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Characterization of the 90 kDa heat shock protein (HSP90)-associated ATP/GTPase

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Abstract. The 90 kDa heat shock protein (HSP90) is an ATP-binding molecular chaperone with an associated ATPase activity having nucleoplasmin and HSP70-binding homology domains and containing Ca-binding EF-hands and a nuclear localization signal. Here we characterize the HSP90-associated ATPase and show that it is (i) a P-type ATPase inhibited by molybdate and vanadate, (ii) able to hydrolyze methylfluorescein phosphate with a 5–6-fold higher affinity, (iii) a 3-times better GTPase than ATPase in the presence of calcium and (iv) HSP27 and F-actin, but not HSP10 can "convert" the HSP90-associated ATPase activity to HSP90 autokinase activity. The HSP90-associated ATP/GTPase may participate in the regulation of complex formation of HSP90 with other proteins, such as F-actin, tubulin and heat shock proteins.

Keywords. Molecular chaperone; Vanadate; molybdate; methylfluorescein phosphate; HSP10; HSP27.

1. Introduction

The 90 kDa heat shock protein (HSP90) is a ubiquitous molecular chaperone believed to play an organizational role in protein traffic (Miyata and Yahara 1991, 1992; Wiech *et al* 1992; Pratt 1993; Jakob and Buchner 1994). In our earlier studies we have demonstrated that HSP90 possesses an ATP-binding site and an ability to phosphorylate itself (Csermely and Kahn 1991). Binding of ATP to HSP90 induces a large conformational change in the protein (Csermely *et al* 1993). Unlike the HSP60 and HSP70 chaperone families (Martin *et al* 1993; Palleros *et al* 1993), ATP does not seem to play an important role in the chaperone function of HSP90 in several model systems (Miyata and Yahara 1992; Wiech *et al* 1992; Jakob and Buchner 1994). However, ATP induces the dissociation of HSP9O from F-actin (Kellermayer and Csermely 1995) which indicates that ATP may modulate the complex formation of HSP90 with some other proteins as well. HSP90 preparations display an ATPase activity (Nadeau *et al* 1992, 1993). In our present paper we characterize this HSP90-associated ATPase and show that it prefers GTP to ATP in the presence of calcium and can be modulated by HSP27 and F-actin, but not by HSP10 and HSP70.

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2. Materials and methods

2.1 *Chemicals*

The chemicals used for polyacrylamide gel electrophoresis (PAGE), Econo-Pac HTP cartridges were from Bio-Rad (Richmond, CA, USA). Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow and Sephacryl S-200 were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). HSP10 was expressed in *Escherichia coli,* (Ryan *et al* 1994, 1995). F-actin was kindly provided by MSZ Kellermayer (Central Research Laboratory, University Medical School of Pécs, Hungary). Recombinant human HSP90 was a kind gift of Drs Yoshihiko Miyata and Ichiro Yahara (Tokyo Metropolitan Institute of Medical Sciences, Japan). HSP27, HSC70 and HSP70 were purchased from StressGen (Victoria B C, Canada). [γ-^{32}P] ATP (370 TBq/mmol), and [γ-^{32}P]GTP (370 TBq/mmol) were from the Hungarian Institute of Isotopes (Budapest, Hungary). All the other chemicals used were from Sigma Chemicals Co. (St. Louis, Mo, USA).

2.2 *Isolation of HSP90*

The 90 kDa heat shock protein was purified from human placenta with consecutive chromatographies on Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow, Sephacryl S-200 and Econo-Pac HTP as described earlier (Csermely and Kahn 1991). The purity of these HSP90 preparations was more than 95% as judged by silver staining of SDS polyacrylamide gels. In some experiments HSP90 from livers of 3–4 week old male CFLP mice and of 4 month old, male Sprague-Dawley rats was also examined, Protein concentrations were determined according to Bradford (1976).

2.3 *Measurement of ATPase and GTPase activities*

ATPase and GTPase activities were determined in a 0·1 ml reaction medium containing 5 μg HSP90, 0·5 mM [γ-^{32} P] ATP or [γ -$^{32}1^{3}$] GTP (200,000 cpm/sample, 40 cpm/pmol), 5 mM $CaCl_2$ (if not otherwise indicated), and 50 mM Hepes, pH 7·4. After an incubation of 20 min at 25°C, the amount of [^{32} P]-inorganic phosphate was determined by extraction of its phosphomolybdate complex to organic phase and liquid scintillation counting (Shacter 1984).

2.4 *Measurement of methylfluorescein phosphatase activity*

Hydrolysis of methylfluorescein phosphate was monitored by the method of Hill *et al* (1968). Human HSP90 (5 μg) sample was incubated in the presence of 5 mM $CaCl_2$, 50mM Hepes, pH 7·4, and 20 μM 3-O-methylfluorescein phosphate at 25°C. The fluorescence of the sample was monitored in a Hitachi F2500 spectrofluorimeter using an excitation/emission wavelength pair of 470/510 nm with 5 nm slits.

2.5 *Autophosphorylation of HSP90*

Human HSP90 (5 μg) was incubated in the presence of 50 mM Hepes, pH 7·4, 200 μM of [γ-^{32}P] ATP (5–6,000 cpm/pmol) and 10 mM of $CaCl_2$ for 20 min at 37°C. The

reaction was stopped by boiling for 5 min in the presence of Laemmli buffer (Laemmli 1970) containing 10 mM EDTA, and 20 mM DTT. Samples were analysed by SDS-PAGE and autoradiography. The radioactivity of the HSP90 bands was quantitated by densitometry of the autoradiograms.

3. Results and discussion

3.1 *Structural analysis of HSP90*

In spite of the fact that the primary structure of HSP90 has been described more than ten years ago (Farrelly and Finkelstein 1984), relatively little is known about the functional role of various segments of the protein. Besides the detailed investigation of the putative HSP90 binding regions for steroid receptors and for pp60$^{v\text{-}src}$ (Cadepond *et al* 1993; Sullivan and Toft 1993; Tbarka *et al* 1993; Nathan and Lindquist 1995), an HSP90 binding site for calmodulin (Minami *et al* 1993) and a C-terminal HSP90 dimerization site (Minami *et al* 1994) have also been estiblished (figure 1A). The dimerization site may overlap with the epitope of the AC-88 monoclonal anti-HSP90 antibody which shows a preference for the uncomplexed protein. Binding of AC-88 to HSP90 interferes with the binding of several proteins, including F-actin, which may indicate the involvement of the C-terminal region of HSP90 in protein binding (Schlatter *et al* 1992; Sullivan and Toft 1993; Kellermayer and Csermely 1995).

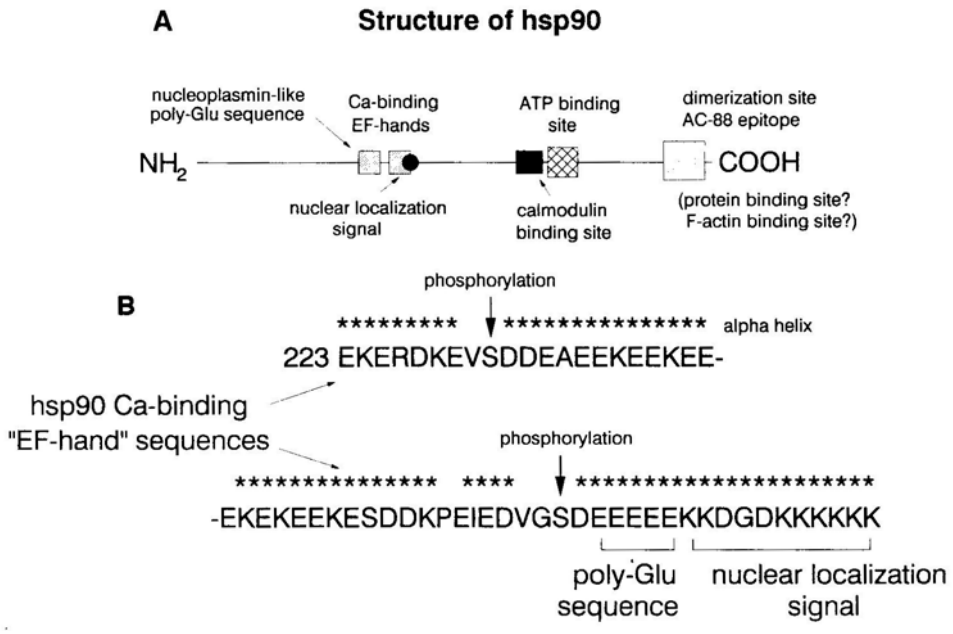

Figure 1. Structural features of hsp90. (**A**) A sketch of HSP90 primary structure denoting the position of various binding sites, and homology domains. The alignment of the putative ATP-binding site, and the determination of the calmodulin-binding site and the dimerization site were as described by Csermely and Kahn (1991) and Minami *et al* (1993, 1994) respectively (**B**) Homology of HSP90 segments with nucleoplasmin, Ca-binding EF-hands and with the bipartite nuclear localization signal.

Our earlier findings (Csermely and Kahn 1991) indicated the presence of at least one conserved ATP-binding site in the C-terminal half of HSP90. Comparison of HSP90 primary structure with the polyglutamic acid sequence of nucleoplasmin, which plays an important role in the assembly of nucleosomal structure (Dingwall *et al* 1987), reveals its similarity with a highly charged region in the N-terminal half of HSP90 (figure 1B). In agreement with this homologous sequence, circular dichroism measurements indicated that HSP90 may have a nucleoplasmin-like activity (Csermely *et al* 1994).

HSP90 is a calcium binding protein (Kang and Welch 1991; Minami *et al* 1993). Adjacent to its nucleoplasmin-like region, two α-helix pairs are predicted by the methods of Chou and Fasman (1974) and Garnier *et al* (1978) showing a high similarity with the calcium binding EF-hand motif (Kretsinger 1987; Renner *et al* 1993). The putative HSP90-EF-hands contain the two major *in vivo* phosphorylation sites of HSP90 which can be phosphorylated *in vitro* by casein kinase II (Lees-Miller and Anderson 1989), a kinase known to form a complex with HSP90 (Miyata and Yahara 1992). An overlap of the phosphorylation sites and the putative calcium binding sites suggests that phosphorylation of the major phosphorylation sites may be a requirement for calcium binding of HSP90. As a possible consequence of this, the Ca-dependent autophosphorylation of HSP90 requires the occupancy of the major phosphorylation sites (Csermely and Kahn 1991).

A small portion of HSP90 is known to reside and/or translocate to the cell nucleus in resting cells and after heat shock (Collier and Schlessinger 1986; Gasc *et al* 1990; Morcillo *et al* 1993). Nuclear transport of HSP90 may be mediated by a bipartite nuclear localization sequence (Dingwall and Laskey 1991) present next to the EF-hand-like structures (figure 1B). Addition of an extra nuclear localization signal to HSP90 shifts cytoplasmic steroid receptor mutants to the nucleus (Kang *et al* 1994). Addition of an extra nuclear localization signal to wild type steroid receptors "docks" them inside the nucleus and thus inhibits their nucleo-cytoplasmic shuttle (Madan and DeFranco 1993). The HSP90 nuclear localization signal may participate in the nucleo-cytoplasmic shuttle of steroid receptors.

The central, highly charged region of HSP90 has been proposed to participate in its association with steroid receptors (Cadepond *et al* 1993; Tbarka *et al* 1993) and with casein kinase II (Miyata and Yahara 1995). Alternating lysine and glutamic acid residues ("KEKE-motifs") may be generally involved in protein-protein interactions (Realini *et al* 1994). HSP90 also harbours consensus sequences (DEAD box) of helicases and displays a helicase activity (Szántó *et al* 1995).

3.2 *The HSP90-associated ATPase is a P-type ATPase*

Nadeau *et al* (1992, 1993) demonstrated the presence of an ATPase activity in purified *Trypanosoma* and human HSP90 preparations. Later the identity of this ATPase with (mammalian) HSP90 was questioned on the basis of negligible ATPase activity of highly purified and recombinant HSP90 (Nadeau *et al* 1994; Shi *et al* 1994). In agreement with the idea that more than 95% pure HSP90 preparations do not display considerable ATPase activity, the ATPase activity of mouse, rat or recombinant human HSP90 was less than 50 pmol phosphate/min/mg protein (Csermely and Kahn 1991; and data not shown). However, approximately 95% pure human placental

HSP90 displays an ATPase activity of 5·3 ± 3 nmol phosphate/min/mg protein in the presence of Mg^{2+} ions (k_{cat} = 0·6 min^{-1}) which is still significantly smaller than the ATPase activity reported by Nadeau *et al* (1993). This ATPase activity is lost if HSP90 is purified further by SDS-PAGE using slab gel (Laemmli 1970) or the BioRad PrepCell apparatus and electroeluted or renatured by the method of Kameshita and Fujisawa (1989). In some experiments the HSP90-associated ATPase activity could be partially regained by the recombination of the SDS-PAGE fractions, which may indicate that either HSP90 is able to activate a contaminating ATPase or the latent ATPase of HSP90 can be activated by some associated proteins. So far we were not successful to work out a reproducible method to regain the HSP90-associated ATPase activity. This difficulty may arise from the irreversibility of binding of HSP90 to its "partner", a phenomenon requiring special conditions for the reconstitution of the HSP90-steroid receptor complex (Pratt 1993) or from a partial (and more less irreversible) denaturation of HSP90 or its partner by detergent treatment. Consistent with this "detergent-sensitivity", the HSP90-associated ATPase can be inactivated also by nonionic detergents, such as Tween-20. Since from our experiments it is not certain that the ATPase activity of highly purified human HSP90 preparations comes from HSP90, we use the term "HSP90-associated ATPase" to denote this activity.

Vanadate and molybdate bind to HSP90 and induce a conformational change in the protein similar to that caused by ATP (Csermely *et al* 1993). Molybdate binding of HSP90 seems to lock the protein to its target, such as the steroid receptor or the src tyrosine kinase (Hutchison *et al* 1992). Both molybdate and vanadate were good inhibitors of the HSP90-associated ATPase having 60 and 90% maximal inhibition at 0·1 mM final concentration, respectively (figure 2). The inhibition by these ATP analogues displayed similar concentration dependence to that of their binding to HSP90 (Csermely *et al* 1993). On the contrary, the maximal inhibition of the HSP90-associated ATPase by nitrate or azide anion was approximately 20–25% even at millimolar concentrations which shows that the ATPase is a P-type, but not an F- or V-type ATPase (Pedersen and Carafoli 1987).

3.3 *Characterization of the HSP90-associated ATPase*

3-O-methylfluorescein phosphate, a fluorescent molecule for the measurement of the phosphatase reaction of ATPases (Hill *et al* 1968) is also a good inhibitor of the HSP90-associated ATPase activity (figure 3). Its inhibitory effect is comparable with that of vanadate (see figures 2 and 3). On the contrary, ATP acts as a poor inhibitor of the methylfluorescein phosphatase reaction showing that the adenine-binding pocket of the HSP90-associated ATPase has a strong preference for fluorescein to adenine and thus the HSP90 ATP-binding site may be more hydrophobic than that of the "usual" P-type ATPases.

The k_M of ATP-hyrolysis is comparable to the value described by Nadeau *et al* (1993) and to the half-maximal ATP concentrations of the ATP-induced conformational change and of the autophosphorylation (table 1). The k_M of the methylfluorescein phosphatase reaction is 5–6 times smaller, showing the high affinity of the ATPase to fluorescein

The HSP90-associated ATPase displays an approximately doubled activity in the Presence of Ca^{2+} compared to that in the presence of Mg^{2+} at 25°C (figure 4). This

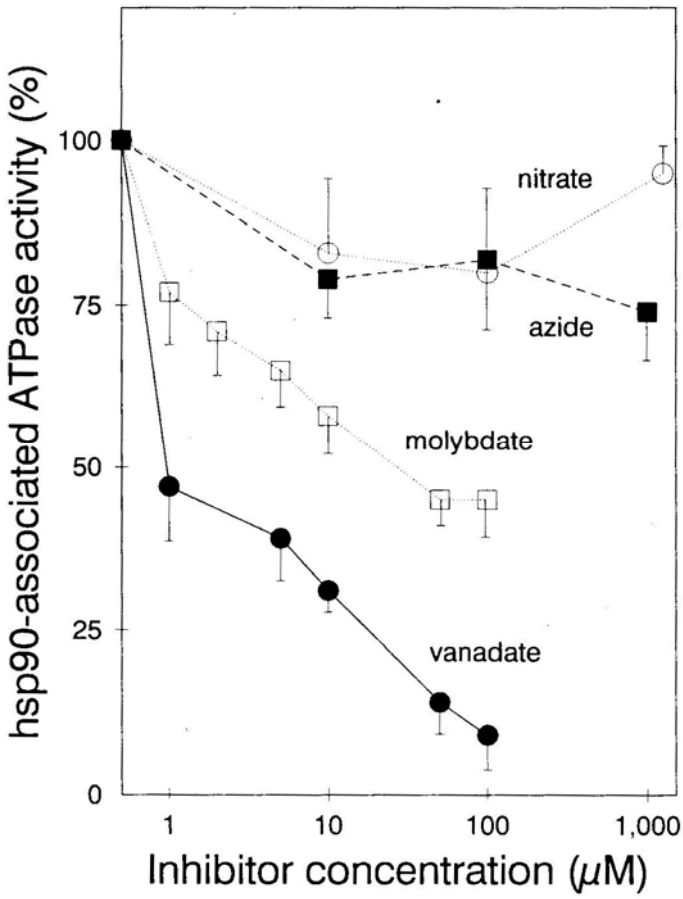

Figure 2. Inhibition of hsp90-associated ATPase activity. Human placental hsp90 was purified and its ATPase activity was measured as described in § 2. Inhibitors were added at final concentrations indicated. Data represent mean ± SD of three independent experiments.

unusual preference for Ca^{2+} ions is similar to the ATP-binding and autophosphorylation of HSP90 (Csermely and Kahn 1991). The ATPase activity is relatively heat-sensitive having a half-maximal inhibition around 60°C.

3.4 *Preference of the HSP90-associated* ATPase *for GTP in the presence of calcium*

GTP is able to substitute ATP in the autophosphorylation of HSP90 (Csermely and Kahn 1991). Similarly, the HSP90-associated ATPase displays a GTPase activity commensurate with the ATPase activity in the presence of Mg^{2+},. In the presence of Ca^{2+}, however, the HSP90-associated nucleotide triphosphatase is 3 times more active with GTP than with ATP (table 2).

In summary, we would like to emphasize that several features of this enzyme reaction (e.g., its inhibition pattern, ion-selectivity and GTP-preference) are highly similar to those of the HSP90 autophosphorylation. This similarity suggests, although does not

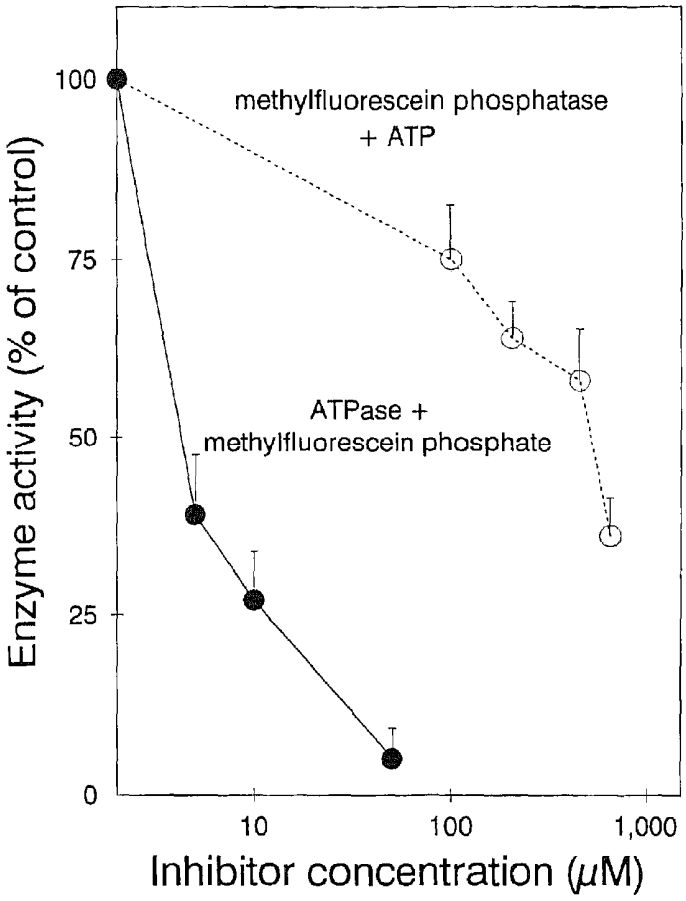

Figure 3. Competition of ATP and methyl-fluorescein-phosphate for the hsp90-associated hydrolytic activity. Methylfluorescein phosphatase and ATPase activity of human placental hsp90 preparations were measured as described in §2. (O), Methylfluorescein phosphatase activity in the presence of ATP; (●), ATPase activity in the presence of methylfluorescein phosphate. Inhibitors were added at final concentrations indicated. Data represent mean ± SD of three independent experiments.

prove that the ATPase activity of highly purified HSP90 preparations is an intrinsic property of HSP90 which is undetectable in the absence of one or more activator proteins.

3.5 *Effect of other proteins on the HSP90-associated ATPase activity*

Following the working hypothesis that HSP90 may show an ATP/GTPase activity in the presence of an activator, which may have been already separated by the end of the purification procedure we analysed the effect of various other proteins on the HSP90-associated ATPase. None of the proteins tested was able to activate the ATPase. On the contrary, HSP27 and F-actin significantly inhibited the ATPase, with a parallel activation of the autophosphorylation of HSP90 (table 3). A similar effect was observed

Table 1. Comparison of apparent k_M values of HSP90/ATP interactions.

Interaction	Apparent k_M (μM)
Conformational change (Csermely *et al* 1993)	240
Autophosphorylation (Csermely and Kahn 1991)	160
ATP hydrolysis	140 ± 12^a
Hydrolysis of methylfluorescein phosphate	25 ± 4^a

[a] k_M values of ATP and methylfluorescein phosphate hydrolysis were determined as described in §2. Data represent mean ± SD of three independent experiments.

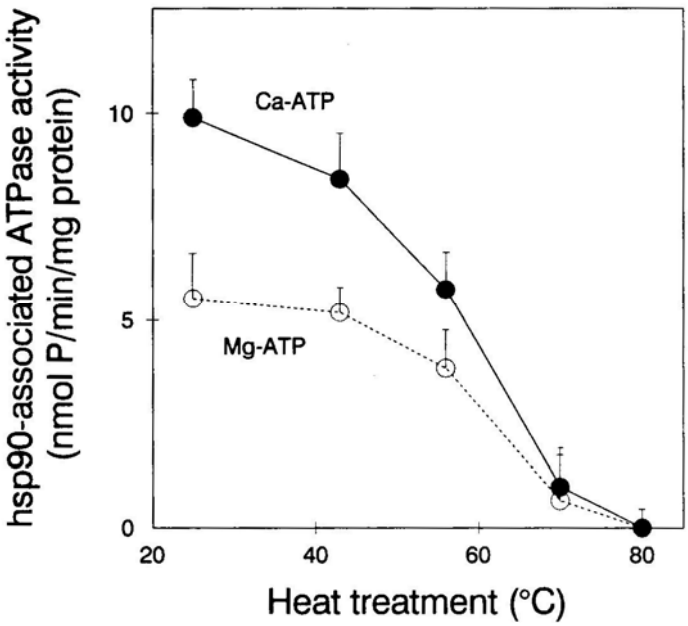

Figure 4. Heat stability of the HSP90-associated ATPase. ATPasc activity of human HSP90 was determined after a heat treatment of 10 min at temperatures indicated as described in § 2. (O), ATPase activity in the presence of Mg^{2+} ions; (●), ATPase activity in the presence of Ca^{2+} ions. Data represent mean ± SD of three independent experiments.

if we added human dnaJ to the system (P Csermely, T Schnaider, Cs Söti, Y Miyata, M E Cheatham, G Nardai and I Yahara, in preparation). Thus these proteins seem to "convert" the HSP90-associated ATPase to HSP90 autokinase activity.

HSP90 has a homologous sequence to the HSP70 binding site of p53 (Lam and Calderwood 1992; table 4), HSP90 and HSP70 are known to form a complex in the "foldosome" (Hutchison *et al* 1994). Despite the expectations, HSP70 does not influence the ATPase activity and autophosphorylation of HSP90. Likewise HSPl0 also did not have a major influence on the HSP90-associated ATPase activity either (table 3).

Summarizing our findings, the characterization of the HSP90-associated ATPase activity indicates that it is (i) a P-type ATPase inhibited by molybdate and vanadate, (ii) able to hydrolyze methylfluorescein phosphate with a 5–6-fold higher affinity and

Table 2. Comparison of HSP90-associated ATPase and GTPase activities.

	(nM/mg HSP90/min)	
Divalent cation (5 mM)	ATPase activity	GTPase activity
Ca^{2+}	12·5 ± 7	38·5 ± 9
Mg^{2+}	5·3 ± 3	4·5 ± 2

ATPase and GTPase activities of human placental hsp90 preparation were measured as described in §2. Data represent mean ± SD of three independent experiments.

Table 3. Effect of other proteins on HSP90-associated ATPase activity and on the autophosphorylation of HSP90.

Protein	ATPase activity (in % of HSP90 alone)	Autophosphorylation (in % of HSP90 alone)
HSP10	125 ± 13	93 ± 18
HSP27	31 ± 6	650 ± 12
F-actin	79 ± 8	493 ± 21
HSP70	97 ± 7	95 ± 4
Bovine serum albumin	90 ± 11	101 ± 3

HSP90-associated ATPase activity and autophosphorylation of HSP90 were measured as described in §2. In the experiments 2μg of the representative protein was added to 2 μg of human placental HSP90. Data represent mean ± SD of three independent experiments.

Table 4. Putative HSP70-binding sequence of HSP90[a].

PLSQETFSGLWKLLPPEDG HSP70-binding site of p53[b].
LVSVTKEGL-EL-PED ATP-binding region of murine HSP90[c].

[a]Homologous amino acids are underlined, gaps in HSP90 sequence are marked with hyphens.
[b]Lam and Calderwood 1992.
[c]Moore *et al* 1989.

(iii) a 3 times more active GTPase than ATPase in the presence of calcium. Furthermore HSP27 and F-actin, but not HSP10 and HSP70 can "convert" the HSP90-associated ATPase to HSP90 autokinase activity. HSP90 may itself carry an ATP/GTPase activity whose manifestation requires the presence of activator protein(s). Further experiments are needed to prove or refute this hypothesis.

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