

## Stimulation of the Weak ATPase Activity of Human Hsp90 by a Client Protein

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Heat shock protein 90 (Hsp90) is a molecular chaperone involved in the folding and assembly of a limited set of “client” proteins, many of which are involved in signal transduction pathways. *In vivo*, it is found in complex with additional proteins, including the chaperones Hsp70, Hsp40, Hip and Hop (Hsp-interacting and Hsp-organising proteins, respectively), as well as high molecular mass immunophilins, such as FKBP59, and the small acidic protein p23. The role of these proteins in Hsp90-mediated assembly processes is poorly understood. It is known that ATP binding and hydrolysis are essential for Hsp90 function *in vivo* and *in vitro*.

Here we show, for the first time, that human Hsp90 has ATPase activity *in vitro*. The ATPase activity is characterised using a sensitive assay based on a chemically modified form of the phosphate-binding protein from *Escherichia coli*. Human Hsp90 is a very weak ATPase, its activity is significantly lower than that of the yeast homologue, and it has a half-life of ATP hydrolysis of eight minutes at 37 °C. Using a physiological substrate of Hsp90, the ligand-binding domain of the glucocorticoid receptor, we show that this “client” protein can stimulate the ATPase activity up to 200-fold. This effect is highly specific and unfolded or partially folded proteins, which are known to bind to Hsp90, do not affect the ATPase activity. In addition, the peroxisome proliferator-activated receptor, which is related in both sequence and structure to the glucocorticoid receptor but which does not bind Hsp90, has no observable effect on the ATPase activity.

We establish the effect of the co-chaperones Hop, FKBP59 and p23 on the basal ATPase activity as well as the client protein-stimulated ATPase activity of human Hsp90. In contrast with the yeast system, human Hop has little effect on the basal rate of ATP hydrolysis but significantly inhibits the client-protein stimulated rate. Similarly, FKBP59 has little effect on the basal rate but stimulates the client-protein stimulated rate further. In contrast, p23 inhibits both the basal and stimulated rates of ATP hydrolysis.

Our results show that the ATPase activity of human Hsp90 is highly regulated by both client protein and co-chaperone binding. We suggest that the rate of ATP hydrolysis is critical to the mode of action of Hsp90, consistent with results that have shown that both over and under-active ATPase mutants of yeast Hsp90 have impaired function *in vivo*. We suggest that the tight regulation of the ATPase activity of Hsp90 is important and allows the client protein to remain bound to Hsp90 for sufficient time for activation to occur.

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**Keywords:** molecular chaperone; heat shock; protein folding; immunophilin; nuclear receptor

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Abbreviations used: Hsp, heat shock protein; Hip, Hsp-interacting protein; Hop, Hsp70-organising protein; PPAR, peroxisome proliferator-activated receptor; TPR, tetratricopeptide-repeating unit; GA, geldanamycin; PBP, phosphate-binding protein; hGR, human glucocorticoid receptor; LBD, Ligand-binding domain; RCMLA, reduced carboxymethylated  $\alpha$ -lactalbumin; GST, glutathione.

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## Introduction

Heat shock protein 90 (Hsp90) is an abundant, cytosolic molecular chaperone, essential in eukaryotes. Among the major heat-shock proteins, Hsp90 appears to be unique in its function: it is not required for the maturation or maintenance of most proteins *in vivo*, but has a specific set of "client" or substrate proteins.<sup>1-5</sup> It has been shown to be involved in the assembly and maturation pathways of a number of critical cellular complexes, many of which are involved in signal transduction pathways.<sup>2,3</sup> For example, client proteins include steroid receptors, non-receptor tyrosine kinases such as the src family, cyclin-dependent kinases cdk4 and cdk6, the cystic fibrosis transmembrane regulator and nitric oxide synthase.<sup>2,3</sup> Other client proteins are not involved in signal transduction and have quite different cellular functions, e.g. telomerase.<sup>6</sup> Hsp90 is essential for the assembly of complexes involving these proteins and reduced levels lead to compromised function in all the cases studied so far.<sup>7-9</sup> In contrast to other molecular chaperones, Hsp90 therefore appears to be relatively specific, the origins of such specificity, however, are not known.

Hsp90 has been shown to bind to short hydrophobic peptides and can maintain unfolded proteins in folding-competent states.<sup>5,10</sup> It is unclear whether, or how, these activities relate to its function *in vivo*. It has been proposed that Hsp90 binds to more highly structured regions of its client proteins,<sup>11</sup> in contrast to other molecular chaperones such as Hsp70, which bind to polypeptides that are largely unfolded and have extended conformations. The degree to which the Hsp90-bound client proteins have native-like structure is unclear. It has been proposed that the role of Hsp90 is to maintain the client protein in a conformation that is poised for activation either by a conformational change or by binding of a cofactor, ligand or partner protein.

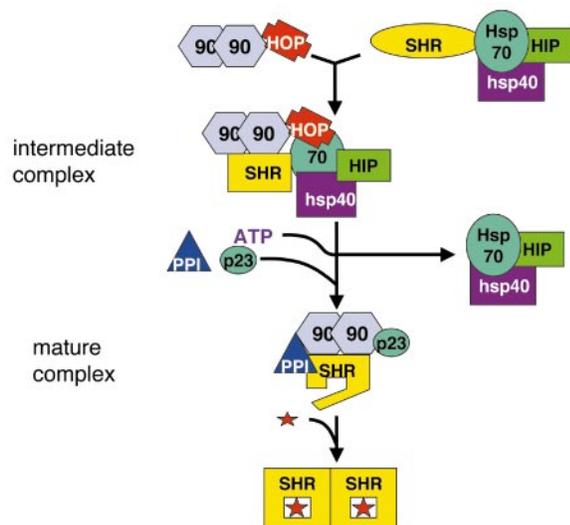
It is interesting to note how highly specific Hsp90 appears to be, it will bind to and activate some members of a protein family but does not bind to, nor is it required for the activation of, other members of the same family. For example, Hsp90 is essential for the correct assembly and maturation of active steroid receptors, including the glucocorticoid, oestrogen and progesterone receptors;<sup>12</sup> however, it does not bind to related members of the family such as the nuclear receptors, including the retinoic acid, vitamin D3 and peroxisome proliferator-activated (PPAR) receptors. In addition, whilst high levels of cellular Hsp90 are essential for the correct maturation of v-src, much lower levels are required for the cellular homologue, c-src.<sup>9,13</sup> As a result of these and other examples it has been suggested that Hsp90 may selectively bind to relatively unstable proteins.<sup>1</sup> Recently, however, we have shown that the glucocorticoid receptor, a client protein of Hsp90, is very stable, probably at least as stable as one of its

nuclear counterparts PPAR (S.H.M. & S.E.J., unpublished results). Thus, it is likely that other factors are as important in determining the recognition of client proteins by Hsp90.

As with other molecular chaperones, Hsp90 does not work alone and a number of other proteins are involved. *In vivo*, Hsp90 can be isolated in complex with other molecular chaperones such as Hsp40, Hsp70, Hip and Hop (the Hsp70-interacting and Hsp-organising proteins, respectively), the high molecular mass immunophilins FKBP59 and cyclophilin 40, the acidic protein p23 and the 50 kDa cdk-associated protein cdc37, as well as with its client proteins. For a recent review, see Pearl & Prodromou.<sup>4</sup> Many of these proteins, including Hop and the immunophilins, contain tetratricopeptide-repeating unit (TPR) domains that bind to a conserved MEEVD motif found at the C terminus of Hsp90.<sup>14</sup> The role of these proteins in Hsp90-mediated assembly processes is poorly understood.

Several groups have proposed schemes by which Hsp90 and associated proteins participate in the maturation and activation of client proteins.<sup>15,16</sup> Perhaps the best characterised of systems is the Hsp90-mediated assembly of steroid receptors into high-affinity, ligand-binding states. Considerable work has been done on the assembly pathway of the progesterone receptor by Smith and co-workers,<sup>17</sup> and on the glucocorticoid receptor by Pratt and co-workers.<sup>15,18</sup> Figure 1 illustrates the main steps in the proposed pathway. It is thought that the client protein first interacts with Hsp70, possibly through an initial interaction with Hsp40.<sup>19</sup> Hop, which binds simultaneously to Hsp70 and Hsp90 through its N and C-terminal TPR domains, then brings the client protein into contact with Hsp90. This state, in which the client protein is associated with Hsp70, Hop and Hsp90 (and possibly Hsp40 and Hip) is referred to as the intermediate complex (Figure 1). The client protein cannot interact with its cognate cofactor, ligand or partner protein in this state. In the next step on the pathway, Hsp70 and Hop dissociate from the intermediate complex, and immunophilins and/or p23 then bind to form a stable, mature complex. It is in this state that the client protein is capable of being activated by the binding of ligand (in the case of steroid receptors) or partner proteins (in the case of cdk4/6), etc. ATP binding and hydrolysis are essential for the formation of both the intermediate and mature complexes.<sup>19</sup> Although this scheme was first proposed some years ago,<sup>16</sup> many aspects of the mechanism are still not understood. For example, little is known about the conformational changes that occur in the client protein, or the role and mechanism by which Hsp90 and co-proteins bring about these conformational changes.

Hsp90 consists of highly conserved N and C-terminal domains connected by a highly charged linker. Crystal structures have been determined for the isolated N-terminal domains of human and yeast Hsp90 and for complexes of these with nucleotides and the inhibitors geldanamycin and



**Figure 1.** General model for the chaperone-mediated assembly of steroid hormone receptors (SHR) into high-affinity, ligand-binding states.<sup>12,16</sup> The client protein, in this case the steroid hormone receptor (SHR), is shown in yellow. An intermediate complex is formed that comprises the client protein and Hsp90, Hsp40, Hsp70, Hip and Hop. The mature complex forms in an ATP-dependent step in which Hsp70 and associated proteins dissociate and the small acidic protein p23 and a high molecular mass immunophilin such as FKBP59 or cyclophilin 40 bind (represented by blue triangle PPI in the diagram). In the mature complex, the client protein is in a state capable of being activated, in this case, by the binding of a steroid ligand (red star).

radicol, see Pearl & Prodromou<sup>4</sup> for a recent review. The crystal structure of the yeast Hsp90 N-terminal domain with bound nucleotide shows structural homology with type II topoisomerase DNA gyrase B<sup>20</sup> and MutL.<sup>21</sup>

Although there was some debate about whether Hsp90 bound ATP and possessed ATPase activity,<sup>22</sup> it has now been clearly established that Hsp90 ATP binding and hydrolysis are essential for its function both *in vitro*<sup>23</sup> and *in vivo*.<sup>24,25</sup> Interestingly, it has been shown that mutants of yeast Hsp90 that have either reduced or enhanced rates of ATP hydrolysis have impaired function *in vivo*.<sup>26</sup> Thus, the level of ATPase activity is critical to the function of Hsp90. Recently, it has been shown that the client protein can be dissociated from the mature complex by the addition of ATP.<sup>27</sup> However, it is unclear from these experiments whether the displaced client protein is in an active state, or whether it needs to remain bound to Hsp90 in order for activation to occur. Clearly, ATP binding and hydrolysis are key steps in driving conformational changes in the client protein, and regulation of the ATPase activity is therefore of importance.

Several groups have studied the function of Hop, which binds to Hsp70 and Hsp90 through its

N and C-terminal TPR domains. The yeast homologue of Hop, Sti1, has been shown to be dimeric and has a binding affinity to yeast Hsp90 in the submicromolar range.<sup>28</sup> There is some evidence that there is a significant conformational change on binding; however, it is not known whether this conformational change is in Hsp90 or Hop. In the presence of Hop, the binding of the inhibitor geldanamycin (GA), which mimics the binding of ADP, is weakened slightly, leading to the suggestion that Hop hinders access of ATP to its binding site.<sup>28</sup> Sti1 has been shown to inhibit the intrinsic ATPase activity of yeast Hsp90.<sup>28</sup>

The high molecular mass immunophilins (FKBP52, FKBP54, FKBP59 and cyclophilin 40) also contain TPR domains that bind to the conserved MEEVD motif at the C terminus of Hsp90. As with Hop, they bind with submicromolar affinities.<sup>28</sup> There is no evidence to suggest that there are any significant conformational changes on binding, nor that they bind to the N-terminal region of Hsp90 or affect ATP binding. It has been suggested that their role may be to displace Hop from Hsp90, thus reversing its effects.<sup>28</sup>

The small acidic protein p23 has been studied by a number of groups. Recently, the crystal structure of a C-terminally truncated form of p23 was solved and the protein shown to have a  $\beta$ -sandwich type structure.<sup>29</sup> It has been shown to bind to regions of Hsp90 encompassing both N and C-terminal domains, and is thought to bind specifically to the ATP-bound state of Hsp90.<sup>30</sup> The role of p23 remains unclear and different roles have been suggested. In one study, p23 appears to stabilise the interaction between the Hsp90 heterocomplex and the client protein,<sup>31</sup> whilst other studies have proposed a role for p23 as a client protein release factor linking ATP hydrolysis with client protein release.<sup>27</sup>

To date, all of the *in vitro* studies on the ATPase activity of Hsp90 have been performed on Hsp82, the yeast homologue.<sup>24,25,32</sup> There is no report on the activity of human Hsp90.<sup>2</sup> Here, we present results for the first time on the ATPase activity of human Hsp90 and compare it with that of yeast Hsp90. We present novel results on the effect of a client protein, the ligand-binding domain of the glucocorticoid receptor, on the ATPase activity of human Hsp90. We show that a client protein can stimulate the ATPase activity of Hsp90 up to 200-fold, and that this effect is specific. In addition, we characterise the effect of the human co-chaperones, Hop, FKBP59 and p23, on the basal and client-protein stimulated rates of ATP hydrolysis by human Hsp90.

## Results

### ATPase activity of human Hsp90

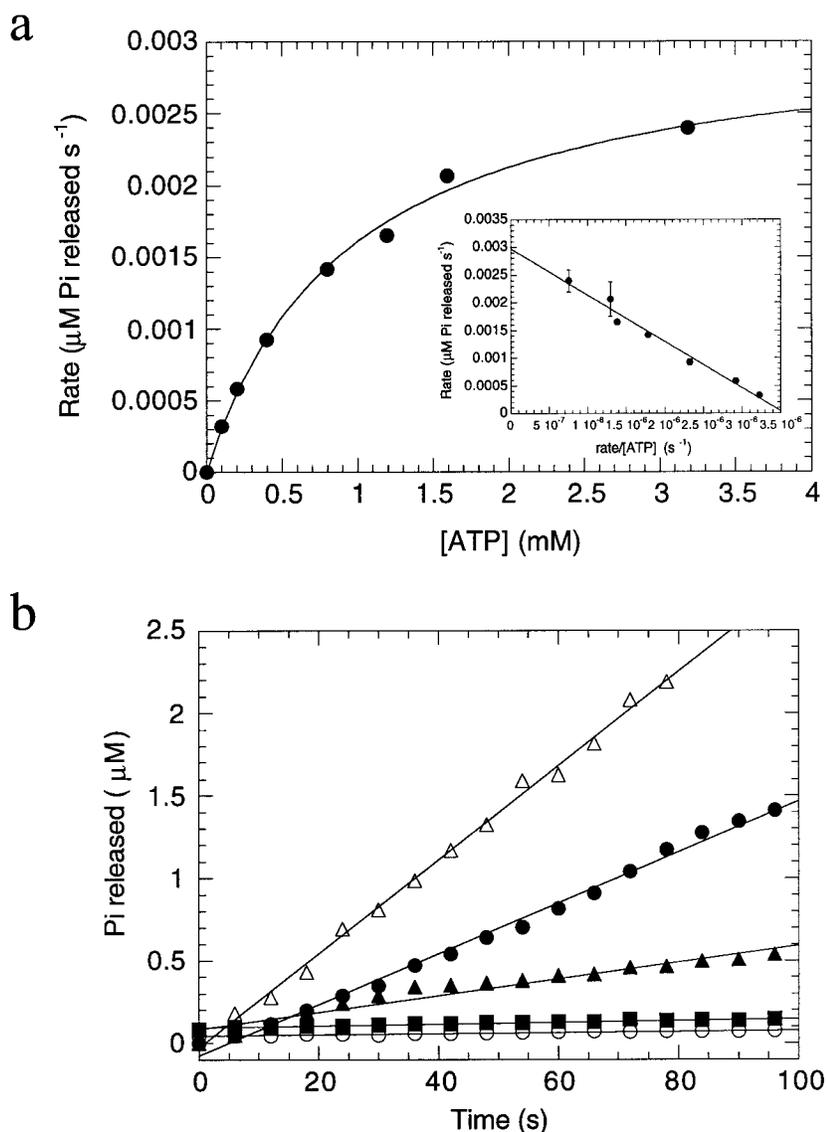
A sensitive assay for the detection of inorganic phosphate was used to measure the weak ATPase activity of recombinantly expressed human Hsp90.

This assay, developed by Webb and co-workers,<sup>33</sup> uses a chemically-modified version of the phosphate-binding protein from *Escherichia coli* (PBP). This undergoes a large change in fluorescence on binding phosphate. The hydrolysis of ATP and release of ADP and inorganic phosphate by human Hsp90 is shown in Figure 2(a). Although the rate is low under the conditions used, it is reproducible and accurate measurements can be made.

In order to verify that this weak ATPase activity was not due to the presence of contaminating ATPases, the rate was measured in the presence of a specific inhibitor of Hsp90, geldanamycin. No ATPase activity, above background, was observed in the presence of 60  $\mu\text{M}$  geldanamycin (data not shown). The  $\text{IC}_{50}$  is approximately 10  $\mu\text{M}$  (data not shown) similar to that measured for yeast Hsp90.<sup>24</sup>

Figure 2(a) shows the rate of inorganic phosphate production as a function of ATP concentration and a Michaelis-Menten analysis was used

to obtain values for  $k_{\text{cat}} = 1.50(\pm 0.1) \times 10^{-3} \text{ s}^{-1}$  ( $0.089(\pm 0.004) \text{ min}^{-1}$ ), and  $K_m = 840(\pm 60) \mu\text{M}$  at  $37^\circ\text{C}$ . Values for  $K_m$  reported for yeast Hsp90 vary between 100 and 830  $\mu\text{M}$ .<sup>24,25,27</sup> The value for human Hsp90 is within this range. The  $k_{\text{cat}}$  for human Hsp90 is some four- to fivefold lower than for the yeast protein M.<sup>24,25,27</sup> The values for yeast Hsp90 are based on a different assay, one that uses pyruvate kinase and lactate dehydrogenase to couple ADP production to the oxidation of NADH, which can be monitored spectrophotometrically. It is possible to use this assay for yeast Hsp90, as it is more active than our human protein. In order to verify that our assay gives accurate results, we tested the more active yeast Hsp90 in our assay. The  $k_{\text{cat}}$  of yeast Hsp90 was  $8.3(\pm 0.3) \times 10^{-3} \text{ s}^{-1}$  ( $0.50(\pm 0.02) \text{ min}^{-1}$ ), similar to that reported previously.<sup>24,25,27</sup> Thus, we are confident that the lower levels of ATPase activity measured for human Hsp90 here are reliable and significant.



**Figure 2.** Stimulation of the ATPase activity of human Hsp90 by hGR-LBD. (a) Michaelis-Menten kinetics of the ATPase activity of 2  $\mu\text{M}$  human Hsp90 $\beta$  were measured using the MDCC-PBP assay as described in Methods. The best fit of the data to a standard Michaelis-Menten equation is shown by the continuous line. Data were transformed into an Eadie-Hofstee plot (inset) which yields  $k_{\text{cat}} = 1.50(\pm 0.1) \times 10^{-3} \text{ s}^{-1}$  ( $0.089(\pm 0.004) \text{ min}^{-1}$ ) and a  $K_m = 840(\pm 60) \mu\text{M}$  at  $37^\circ\text{C}$ . (b) The initial ATPase rate of 2  $\mu\text{M}$  Hsp90 (open circle) is stimulated by addition of 2  $\mu\text{M}$  (filled squares), 4  $\mu\text{M}$  (filled triangles), 6  $\mu\text{M}$  (filled circles) or 10  $\mu\text{M}$  (open triangles) hGR-LBD.

### The effect of the ligand-binding domain of the glucocorticoid receptor on the ATPase activity of human Hsp90

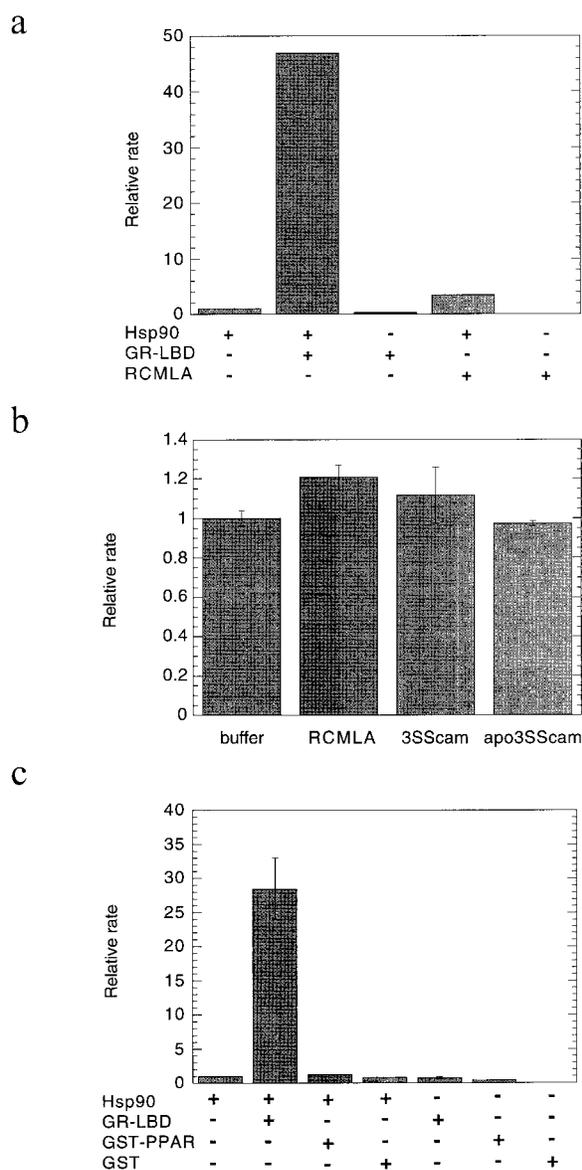
The glucocorticoid and progesterone receptors and, to a lesser extent, the oestrogen receptor, have been shown to require Hsp90 in order to assemble and maintain a high-affinity, ligand-binding state.<sup>7,8,15</sup> The interaction between Hsp90 and the glucocorticoid receptor has been shown to localise to the ligand-binding domain of the receptor.<sup>18</sup> This domain was therefore chosen as our "client" or "substrate" protein. We used a recombinantly expressed form of the ligand-binding domain of human glucocorticoid receptor (hGR-LBD), which we have shown can be refolded *in vitro*, in the presence of small amounts of the detergent Zwittergent, to a dimeric state which is compact and which has significant structure (S.H.M. & S.E.J., unpublished results).

Figure 2(b) shows the effect of hGR-LBD on the initial rates of ATP hydrolysis by human Hsp90. Remarkably, rate enhancements of up to 200-fold are observed. Controls showed that detergent alone had little effect on activity. In comparison, the ligand-binding domain stimulated the basal ATPase activity of yeast Hsp90 only modestly (by a factor of 2-3) under the same conditions (data not shown). In order to investigate this effect in more detail, Michaelis-Menten kinetics of the ATPase activity were determined and values of  $k_{cat}$  and  $K_m$  measured. Addition of hGR-LBD stimulates  $k_{cat}$  13-fold and there is also an approximately twofold decrease in  $K_m$  to 500( $\pm$ 100)  $\mu$ M at 37 °C.

### Specificity of the stimulation of ATPase activity

Hsp90 has been shown to bind many unfolded proteins and short peptides, in some cases keeping them in a folding-competent state and thus reducing overall aggregation.<sup>10,11</sup> In these studies, non-physiological protein substrates were used. As a result of these observations, we tested to see whether non-native proteins would also stimulate the ATPase activity of human Hsp90. Reduced carboxymethylated  $\alpha$ -lactalbumin (RCMLA) was chosen as a model of a non-native substrate protein, as it has been shown to be extensively unfolded.<sup>34</sup> Figure 3(a) shows the effect of RCMLA on Hsp90 ATPase activity. Although a very small increase in activity is observed, it is some 15 fold lower than that obtained with the cognate substrate hGR-LBD.

In addition to unfolded proteins, some Hsp90 homologues have been shown to bind to partially folded proteins. For example, the endoplasmic reticulum resident Hsp90 homologue, Grp94, has been reported to bind to late-folding intermediates of immunoglobulin chains.<sup>35</sup> To investigate if cytoplasmic Hsp90 also has a preference for partially structured substrates, 3SScam and apo-3SScam  $\alpha$ -lactalbumin were assayed. Both of these forms of  $\alpha$ -lactalbumin, in which three out of the four native disulphide bonds are formed, have been



**Figure 3.** Specificity of the substrate-stimulated Hsp90 ATPase activity. The ATPase activity of 2  $\mu$ M human Hsp90 was assayed in the presence of a fivefold molar excess of (a) hGR-LBD or RCMLA, which is known to be extensively unfolded;<sup>34</sup> (b) RCMLA; 3SScam and apo-3SScam (3SScam has three out of the four native disulphide bonds formed and Ca<sup>2+</sup>-bound, whilst apo-3SScam has three out of its four native disulphide bonds formed but no Ca<sup>2+</sup> bound). Both of these are known to adopt partially structured, molten globule-like states with differing degrees of secondary and tertiary structure;<sup>34</sup> (c) GST-PPAR or GST alone. All proteins were assayed in the absence of Hsp90 to quantify any contaminating ATPase activity. ATPase activities are expressed relative to that of Hsp90 in the absence of any added protein.

shown to adopt partially folded states similar to molten globule-like structures.<sup>34</sup> Neither protein significantly affected the ATPase activity of human Hsp90 (Figure 3(b)).

In order to investigate what appears to be the specific stimulation of the ATPase activity of human Hsp90 further, a protein related to the glucocorticoid receptor was studied. The nuclear receptor PPAR does not require Hsp90 for ligand binding *in vivo*. However, PPAR shares a significant degree of sequence homology with the glucocorticoid receptor and the ligand-binding domains of all members of the steroid and nuclear receptor superfamily have similar structures. Thus, this is the most stringent test of specificity. The effect of the ligand-binding domain of PPAR on the ATPase activity of Hsp90 is shown in Figure 3(c). There is no significant effect.

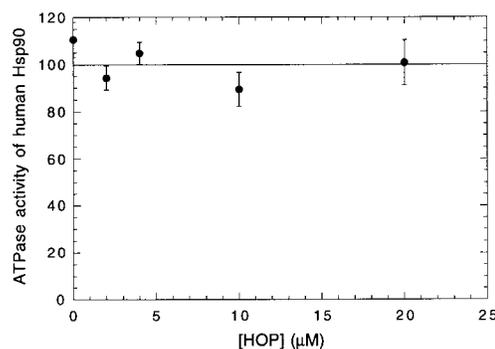
### The effect of co-chaperones, Hop, FKBP59 and p23, on the basal and stimulated ATPase activity of Hsp90

Yeast co-proteins such as the TPR-containing proteins Sti1 (p60/Hop in humans) and the high molecular mass cyclophilins Cpr6 and Cpr7 (Cyp40 in humans) are known to modulate the ATPase activity of yeast Hsp90 in the absence of any client protein.<sup>28</sup> Sti1 has been shown to inhibit the ATPase activity, whereas Cpr6 can reverse the effects of Sti1 by displacing it from Hsp90. We tested the effects of the human homologues on the basal and client-protein stimulated ATPase activity of human Hsp90. We used a recombinant form of human Hop which, like yeast Sti1, is dimeric, as shown by size-exclusion chromatography (data not shown). The results for human Hop are shown in Figure 4(a). In contrast to Sti1, Hop has little effect on the basal ATPase activity of human Hsp90 but shows a significant inhibition of the substrate-stimulated rate (Figure 4(b)). The effective  $IC_{50}$  is approximately 0.4  $\mu$ M, typical of the binding affinity measured for Sti1 and yeast Hsp90.<sup>28</sup>

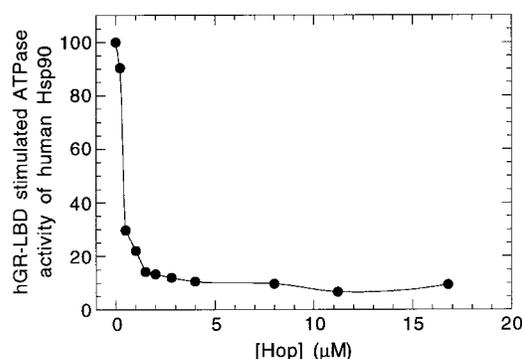
We also assessed the effect of human FKBP59, a high molecular mass immunophilin containing a C-terminal TPR domain similar to the yeast cyclophilins Cpr6 and Cpr7. Both FKBP59 and Cpr6 and 7 possess an N-terminal domain that has peptidyl-prolyl isomerase activity and a C-terminal TPR domain that binds to the same site on Hsp90. The results of FKBP59 on the basal and substrate-stimulated rates of ATP hydrolysis by Hsp90 are shown in Figure 5(a). Whilst it has little effect on the basal rate, it further stimulates the substrate-stimulated rate. It is important to note that FKBP59, either by itself or in combination with hGR-LBD, has little detectable ATPase activity.

The effect of the small acidic co-chaperone p23 on the ATPase activity was also measured. p23 is known to interact with Hsp90 in a nucleotide-dependent manner, with increased association when Hsp90 is in the ATP-bound state.<sup>23</sup> It has been reported that the yeast homologue, Sba1, has no effect on the ATPase activity of yeast Hsp90.<sup>25,27</sup> Figure 5(b) shows the results of p23 on the basal and substrate-stimulated ATPase rates of human Hsp90. In contrast with the yeast results, human

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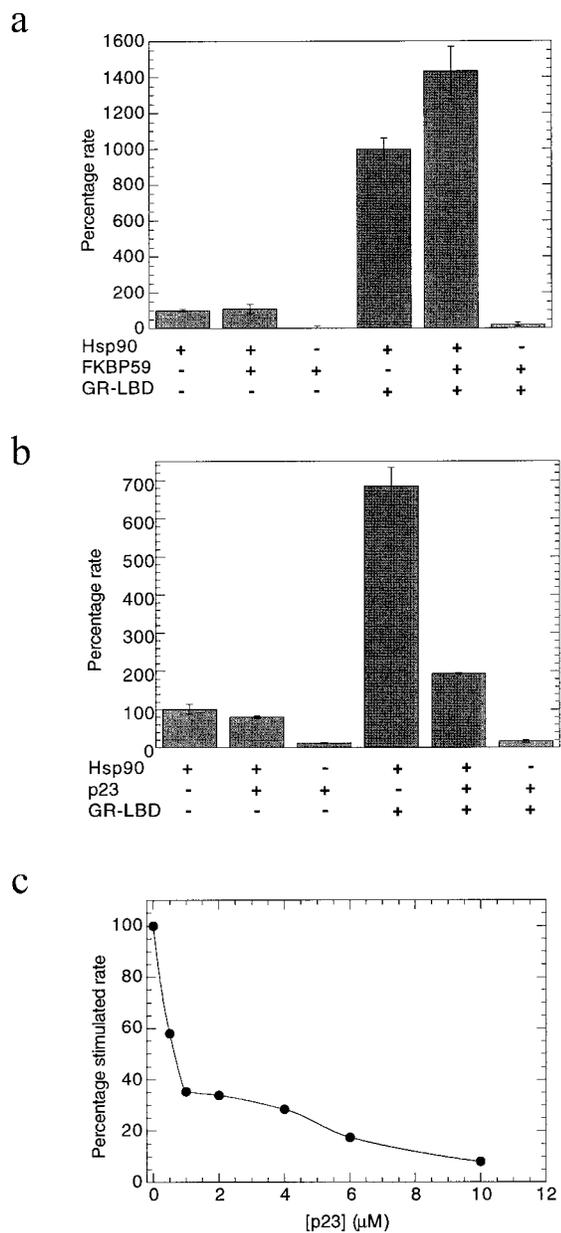
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**Figure 4.** Effect of Hop on the intrinsic and substrate-stimulated ATPase activity of Hsp90. (a) The effect of Hop on the ATPase activity (as measured from initial rates of ATP hydrolysis) of 2  $\mu$ M human Hsp90; (b) The effect of Hop on the substrate-stimulated ATPase activity of 2  $\mu$ M human Hsp90 (a fivefold molar excess of hGR-LBD was used). The (a) unstimulated or (b) stimulated activity in the absence of Hop is set at 100%.

p23 shows a small but significant suppression of the basal ATPase activity (to 71( $\pm$ 5)% initial rate), an effect that is observed consistently over many assays. Significantly, p23 dramatically inhibits the substrate-stimulated rate with a reduction in ATPase activity to near basal levels (Figure 5(c)).

Although p23 does not contain a TPR domain, it binds to both C and N-terminal regions of Hsp90 and may be displaced by TPR-containing proteins such as Hop and FKBP59. As we have shown that FKBP59 further stimulates the substrate-stimulated rate of ATP hydrolysis, we tested the combined effects of p23 and FKBP59. The association of FKBP59 with Hsp90 does not overcome p23 inhibition (Figure 5(c)). Even at a tenfold molar excess of FKBP59 over p23, the substrate-stimulated ATPase activity of Hsp90 is suppressed.



**Figure 5.** Regulation of Hsp90 ATPase activity by co-chaperones. The ATPase activity of 2  $\mu$ M human Hsp90 was assayed in the presence or absence of a fivefold molar excess of hGR-LBD and (a) FKBP59, (b) p23 or (c) fivefold molar excess of FKBP59 with increasing concentrations of p23. In (a) and (b), the unstimulated Hsp90 ATPase activity is set at 100%. In (c), the stimulated activity in the presence of both hGR-LBD and FKBP59 but in the absence of p23 is set at 100%.

## Discussion

### Human Hsp90 is a weak ATPase

ATP binding and hydrolysis have been shown to be essential for the function of Hsp90 *in vivo* in *Saccharomyces cerevisiae*.<sup>24,25</sup> This has been demonstrated in higher eukaryotes using an *in vitro* assay

for ligand binding to the progesterone receptor.<sup>23</sup> In this case, recombinantly expressed mutants of chicken Hsp90 that were deficient in either ATP binding or hydrolysis were used.<sup>23</sup> The ATPase activity of yeast Hsp90 has now been measured *in vitro* by a number of different groups.<sup>24,25,32</sup> In comparison, there is no report, to date, on the ATPase activity of Hsp90 from higher eukaryotes, including human Hsp90. Previous studies have been unable to detect any ATPase activity for human Hsp90.<sup>36</sup>

Here, we have shown that human Hsp90 possesses ATPase activity that we can measure *in vitro* using a sensitive assay (Figure 2(a) and (b)). In comparison to the yeast homologue, the ATPase activity of human Hsp90 is low. It is likely that this is one of the reasons why the activity of human Hsp90 has not been characterised before now. It has been reported that human Hsp90 could not be expressed in an enzymatically active form in *E. coli*.<sup>27</sup> However, our results show that an active form of human Hsp90 can be made recombinantly in *E. coli* and suggest that previous difficulties may have been due to detecting such intrinsically low levels of ATPase activity. With such low levels of activity it is essential to show that these are not due to contaminating ATPases. Using geldanamycin, a specific inhibitor of Hsp90, we have shown that the activity we measure is from human Hsp90.

The half-life for ATP hydrolysis is eight minutes at 37 °C, making human Hsp90 one of the weakest ATPases known. It is interesting to note that, although human Hsp90 exhibits a lower basal rate of ATPase activity than yeast Hsp90, human Hsp90 can substitute for yeast Hsp90 *in vivo*.<sup>7</sup>

### Client proteins stimulate the ATPase activity

Given that human Hsp90 has been shown to interact with a limited set of client proteins *in vivo*, it was important to study the effect of a physiologically relevant protein substrate on the intrinsic ATPase activity of human Hsp90. This was borne out by the fact that a model unfolded protein (RCMLA) and a "molten globule-like" partially folded protein (apo-3SScam  $\alpha$ -lactalbumin) had minimal effects on the activity (Figure 3). In contrast, a physiological substrate, hGR-LBD, known to be dependent on Hsp90 for ligand-binding, has a dramatic effect on the ATPase activity, stimulating it up to 200-fold (Figure 2(b)). In the presence of such a client protein, the ATPase activity of human Hsp90 becomes comparable with that of the yeast homologue. It is interesting to note that we also observe an effect of hGR-LBD on the ATPase activity of yeast Hsp90 but to a lesser degree (data not shown). This perhaps reflects the higher basal ATPase activity of yeast Hsp90.

Perhaps the most stringent test of specificity was with PPAR. This is a nuclear receptor related in sequence and structure to the glucocorticoid receptor but not known to interact with Hsp90. Again,

there was no observable effect on the ATPase activity (Figure 3(c)). Thus, the stimulation of ATPase activity is highly specific, correlating with the interaction of Hsp90 with a select set of client proteins *in vivo* and not all *de novo* folding polypeptides.

The results we have obtained for human Hsp90 are in sharp contrast to the promiscuous binding properties of other molecular chaperones such as GroEL and Hsp70. In these cases, the ATPase activities are also stimulated by substrate binding<sup>37</sup> but many unfolded or partially folded proteins act as substrates. It has been estimated that some 10–15% of cellular proteins interact with these chaperones.<sup>38–40</sup> In comparison, Hsp90 appears to be highly selective.<sup>2</sup>

These results are similar to those reported for DNA gyrase B and MutL which are structurally homologous to Hsp90.<sup>42</sup> In these cases, the binding of substrate also enhances the rate of ATP turnover.<sup>21,41</sup> Thus, it appears likely that these proteins, which all belong to the GHKL ATPase/kinase superfamily,<sup>42</sup> share a common mechanism.

### Co-chaperones regulate both the basal and stimulated ATPase activities

It is known that some of the co-chaperones associated with Hsp90 affect the basal rate of ATP hydrolysis by yeast Hsp90.<sup>28</sup> We have now established the effects of these proteins on the basal ATPase activity of human Hsp90 as well as the client protein-stimulated rate. Interestingly, some of the co-chaperones have different effects on the basal *versus* the client protein-stimulated rates (Figures 4 and 5). In addition, there are differences between the effect of human co-chaperones on the ATPase activity of human Hsp90 and results obtained for the homologous yeast proteins.<sup>27,28</sup>

The TPR-containing protein Hop binds to both Hsp70 and Hsp90, and is thought to act as a bridging agent enabling the client protein to be transferred from Hsp70 to Hsp90.<sup>14</sup> Recently, it has been shown that Sti1, the yeast homologue of Hop, inhibits the ATPase activity of yeast Hsp90.<sup>28</sup> In contrast, we find that human Hop has little effect on the basal ATPase activity of human Hsp90 (Figure 4(a)). However, Hop strongly inhibits the client protein-stimulated rate (Figure 4(b)). It is perhaps somewhat surprising that human Hop does not inhibit the basal ATPase activity of human Hsp90. However, this may reflect the fact that human Hsp90 is relatively inactive compared with yeast Hsp90 in the absence of a client protein.

The differing effects of Hop on the basal and stimulated ATPase activities strongly suggests a change in rate-determining step. Pearl and co-workers have suggested that the rate-determining step for the hydrolysis of ATP by yeast Hsp90 is dimerisation of the N-terminal ATPase domains.<sup>26</sup> In addition, results from kinetic competition studies on yeast Hsp90 have led to the proposal that either a conformational change (such as dimerisa-

tion) or ATP hydrolysis itself is rate-limiting.<sup>32</sup> By analogy, we propose that the rate-determining step of ATP hydrolysis by human Hsp90 in the absence of a client protein is also dimerisation of the N-terminal ATPase domains. If this is the case, then Hop association may not have any effect on this step and hence the overall basal rate. In the presence of a client protein, however, we suggest that the dimerisation step is accelerated to such a degree that another step in the mechanism becomes rate-limiting. Then it is possible that Hop affects the kinetics of this new rate-determining step. Such a change in rate-determining step is not unprecedented and it has been shown for other molecular chaperones that the binding of substrate or co-proteins can change the kinetics of ATP hydrolysis. For example, it has been found that the rate-determining step for the ATPase activity of the *E. coli* Hsp70 chaperone DnaK is the cleavage of the  $\gamma$ -phosphate group of ATP. The Hsp40 (DnaJ) co-chaperone stimulates this step to such an extent that the rate of dissociation of ADP and subsequent association of ATP becomes limiting.<sup>43</sup>

The high molecular mass immunophilins FKBP59 and cyclophilin 40 both contain a C-terminal TPR domain that binds competitively with Hop to the C-terminal domain of Hsp90. Pearl and co-workers have shown that the yeast homologues of cyclophilin 40 (cpr6/7) have no effect on the basal ATPase activity of yeast Hsp90;<sup>28</sup> however, they can displace Sti1 (Hop), thus removing its inhibitory effects. Figure 5(a) shows the effect of human FKBP59 on the ATPase activity of human Hsp90. As with Hop, there is little effect of FKBP59 on the basal rate of ATP hydrolysis. However, FKBP59 does have an effect on the client-protein stimulated ATPase activity, stimulating it further. Although the effect is not large, it is reproducible. The functional significance of this is not understood.

It is of interest that the two TPR-containing proteins, Hop and FKBP59, have different effects on the ATPase activity of human Hsp90, as has been reported for the yeast proteins.<sup>28</sup> This has been attributed to the fact that Hop interacts with both N and C-terminal domains of Hsp90, thereby affecting the ATP-binding site, whereas the immunophilins interact with the C-terminal domain alone. This supports the proposal that the role of the immunophilins may be to displace Hop, thus removing its inhibitory effect and allowing ATP hydrolysis to occur.<sup>28</sup>

We have also studied the effect of the co-chaperone p23 on the ATPase activity of human Hsp90. p23 is a small acidic protein known to associate with the mature complexes of Hsp90 with steroid receptors in an ATP-dependent manner.<sup>30</sup> It is thought that dimerisation of the N-terminal domains is necessary for p23 binding to occur.<sup>26</sup> Recent work has shown that the yeast homologue, Sba1, has no effect on the ATPase activity of yeast Hsp90.<sup>27</sup> In contrast, we find that human p23 inhibits the basal and stimulated rates of ATP hydroly-

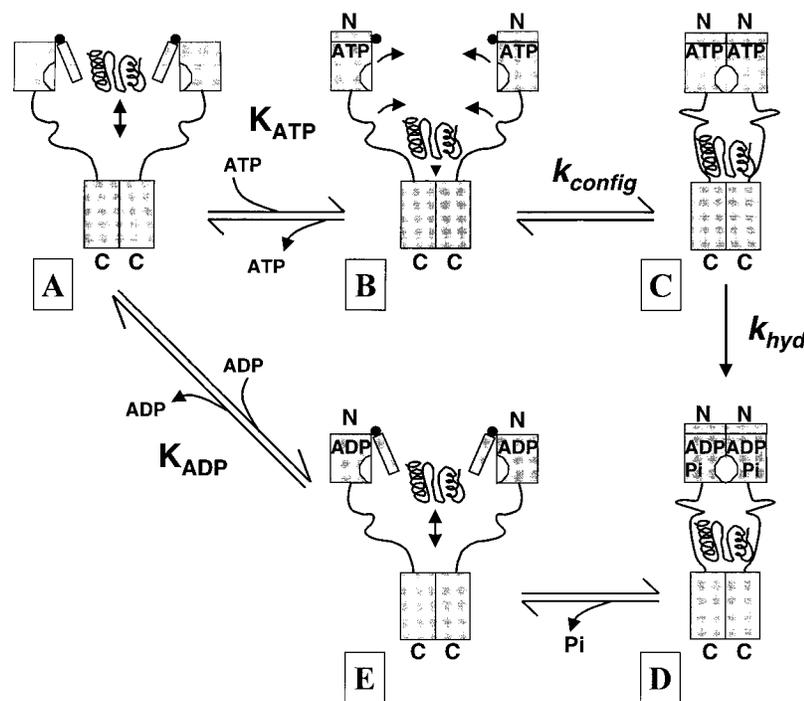
sis by human Hsp90 (Figure 5(b)) and it inhibits the ATPase activity in the presence of client protein and FKBP59 (Figure 5(c)). The apparent  $IC_{50}$  is approximately micromolar, suggesting that p23 binds to Hsp90 with an affinity in the same range as those measured for St1 and cpr6 or cpr7.

Although the role of p23 remains unclear, our results are consistent with previous results, which have shown that p23 binds to and stabilises mature complexes of Hsp90 with steroid receptors by binding to the ATP-bound form of Hsp90.<sup>23</sup> It is thought that it maintains Hsp90 in a closed conformation (with the N-terminal domains dimerised), a state in which the client protein remains bound. p23 inhibits the ATPase activity directly (Figure 5(b) and (c)), thereby slowing complex dissociation.

### Nucleotide status as a regulator of Hsp90 function

By analogy with DNA gyrase B,<sup>20</sup> and based on cross-linking experiments with ATP and non-hydrolysable analogues on yeast Hsp90, a mechanism for the action of Hsp90 has been proposed.<sup>26</sup>

In this mechanism, ATP binding and hydrolysis drives the opening and closing of a molecular clamp, formed when Hsp90, already a dimer through interactions in the C-terminal domain, also dimerises through its N-terminal domains.<sup>26</sup> The detailed mechanism is shown in Figure 6. In the nucleotide-free or ADP-bound state, Hsp90 is in an "open" conformation (A or E). It is thought that Hop binds to this state, thereby facilitating transfer of the client protein from Hsp70 to Hsp90. Binding of Hop to this state inhibits the ATPase activity of yeast Hsp90<sup>26</sup> and the client protein-stimulated activity of human Hsp90 (Figure 4(b)). Binding of Hop to yeast Hsp90 has been shown to change the affinity of Hsp90 to geldanamycin, leading to the suggestion that it disrupts the ATP-binding site.<sup>28</sup> However, this has not been shown directly. ATP binding causes a change in conformation, allowing the N-terminal domains to dimerise, thereby encapsulating the client protein (C) in a manner similar to the encapsulation of DNA by DNA gyrase B.<sup>20</sup> All evidence so far suggests that p23 binds to this state, thereby stabilising the heterocomplex and, for human Hsp90, inhibiting ATP hydrolysis.



**Figure 6.** Model of the mechanism of ATP hydrolysis by human Hsp90. Client protein stimulation of Hsp90 ATPase activity is consistent with a model that has been proposed for Hsp90 function<sup>26</sup> similar to that for the analogous protein DNA gyrase B.<sup>20</sup> (a) In the absence of any nucleotide Hsp90 is dimerised only through its C-terminal domains in an "open" conformation allowing the association of client protein. (b) The binding of ATP to the N-terminal domains causes a conformational change resulting in the closure of a lid over the ATP-binding site that reveals a dimerisation motif. (c) The N-terminal domains dimerise to form the "closed" state entrapping the client protein. (d) A conformational change occurs either after hydrolysis of ATP and (e) release of inorganic phosphate or, in the ADP-bound state in which the N-terminal domains are dissociated, allowing the client protein to dissociate.<sup>27</sup> Dissociation of ADP regenerates the nucleotide-free state. A kinetic analysis of yeast Hsp90<sup>32</sup> has shown that the rate of conformational change ( $B \rightarrow C$ ,  $k_{config}$ ) or the rate of hydrolysis ( $C \rightarrow D$ ,  $k_{hyd}$ ) could be rate-limiting. Results from cross-linking experiments coupled with mutagenesis studies have led to the proposal that dimerisation of the N-terminal domains is the rate-limiting step in ATP turnover.<sup>26</sup>

ATP hydrolysis and the subsequent release of inorganic phosphate and/or ADP results in the dissociation of the N-terminal domains, thereby allowing release of the client protein. In the absence of client protein or co-chaperones, it is thought that the rate-determining step in the ATP hydrolysis cycle is the dimerisation of the N-terminal domains<sup>26,44</sup> (B → C, Figure 6). However, it is unclear what the rate-determining step is in this cycle when client protein or co-chaperones are bound. There is precedence from studies on other ATP-dependent chaperones that binding of substrate protein or co-chaperones changes the kinetics of ATP hydrolysis. For example, GrpE stimulates the rate of the nucleotide exchange by DnaK up to 5000-fold.<sup>45</sup> At present, it is unclear exactly which step in the ATP hydrolysis cycle of human Hsp90 is rate-limiting and how that differs from yeast Hsp90.<sup>32</sup> A difference in the respective rate-determining steps may explain why (i) human Hsp90 is a weaker ATPase than yeast Hsp90, (ii) the differential effect of Hop on the basal and stimulated ATPase activity of human Hsp90, and (iii) yeast p23 (Sba1) has no effect on the ATPase activity of yeast Hsp90<sup>27</sup> compared to the inhibitory effect of human p23 on the ATPase activity of human Hsp90 (Figure 5(c)). Only a full kinetic analysis of human Hsp90 will be able to answer these questions.

If the dynamics of ATP binding, hydrolysis and ADP dissociation regulate the client protein binding in Hsp90,<sup>27</sup> then the turnover of ATP must correlate with the cycling of Hsp90 complexes *in vivo*. For instance, some temperature-sensitive mutants of yeast Hsp90 that have either increased or decreased ATPase activity are defective *in vivo*,<sup>8,26</sup> as the association between client protein and Hsp90 is either too long or too short to promote folding and assembly. This suggests that the timing mechanism of client protein association needs to be finely tuned. As we have shown here, the ATPase activity of human Hsp90 can be both up-regulated and down-regulated by the binding of client protein and co-chaperones.

## Materials and Methods

### Expression and purification

All proteins were expressed in *E. coli*. hGR-LBD was expressed and purified as described (S.H.M. & S.E.J., unpublished results). The clone for *E. coli* PBP A197C mutant was expressed and purified as described.<sup>33</sup> Hexahistidine human Hsp90 $\beta$  was purified using a nickel/iminodiacetic acid/agarose column (Sigma). The column was washed with 10 mM MgATP in 50 mM Tris-HCl (pH 8.0) and then 50 mM Tris-HCl (pH 8.0). The fusion protein was eluted with 250 mM imidazole, 50 mM Tris-HCl (pH 7.5). Hsp90 was bound to a Mono Q HR10/10 column (Pharmacia) equilibrated in 50 mM Tris-HCl (pH 7.5), 1 mM DTT and then eluted with a linear gradient of 0–0.5 M NaCl over 90 ml. Pooled Hsp90 was further purified on a G200 Sephadex HR26/60 gel-filtration column, pre-equilibrated in 50 mM Tris-HCl

(pH 7.4), 500 mM NaCl, 1 mM DTT. Human Hop was expressed as a fusion with a chitin-binding domain, separated by an intein. The fusion was bound to chitin-Sepharose (NEB) in 20 mM Hepes (pH 8.0), 500 mM NaCl, 1 mM EDTA and cleaved by addition of 50 mM DTT. The released Hop was further purified on a Mono Q HR10/10 column (Pharmacia) pre-equilibrated in 50 mM Tris-HCl (pH 8.0), 1 mM DTT, eluting with a linear gradient of 0–1 M NaCl over 180 ml and by gel-filtration on a G200 Sepharose HR26/60 column in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT. Human p23 was expressed as a hexahistidine fusion and purified on nickel/iminodiacetic acid/agarose column as described for Hsp90 above, except that the protein was cleaved *in situ* with bovine thrombin (Sigma). The eluted untagged p23 was purified by gel-filtration on a G75 Sepharose HR26/60 in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM glycerol, 1 mM DTT. The pooled p23 was further purified on a Mono Q HR10/10 column (Pharmacia) equilibrated with 20 mM piperazine (pH 5.2), 10 mM glycerol, 1 mM DTT. Bound protein was eluted with a linear salt gradient over 180 ml from 0–1 M NaCl. FKBP59 was expressed as a GST-fusion and purified on GSH-Sepharose (Pharmacia) equilibrated in phosphate-buffered saline (PBS). Unbound material was washed from the column with 10 mM MgATP in PBS and then with PBS, 1 mM DTT. FKBP59 was eluted from the column following cleavage from GST with bovine thrombin. The protein was subsequently purified by gel-filtration on a G200 Sepharose HR26/60 column equilibrated in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and then on a Mono Q HR10/10 column (Pharmacia) binding in 20 mM bis-Tris (pH 6.2), 1 mM DTT and eluting with a linear gradient from 0–1 M NaCl over 180 ml. PPAR was purified as described for FKBP59 without thrombin cleavage. All proteins were dialysed extensively against Tris-HCl (pH 7.4), 1 mM DTT. The purity of the proteins were determined by SDS-PAGE, mass spectrometry and N-terminal protein sequencing to be greater than 95% (data not shown). Protein concentrations were determined spectrophotometrically.

### ATPase assay

ATP hydrolysis was measured by the relative fluorescence emission at 465 nm of A197C PBP labelled with *N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC, Molecular Probes) upon binding inorganic phosphate.<sup>33</sup> An excitation wavelength of 425 nm was used in either a SLM Aminco Bowman Series 2 luminescence spectrometer or Molecular Devices SpectraMax plate reader. The fluorescence change ( $\Delta F$ ) of MDCC-PBP upon binding was standardised by addition of accurate concentrations of inorganic phosphate. The data were fit to the following equation:

$$\Delta F = \Delta F_{\max} - K_d \times \frac{\Delta F}{[L]} \quad (1)$$

where  $[L]$  is ligand concentration and  $K_d$  is the dissociation constant. Typically, 1  $\mu$ M MDCC-PBP was used to measure the inorganic phosphate released by 2  $\mu$ M Hsp90 in 50 mM Tris-HCl (pH 7.4), 6 mM MgCl<sub>2</sub>, 20 mM KCl, 1 mM DTT with 1 mM ATP, at 37 °C. Contaminating inorganic phosphate was removed prior to the addition of Hsp90 by incubation for 20 minutes with 140  $\mu$ M 7-methylguanosine (Sigma) and 1 unit  $\mu$ l<sup>-1</sup> of purine nucleoside phosphorylase (Sigma), which was inhibited during the measurement by addition of acyclo-

vir diphosphate.<sup>46</sup> The background ATPase activity was determined by addition of geldanamycin (Sigma) to Hsp90 at a final concentration of 60  $\mu$ M. All assays were carried out in triplicate.

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