed by motifs of the yeast Hsp90 polypeptide sequence, which are absolutely conserved in all Hsp90s and also occur in the N-terminal ATP-binding domains of type II DNA topoisomerases, and in MutL DNA mismatch repair proteins (Bergerat *et al*., 1997).

Here we demonstrate *in vitro* an inherent ATPase activity of Hsp90, which is sensitive to inhibition by the specific inhibitor geldanamycin and which is essential for the function of this molecular chaperone *in vivo*. These results suggest that protein folding mediated by Hsp90 operates via an 'active' ATP-coupled mechanism.

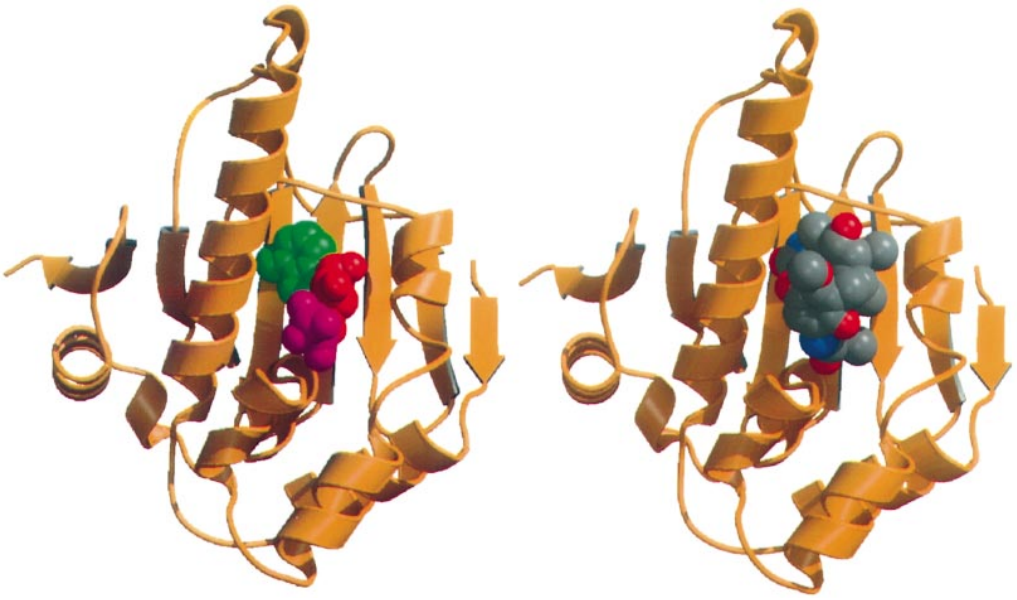

Fig. 1. Co-localization of ATP/ADP- and geldanamycin-binding sites. Cartoons of crystal structures of yeast Hsp90 N-domain complexed with Mg^{2+}-ADP (left) and geldanamycin (right). The ligands are shown as space-filling models. Full details of the yeast Hsp90 N-terminal domain–geldanamycin complex will be published elsewhere, but the essential features of the complex are very similar to those described for the human Hsp90–geldanamycin complex (Stebbins *et al*., 1997). Figures were generated using MOLSCRIPT (Kraulis, 1991) and Raster3D (Merrit and Murphy, 1994).

Results

In vitro ATPase activity of Hsp90

The *Saccharomyces cerevisiae* Hsp90 isoform Hsp82, purified from an overproducing yeast strain to crystalline quality (Prodromou *et al*., 1996), was assayed for ATPase activity using a sensitive coupled enzyme assay (see Materials and methods). The pyruvate kinase (PK)–lactate dehydrogenase (LDH) coupled assay converts any contaminating ADP to ATP prior to the addition of a putative ATPase. As ADP binds to the nucleotide-binding domain of Hsp90 with ~5-fold greater affinity than ATP (Prodromou *et al*., 1997b), its removal eliminates the possibility of ADP inhibition, which may have compromised other studies. At 37°C, which constitutes moderate heat-shock conditions for *S.cerevisiae*, we observed an ATPase activity for yeast Hsp90 of 5000 pmol/min/mg corresponding to a k_{cat} of 0.4/min, with a K_M of ~100 μM. At a normal growth temperature of 30°C, the activity was reduced by ~4-fold, but was stimulated further at 43°C to nearly 1.0/min.

To demonstrate that the ATPase activity we observe is specific to Hsp90, and not due to contaminating protein kinases or other ATPases, we have taken advantage of the observation that the ATP-binding site in Hsp90 revealed by structural and biochemical studies (Grenert *et al*., 1997; Prodromou *et al*., 1997b) is also the binding site for the ansamycin antibiotic geldanamycin (Stebbins *et al*., 1997) (Figure 1). This compound binds to the yeast Hsp90 N-terminal domain with a K_d of 0.5 μM, making specific interactions with many of the residues that interact with ATP, and is expected, therefore, to be a potent inhibitor of ATP binding. Addition of geldanamycin did not interfere with the function of the coupled enzyme assay, but effectively abolished the Hsp90-associated ATPase activity at a concentration of 15 μM (Figure 2).

We have also measured an ATPase activity (6500 pmol/min/mg equivalent to k_{cat} 0.47/min at 37°C) in the *Escherichia coli* Hsp90 homologue HtpG, again purified to crystalline quality from an overexpressing *E.coli* strain. This activity was also sensitive to inhibition by geldanamycin, but was only slightly stimulated at 43°C. The presence of this activity in Hsp90 purified from entirely different backgrounds is further evidence that it is not an artefact due to a co-purified contaminant, and suggests that the ATPase activity is probably a general property of prokaryotic and eukaryotic Hsp90s.

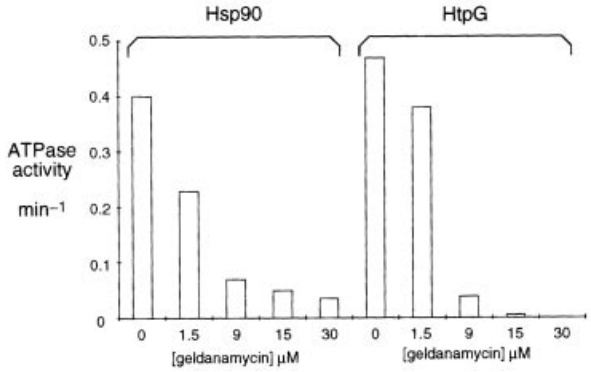

Fig. 2. Geldanamycin-sensitive ATPase activities. ATPase activities (expressed in mol/min/mol) for yeast Hsp90 and *E.coli* HtpG at 37°C, with the addition of geldanamycin. The yeast Hsp90 samples display a residual geldanamycin-insensitive ATPase activity of 10% of the total activity. Geldanamycin concentrations are in micromoles. The geldanamycin has an IC_{50} in this assay of 3–5 μM.

Although the turnover rates we observe for the inherent ATPase of Hsp90s are low by the standards of many ATP-hydrolysing enzymes, they are of the same order of magnitude as the inherent ATPase activity of Hsp70s (O'Brien and McKay, 1995; Bukau and Horwich, 1998). With Hsp70, the ATPase activity is markedly stimulated by client protein substrates and co-chaperones (Jordan and

McMacken, 1995; McCarty *et al*., 1995; Theyssen *et al*., 1996). It remains to be determined whether the ATPase activity of Hsp90 is modulated similarly by the presence of Hsp90-dependent client proteins, and/or by the co-chaperones that are needed for Hsp90 action in both *in vivo* and *in vitro* reconstituted systems (Dittmar and Pratt, 1997).

While this manuscript was in preparation, Scheibel *et al*. (1998) reported an ATPase activity in yeast Hsp90 which was also sensitive to inhibition by geldanamycin. However, the turnover reported in that study is only ~20% of that we observe at 30°C. While we are not in a position to reconcile these differences, we note that that study utilized a radioactive non-regenerating assay, and may have been subject to inhibition by ADP (see above, and Materials and methods), which is inevitably present in such an assay.

In vivo ATP dependence

The existence of an inherent ATPase activity in Hsp90 suggests that many, if not all, of the biological functions of Hsp90 are directly ATP dependent. To test this hypothesis, we have determined the effect of mutations in residues implicated in ATPase activity on Hsp90 function *in vivo*. *Saccharomyces cerevisiae* has two genes for the Hsp90 protein, *HSC82*, which is constitutively expressed, and *HSP82*, which is normally expressed at a lower level than *HSC82* but is strongly activated by heat shock. The encoded protein sequences are 97% identical. Deletion of either of these genes still produces viable cells, but deletion of both is lethal (Borkovich *et al*., 1989). We constructed a haploid *S.cerevisiae* strain (PP30) that has the coding regions of both *HSC82* and *HSP82* completely deleted, but which is viable because it contains an episomal *URA3* plasmid carrying a wild-type copy of *HSC82* (see Materials and methods). Wild-type or mutant versions of an Hsp90-encoding gene can then be introduced on single-copy plasmids with *LEU2* positive selection, and the functionality of the expressed Hsp90 protein determined by its ability to maintain cell viability when the *URA3* plasmid that bears the wild-type *HSC82* gene is deselected on 5-fluoro-orotic acid (5-FOA) plates.

From the crystal structures of the N-terminal domain of the yeast Hsp90 chaperone with bound nucleotides (Prodromou *et al*., 1997b), we identified Asp79 as a key residue in adenine nucleotide binding. The carboxylate side chain of this residue makes a hydrogen bond to the exocyclic N6 group of adenine, thereby providing the only direct hydrogen bonding interaction between the protein and the base of the bound nucleotide. The hydrogen-bonding environment of this residue is such that even a subtle mutation to asparagine would disfavour ATP binding by generating a strongly repulsive interaction with the adenine base, with minimal disruption of the structure in the nucleotide-binding site (Figure 3). Furthermore, as this residue lies at the bottom of a deep pocket in the Hsp90 N-terminal domain structure, its mutation cannot directly affect interactions with other domains or associated proteins that are not mediated by a bound nucleotide. Structural homology between the Hsp90 N-terminal domain and the N-terminal ATPase domain of the DNA gyrase B protein (Gerloff *et al*., 1997; Prodromou *et al*., 1997b) aligns Glu33 of Hsp90 with Glu42 of gyrase B.

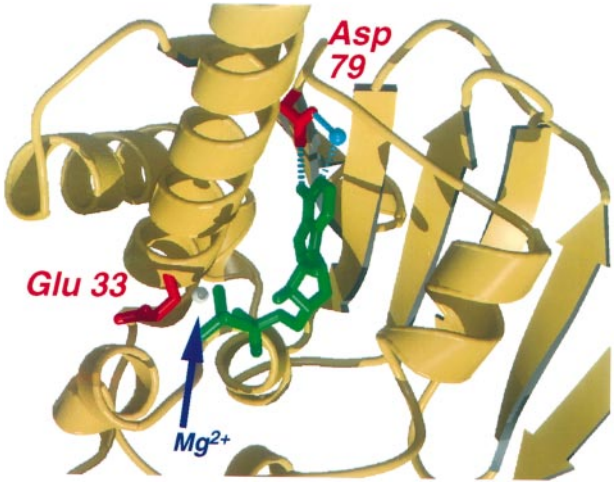

Fig. 3. Residues implicated in ATP binding and hydrolysis. A close-up of the Hsp90 N-terminal domain showing the location of Asp79 and Glu33 (in red) in the Hsp90 nucleotide-binding site. The bound water forming part of the Asp79–adenine interaction is shown as a blue sphere, and the Mg^{2+} ion which is essential for nucleotide binding is shown as a white sphere.

Glu42 functions as a general base in the ATPase mechanism of DNA gyrase B (Jackson and Maxwell, 1993), and mutation to alanine substantially reduces supercoiling and DNA-dependent ATPase activity, but does not prevent nucleotide binding (Jackson and Maxwell, 1993). The structural homology between the ATP-binding sites in gyrase B and Hsp90 suggests that Glu33 might play a similar role in the ATPase activity of Hsp90. Thus, mutations of Asp79 and Glu33 would be expected to compromise Hsp90 function *in vivo* if ATP binding and hydrolysis are indeed essential.

HSP82 alleles with Asp79Asn, Asp79Arg, Asp79Trp and Glu33Ala mutations all failed to maintain yeast cell viability when the wild-type *HSC82* gene was deselected on 5-FOA plates, whereas cells with the wild-type *HSP82* remained perfectly viable (Figure 4A and B). To verify that the targeted mutations were indeed responsible for the loss of viability, the mutant genes were reverted to wild-type by a second round of site-directed mutagenesis. All the reverted *HSP82* alleles conferred full viability on 5-FOA plates. When co-expressed in the presence of wild-type *HSC82*, the mutant genes directed the synthesis of a full-length *HSP82* product when induced by heat-shock, indicating that these mutations do not simply prevent expression of the protein (Figure 4C).

A functional *HSC82* gene is sufficient to maintain viability in the presence of Asp79 or Glu33 mutant *hsp82* alleles. However, these cells expressing both wild-type Hsc90 and a putative ATP binding-defective or ATPase-defective mutant Hsp90 grew less well than similar cells expressing Hsc90 and the wild-type Hsp90, indicating a probable semi-dominance of these Hsp90 mutations. To investigate this further, we constructed a variant of the PP30 strain (PP30a; see Materials and methods) in which Hsp90 function is provided by a *HSP82* gene on the *LEU2* plasmid pHSP82. Mutant or wild-type *HSP82* alleles placed under control of the galactose-inducible *GAL1* promoter were then introduced into this background on a *URA3* plasmid, and their growth under inducing or non-

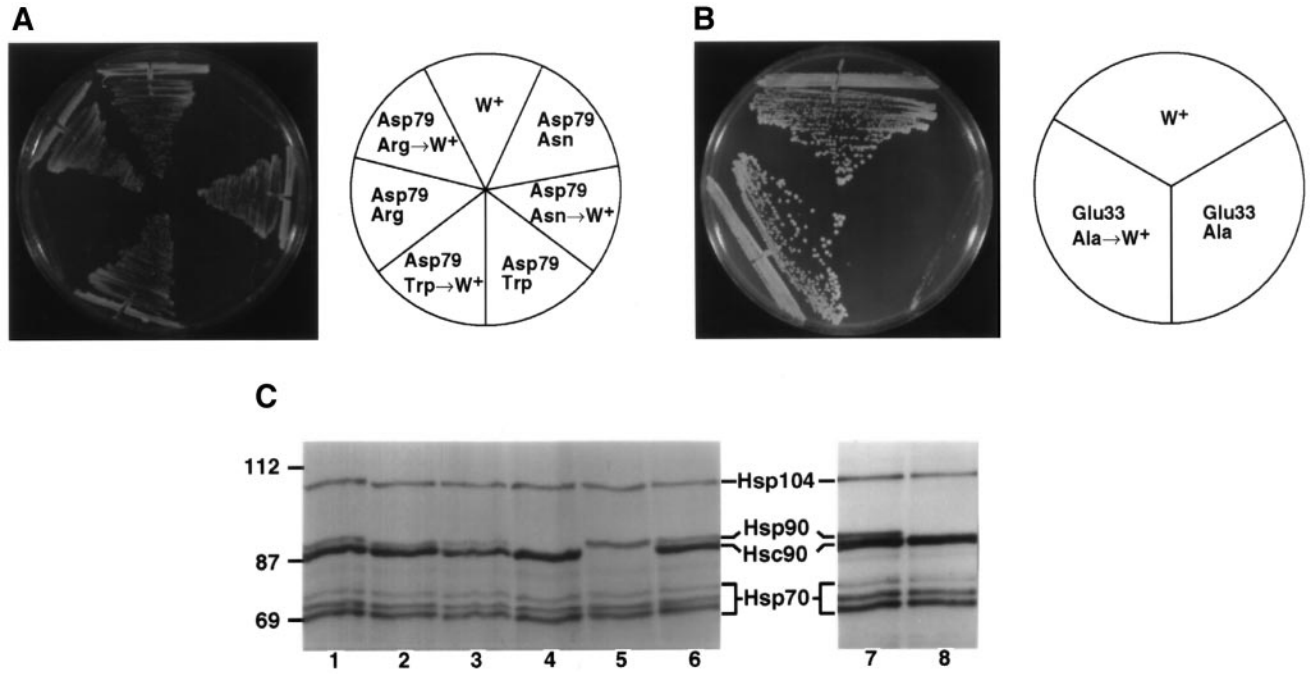

Fig. 4. *In vivo* effects of ATP-binding site mutations. (**A**) Uracil/5-FOA plate showing growth of *S.cerevisiae* strain PP30, harbouring wild-type (W^+), Asp79 mutant or reverted Asp79 mutant (W^+) *HSP82* genes on a centromeric *LEU2* vector (see Materials and methods). Mutations in Asp79 abolish viability on deselection of the *HSC82* gene. Reversion of the mutation restores viability. (**B**) As (A), but for the Glu33Ala *HSP82* mutant. (**C**) SDS–PAGE gel showing heat-shock expression of Hsp90 mutant proteins from a single-copy plasmid. Yeast strains were grown to exponential phase at 28°C and then heat-shocked at 40°C for 10 min to enhance *HSP82* gene expression, labelled, and the gels visualized by fluorography (see Materials and methods). Equivalent amounts of TCA-precipitable protein (500 000 d.p.m.) were loaded in each lane. Molecular weights (kDa) of markers are indicated on the left and the positions of Hsc90, Hsp90, Hsp104 and Hsp70 are indicated on the right. Lane 1, Hsc90 (W^+) + Hsp90 (Asp79Asn); lane 2, Hsc90 (W^+) + Hsp90 (Asp79Trp); lane 3, Hsc90 (W^+) + Hsp90 (Asp79Arg); lane 4, Hsc90 (W^+) alone; lane 5, Hsp90 (W^+) alone; lane 6, Hsc90 (W^+) + Hsp90 (W^+); lane 7, Hsc90 (W^+) + Hsp90 (Glu33Ala); lane 8, as lane 4. Hsp90 and Hsc90 can be readily distinguished (see lanes 4 and 5). All the mutant *HSP82* genes express full-length protein at levels comparable with the wild-type.

inducing conditions compared. Relative to non-inducing conditions (Figure 5A), all strains harbouring a mutant *hsp82* allele showed a marked retardation in growth on galactose substrates, a retardation not shown by isogenic cells expressing the wild-type Hsp90 protein (Figure 5B and C). Thus the expression of mutant Hsp90s that have been shown in other experiments to be ATP binding-defective or ATPase-defective (see below) is not neutral *in vivo*, but exerts strong dominant-negative effects on growth. We have not yet defined the mechanism of this semi-dominance, but it most likely arises through sequestration of essential client proteins and/or co-chaperones into complexes with these inactive Hsp90 dimers, which are then unable to progress through folding. Whether heterodimers between wild-type and defective mutants are also inactive remains to be seen.

In vitro analysis of ATP-binding site mutations

From the roles ascribed to Glu33 and Asp79 by the structural data, and by analogy with the homologous residues in DNA gyrase (Jackson and Maxwell, 1993), mutants of the catalytic residue Glu33 would be expected to bind adenine nucleotides, whereas mutants of Asp79 would not. To verify that the observed functional defects of mutations of these residues *in vivo* was consistent with their predicted biochemical roles, the N-terminal domains for the Asp79Asn and Glu33Ala mutants were assayed for ADP and ATP binding using isothermal titration calorimetry, as described previously for the wild-type domain (Prodromou *et al*., 1997b; see Materials and methods). The Asp79Asn mutant N-terminal domain bound neither ATP nor ADP to any measurable extent, in contrast to the Glu33Ala mutant which retained affinity for ATP comparable with that previously described for the wild-type domain (Prodromou *et al*., 1997b). Although ATP binding by the N-terminal domain was largely unaffected, the Glu33Ala mutation reduced the ATPase activity of the intact protein to $<1\%$ of the level observed for the wild-type. Finally, to verify that the loss of adenine nucleotide binding by the Asp79Asn mutant was due to the local changes in hydrogen bonding possibilities at the bottom of the nucleotide-binding pocket, and not to gross misfolding of the protein as a result of the mutation, the N-terminal domain of the Asp79Asn mutant was crystallized as described previously for the wild-type protein (Prodromou *et al*., 1996) and the structure determined at 2.1 Å resolution. Comparison of the refined Asp79Asn mutant N-terminal domain structure with that of the wild-type chaperone gives a root-mean-square difference between all protein atoms of only 0.9 Å, which indicates that the overall structures are effectively identical within the limits of the method, and have not been altered significantly by the Asp to Asn mutation.

Discussion

The presence of a specific ATPase activity inherent in Hsp90, and the failure of the Hsp90 mutants defective in ATP binding and hydrolysis *in vitro* to maintain cell viability, demonstrates unambiguously the direct ATP

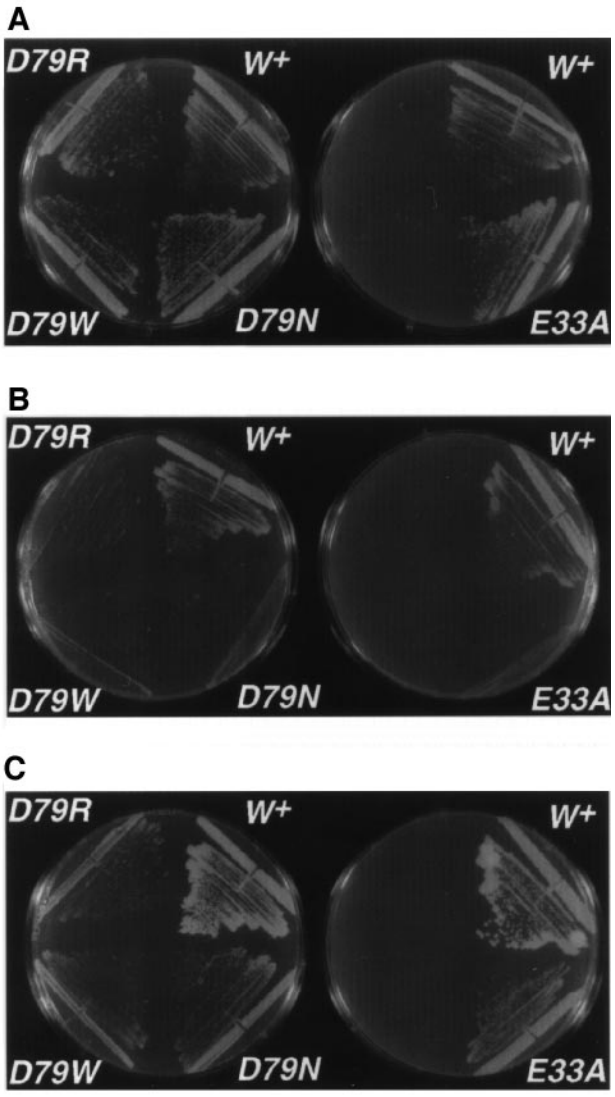

Fig. 5. Semi-dominance of ATP binding/hydrolysis-defective mutants. A strain expressing *HSP82* from its own promoter, PP30(a), was transformed with a vector bearing a wild-type (W^+) or mutant allele (Asp79Asn, Asp79Trp, Asp79Arg and Glu33Ala) of *HSP82* under the control of the galactose-inducible *GAL1* promoter. Under non-inducing conditions of growth on glucose, at 2 days post-inoculation (**A**) all strains grow normally. When grown under inducing conditions on galactose, strains expressing mutant *HSP82* alleles defective in ATP binding and/or hydrolysis, although viable, show clear growth retardation 2 (**B**) and 5 (**C**) days post-inoculation, suggesting a semi-dominant effect of the defective alleles.

dependence of Hsp90 function *in vivo*. All the residues implicated in ATP binding and hydrolysis from structural studies (Prodromou *et al*., 1997b) are absolutely conserved in the known amino acid sequences of the cytosolic Hsp90 and endoplasmic reticulum GRP94 of eukaryotes, and in the HtpG of bacteria, suggesting that ATP dependence is a universal property of the Hsp90 family of molecular chaperones.

A variety of temperature-sensitive mutants of yeast Hsp90 have been identified (Nathan and Lindquist, 1995), some of which correspond to missense mutations within the N-terminal domain of the protein. One of these mutations, Ala41Val, affects a residue which forms part of the hydrophobic binding site for the adenine base of ATP (Prodromou *et al*., 1997b). Mutation to the larger side chain of valine would be expected to constrict the adenine-binding site and decrease the affinity of Hsp90 for ATP. While this mutant only shows mild growth retardation at permissive temperatures, its complete loss of viability at non-permissive temperatures could indicate that ATP binding and hydrolysis are of increased importance to the cellular function of Hsp90 during heat shock. Characterization of ATP binding and hydrolysis by this and other temperature-sensitive mutants is in progress.

Previous *in vitro* studies have shown that isolated Hsp90 displays an apparently ATP-independent chaperone activity *in vitro*, binding denatured proteins and thereby contributing to their renaturation by preventing their aggregation (Wiech *et al*., 1992; Jakob *et al*., 1995; Yonehara *et al*., 1996). Although these studies have been conducted with proteins not known to be dependent on Hsp90 for their folding *in vivo*, and in the absence of the various co-chaperones with which Hsp90 is known to be functionally associated *in vivo*, the lack of a requirement for ATP in this *in vitro* chaperone activity would appear to be in conflict with the ATP dependence of Hsp90 activity *in vivo* that we describe here.

A resolution of this apparent paradox comes from a reassessment of studies of the action of ansamycin antibiotics on Hsp90-mediated protein folding. Geldanamycin and the closely related herbimycin-A have been shown to interfere with the folding and activation of a range of client proteins by Hsp90 (e.g. Whitesell and Cook, 1996; Stancato *et al*., 1997) and thereby promote their degradation (Schneider *et al*., 1996; Whitesell *et al*., 1997). Significantly, inhibition of Hsp90-mediated protein folding by geldanamycin does not block formation of Hsp90 complexes with incompletely folded or denatured client proteins (Smith *et al*., 1995; Dittmar and Pratt, 1997), nor does it disrupt existing Hsp90–client complexes (Chen *et al*., 1997; Dasgupta and Momand, 1997), but rather stabilizes them, leaving unfolded protein associated with Hsp90 and unable to progress through folding (Schneider *et al*., 1996). Given that geldanamycin binds to the same site on Hsp90 as ATP (Grenert *et al*., 1997; Prodromou *et al*., 1997b) and inhibits the ATPase activity of Hsp90 (see above), these observed effects of ansamycins are most consistent with the inhibition of a directly ATP-dependent step in Hsp90-mediated protein folding, occurring after incompletely folded proteins have bound to Hsp90. Thus, the ATP-independent 'passive' chaperone activity of Hsp90 observed *in vitro*, and previously presented as the sum-total of its activity as a molecular chaperone (Buchner, 1996), may instead represent just the first step in an active, and ATP-dependent mechanism *in vivo*. This does not eliminate the possibility that for some proteins, a passive anti-aggregant interaction with Hsp90 *in vivo*, as with citrate synthase and β-galactosidase *in vitro* (Jakob *et al*., 1995; Freeman and Morimoto, 1996), may be sufficient to facilitate folding. However, that the Hsp90-dependent folding of many cell regulatory and signal transduction proteins is blocked by geldanamycin argues strongly that these most biologically significant client proteins demand an active and ATP-dependent chaperone activity of Hsp90.

Direct functional dependence on binding and hydrolysis of ATP moves Hsp90 into the class of 'active' chaperones

typified by Hsp70/DnaK and Hsp60/GroEL, and prompts consideration of functional analogies to these comparatively well-characterized systems. While Hsp60 and Hsp70 themselves are substantially different in their structures and in their mode of interaction with protein substrates, they both utilize ATP in essentially similar ways. Thus both systems are switched between an ATP-bound conformational state with low affinity for substrate proteins and an ADP-bound conformational state with high affinity for substrate, by cycles of ATP binding, ATP hydrolysis and ADP release (Bukau and Horwich, 1998). With Hsp90, there is clear evidence for both binding (Grenert *et al*., 1997; Prodromou *et al*., 1997b; Scheibel *et al*., 1997) and now hydrolysis of ATP. In type II DNA topisomerases, ATP binding and hydrolysis by the homologue of the Hsp90 N-terminal domain provides an ATP-driven motor for the conformational changes accompanying DNA relaxation (Bates and Maxwell, 1997). ATP-induced conformational changes have been reported for Hsp90 (Csermely *et al*., 1993; Sullivan *et al*., 1997). The molecular nature of these ATP-dependent conformational changes in Hsp90 are unknown; however, the ATP-bound state appears to display the significantly decreased hydrophobicity (Sullivan *et al*., 1997) that would be consistent with the low affinity for substrates observed in the ATP-bound states of Hsp60/GroEL and Hsp70/DnaK chaperones. Thus, there is at least a *prima facie* case for a chaperone cycle in Hsp90. The key questions of how binding and hydrolysis of ATP are coupled to conformational changes in Hsp90, how these facilitate protein folding and what roles are played by the various co-chaperones are yet to be answered.

Materials and methods

Strains and plasmids

The *S.cerevisiae* strain PP30 (*a, trp1-289, leu2-3,112, his3-200, ura3-52, ade2-101oc, lys2-801am, hsc82KANMX4, hsp82KANMX4*) was used as the host strain for expression of *HSP82* mutant alleles. Its essential Hsp90 function is provided by the *HSC82* gene of the plasmid pHSC82, a vector derived by cloning the 5.5 kb *Bam*HI fragment from pUTX203 (Borkovich *et al*., 1989) into the *URA3* vector pYEplac195 (Gietz and Sugino, 1988). Plasmid pHSP82 was obtained by cloning the 2.5 kb *Bam*HI *HSP82* fragment from p82-2B (Cheng *et al*., 1992) into the centromeric *LEU2* vector pYCplac111 (Gietz and Sugino, 1988). To generate strain PP30(a), which expresses *HSP82* only, PP30 was transformed with pHSP82, followed by curing for pHSC82 by streaking a transformant on drop-out media without leucine, but containing 5-FOA (0.1%).

Mutagenesis and plasmid construction

Single amino acid changes were generated in the *HSP82* gene of pHSP82 using the QuickChange site-directed mutagenesis kit (Stratagene). The following mutations were introduced and confirmed by sequencing: Asp79Asn, Asp79Arg, Asp79Trp (GAT to AAC, TGG and AGA, respectively) and Glu33Ala (GAA to GCT). Mutants were reverted to wild-type by a second round of mutagenesis, and their ability to support yeast cell viability as the only Hsp90 present was tested in order to verify that the desired mutation was the only change present in each gene. Mutations were confirmed by dye terminator cycle sequencing (ABI).

To illustrate clearly a semi-dominant phenotype, the wild-type and mutant alleles of *HSP82* were placed under the control of an inducible promoter. Alleles were PCR amplified using pHSP82 (or Asp79Asn, Asp79Trp, Asp79Arg and Glu33Ala mutants) and inserted into pYES2 with *URA3* selection for expression under control of the inducible *GAL1* promoter of *S.cerevisiae*.

For expression of yeast *HSP82* mutants in *E.coli*, the coding sequences were PCR amplified from the yeast pHSP82 plasmids and inserted into pRSETA, for expression of an N-terminally fused His_6-tagged protein.

Media and genetic techniques

The *S.cerevisiae* strain PP30 was transformed with single or multicopy vectors bearing wild-type or mutant *HSP82* (Ito *et al*., 1983). Transformants were selected on drop-out media (Rose *et al*., 1990) without uracil and leucine. The ability of *HSP82* mutants to maintain cell viability was assessed by streaking the transformants on drop-out media without leucine, but containing uracil (50 mg/ml) and 5-FOA (0.1%).

For semi-dominance studies, *S.cerevisiae* strain PP30(a) was transformed with pYES2 bearing the wild-type or mutant alleles of *HSP82*. Transformants were maintained on drop-out media without leucine or uracil. Transcription of *HSP82* alleles on pYES2 was induced by streaking cells onto drop-out media without leucine or uracil containing galactose as the sole carbon source.

Radioactive labelling, production of cell extracts and fluorography

Expression of Hsp90 was visualized by pulse labelling yeast cultures with 15 μCi/ml of Trans^{35}S-label (ICN). Cultures growing exponentially at 28°C were shifted to 40°C heat shock for 10 min, and then labelled for 50 min. Quantification of radioisotope incorporation by scintillation counting was carried out as described previously (Panaretou and Piper, 1990). Radiolabelled protein was visualized by SDS–PAGE on 7% gels, followed by fluorography (Chamberlain, 1979).

Expression, purification and crystallization of mutants

Expression, purification and crystallization of the mutant N-terminal domains was as described previously for the wild-type protein (Prodromou *et al*., 1996, 1997a) except for the following modifications. Protein was purified by loading a 60 ml nickel affinity column in 20 mM Tris–HCl pH 8.0 containing 500 mM NaCl (buffer A). The column was then washed with buffer A containing 50 mM imidazole pH 8.0, and mutant N-terminal domain eluted from the column with buffer A containing 300 mM imidazole pH 7.0. The protein was subsequently concentrated by an 80% $(NH_4)_2SO_4$ precipitation and purified further using Superdex 75 gel filtration chromatography. Finally, the sample was concentrated by a second $(NH_4)_2SO_4$ precipitation, dialysed against 3×5 l of 20 mM Tris–HCl pH 7.4 and then concentrated to 40–50 mg/ml using an Amicon centriplus concentrator. Tetragonal bipyramidal crystals similar to those of the native N-terminal domain of Hsp90 (Prodromou *et al*., 1997a) were grown by vapour diffusion in hanging drops with the Asp79Asn mutant. Protein droplets contained 32.5 mM sodium succinate pH 5, 20 mM Tris–HCl pH 7.4, 65 mM $(NH_4)_2SO_4$, 9.75% PEGME 550 and 32 mg/ml protein. Crystallographic data sets were collected at 100 K on a Rigaku/MAR system with CuK radiation, and processed and refined as described previously (Prodromou *et al*., 1996, 1997a).

Hsp90-E33A mutant was expressed from the vector pRSETA in *E.coli* strain BL21(DE3)plysS under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter. Cells were harvested 3 h after induction with 1 mM IPTG, and resuspended in 20 mM Tris pH 7.4 containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 15% glycerol. Following disruption in the presence of protease inhibitors (Boehringer Mannheim), the clarified supernatant was subjected to ion exchange chromatography using Q-Sepharose and eluted with an NaCl gradient. After concentration on an XM50 membrane (Amicon), the protein was loaded onto a Sephacryl 400 HR gel filtration column equilibrated in 20 mM Tris pH 8.0 containing 500 mM NaCl and protease inhibitors. Fractions containing Hsp90E33A were pooled and diluted with 20 mM Tris pH 8.0 to reduce the NaCl concentration to 100 mM and applied to a Talon (Clontech) column equilibrated with 20 mM Tris pH 8.0 and 100 mM NaCl (buffer A). The column was washed with buffer A containing 10 mM imidazole and mutant protein eluted with buffer A containing 600 mM imidazole. The protein subsequently was dialysed against 3×5 l of 20 mM Tris 7.4 and 1 mM EDTA prior to concentrating to 15.7 mg/ml.

Isothermal titration calorimetry (ITC) of nucleotide binding

The titration experiments were performed using the MSC system (MicroCal Inc., MA). In each experiment, 16 aliquots of 15 μl of 1 mM ATP or ADP were injected into 1.3 ml of N-terminal Hsp90 at 100 μM at 25°C, and the resulting data were fit after subtracting the heats of dilution as described previously (Prodromou *et al*., 1997b). Heats of dilution were determined in separate experiments from addition of ATP or ADP into buffer and buffer into protein. Titration data were fit using a non-linear least squares curve-fitting algorithm with three floating variables: stoichiometry, binding constant ($K_b = 1/K_d$) and change of enthalpy of interaction (ΔH^0). Dissociation constants estimated for ATP

binding are: K_d = 97 ± 14 μM for the wild-type N-terminal domain, and K_d = 143 ± 75 μM for the Glu33Ala mutant N- terminal domain. These affinities are close to the limit for ITC and consequently have large apparent errors of measurement. Binding to the Asp79Asn mutant was undetectable.

ATPase assay

The ATPase assay was based on a regenerating coupled enzyme assay (Ali *et al*., 1993), in which the phosphorylation of ADP by PK at the expense of phosphoenol pyruvate is coupled to the reduction of the resulting pyruvate by LDH at the expense of NADH. Oxidation of NADH to NAD^+ produces a loss of optical density at the NADH absorbance maximum of 340 nm, in direct stoichiometry to the amount of ADP phosphorylated. Each 1 ml assay contained 100 mM Tris–HCl pH 7.4, 20 mM KCl, 6 mM $MgCl_2$, 0.8 mM ATP (Sigma), 0.1 mM NADH (Boehringer Mannheim), 2 mM phosphoenol pyruvate (Boehringer Mannheim), 0.2 mg of PK (Boehringer Mannheim), 0.05 mg of L-LDH (Boehringer Mannheim) and between 2 and 3.5 nmol of Hsp90 or HtpG. For the E33A mutant Hsp90, 20 nmol were used in each assay. Sufficient NADH was added to give an initial absorbance of 0.3 at 340 nm prior to addition of Hsp90s or fragments, and activity was detected as a decrease in absorbance. Inhibition of ATPase activity by geldanamycin was achieved by the addition of 1–10 μl of geldanamycin dissolved in dimethylsulfoxide (DMSO) to a final concentration of 1.5, 9, 15 and 30 μM geldanamycin. In control experiments, 1% DMSO present alone did not affect the measured ATPase activities, and stoichiometric re-phosphorylation of ADP directly added to the assay system was unaffected by 1% DMSO or geldanamycin at the maximal concentration used. Geldanamycin was kindly provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. All measurements were made on a Shimadzu UV-240 spectrophotometer.

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