Eur. J. Biochem. **270**, 2421–2428 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03610.x

Comparative analysis of the ATP-binding sites of Hsp90 by nucleotide affinity cleavage: a distinct nucleotide specificity of the C-terminal ATP-binding site

Csaba Sőti[1], Ákos Vermes[1], Timothy A. J. Haystead[2] and Péter Csermely[1]

[1]*Department of Medical Chemistry, Semmelweis University School of Medicine, Budapest, Hungary;*
[2]*Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA*

The 90-kDa heat shock protein (Hsp90) is a molecular chaperone that assists both in ATP-independent sequestration of damaged proteins, and in ATP-dependent folding of numerous targets, such as nuclear hormone receptors and protein kinases. Recent work from our lab and others has established the existence of a second, C-terminal nucleotide binding site besides the well characterized N-terminal, geldanamycin-sensitive ATP-binding site. The cryptic C-terminal site becomes open only after the occupancy of the N-terminal site. Our present work demonstrates the applicability of the oxidative nucleotide affinity cleavage in the site-specific characterization of nucleotide binding proteins. We performed a systematic analysis of the nucleotide binding specificity of the Hsp90 nucleotide binding sites. N-terminal binding is specific to adenosine nucleotides with an intact adenine ring. Nicotinamide adenine dinucleotides and diadenosine polyphosphate alarmones are specific N-terminal nucleotides. The C-terminal binding site is much more unspecific—it interacts with both purine and pirimidine nucleotides. Efficient binding to the C-terminal site requires both charged residues and a larger hydrophobic moiety. GTP and UTP are specific C-terminal nucleotides. 2′,3′-*O*-(2,4,6-trinitrophenyl)-nucleotides (TNP-ATP, TNP-GTP) and pyrophosphate access the C-terminal binding site without the need for an occupied N-terminal site. Our data provide additional evidence for the dynamic domain–domain interactions of Hsp90, give hints for the design of novel types of specific Hsp90 inhibitors, and raise the possibility that besides ATP, other small molecules might also interact with the C-terminal nucleotide binding site *in vivo*.

Keywords: alarmones; Hsp90; molecular chaperone; NAD; nucleotide analogs.

The 90-kDa heat shock protein (Hsp90) is a cytoplasmic chaperone that helps the folding of nuclear hormone receptors and various protein kinases [1–4]. Hsp90 is an ATP-binding chaperone [5,6] and ATP binding induces a conformational change in Hsp90 [7,8]. Assembly of the Hsp90-organized chaperone machinery, the foldosome, with target proteins requires ATP [9,10]; moreover, ATP binding and hydrolysis are essential for the *in vivo* function of Hsp90 [11,12].

Crystallization of the N-terminal domain uncovered a Bergerat-type ATP-binding fold [13], which can also be occupied by geldanamycin (GA) [14] and radicicol [15,16]. These natural antitumor antibiotics abolish Hsp90-dependent folding of immature client proteins, and direct them to proteolysis [17,18].

Recent communications have reported a second ATP-binding site in the C-terminal domain of Hsp90 [19–21]. Our studies demonstrated that the C-terminal nucleotide binding site becomes accessible only after the occupancy of the N-terminal site and is sensitive to cisplatin [20].

The characterization of the nucleotide binding properties of Hsp90 has been hindered for quite a while by the low affinity interactions of nucleotides with this protein, which required the development of new experimental techniques and approaches. More than a decade ago it was been shown by us that Hsp90 has a low affinity ATP/GTP-binding site(s) and is able to autophosphorylate itself using both nucleotides [5]. Later, David Toft and coworkers analyzed the nucleotide specificity of full-length Hsp90 by means of γ-phosphate-linked ATP–Sepharose affinity chromatography. They showed a competition with soluble ADP and ATP, but not with GTP up to 5 mM [9]. On the contrary, recent experiments on N-terminally truncated Hsp90 constructs suggested that GTP, indeed, may bind to the C-terminal domain [19]. Using different fluorescent ATP analogs, including N^6-etheno-ATP, Scheibel *et al.* [6] could not detect a high affinity ATP-binding to Hsp90. However, they could see a weak binding to an ATP-analog spin-labeled on the ribose hydroxyls [6]. Unfortunately, the question, whether GA inhibited this interaction was not addressed. Another study demonstrated that CTP and

Correspondence to P. Csermely, Department of Medical Chemistry, Semmelweis University School of Medicine, Budapest, PO Box260. H-1444 Hungary.
Fax: + 36 1266 7480, Tel.: + 36 1266 2755 extn 4102,
E-mail: csermely@puskin.sote.hu

Abbreviations: AMP-PNP, adenyl-5′-yl-imidodiphosphate; ATPγS, adenosine 5′-[γ-thio]-triphosphate; FSBA, 5′-[*p*-(fluorosulfonyl) benzoyl]-adenosine; GA, geldanamycin; GMP-PNP, guanyl-5′-yl-imidodiphosphate; Hsp, heat shock protein; Hsp90, 90 kDa heat shock protein; OMFP, *o*-methylfluorescein phosphate; TNP-nucleotides, 2′,3′-*O*-(2,4,6-trinitrophenyl)-nucleotides.

(Received 6 February 2003, revised 27 March 2003, accepted 7 April 2003)

NAD affected the tertiary–quaternary structure of the Hsp90 homolog of *Neurospora crassa* [22].

Since the available data in the literature is rather sporadic, and previous experiments obviously could not take into account the existence of the second ATP-binding site on Hsp90, which has been uncovered just recently [19–21], in the present study we undertook a systematic and comparative analysis of the nucleotide specificity of both the N-terminal and C-terminal Hsp90 nucleotide binding sites. In this study we demonstrate that oxidative nucleotide affinity cleavage is a useful technique to characterize the nucleotide binding sites of Hsp90. Using this approach we show that the N-terminal site is fairly specific for adenine nucleotides with an intact adenine ring. On the contrary, the C-terminal site is much more unspecific—it binds both purine and pirimidine nucleotides. Nicotinamide adenine dinucleotides and diadenosine polyphosphate alarmones are specific N-terminal nucleotides, while GTP and UTP are specific C-terminal nucleotides. Our data provide additional evidence for the dynamic domain–domain interactions of Hsp90, help the design of more site-specific Hsp90 inhibitors, and raise the possibility that besides ATP other small molecules might also interact with the C-terminal nucleotide binding site *in vivo*.

Materials and methods

Chemicals

The chemicals used for PAGE, protein determination, blotting membranes, Q2 FPLC and Econo-Pac HTP cartridges were from Bio-Rad. Butyl-Sepharose 4B and DEAE-Sepharose Fast Flow were from Pharmacia LKB Biotechnology Inc. GA was from Gibco-BRL. TNP-nucleotides and etheno-ATP were from Molecular Probes. The ECL bioluminescence kit was from New England Nuclear. The K3725B anti-(C-terminal Hsp90) Ig [23] was a kind gift of T. Nemoto (Department of Oral Biochemistry, Nagasaki University, Nagasaki, Japan), H. Iwanari and H. Yamashita (Institute of Immunology Ltd, Tokyo, Japan). The K41218 anti-(N-terminal Hsp90) Ig [23] was purchased Institute of Immunology Ltd. The PA3-012 anti-(N-terminal Hsp90) Ig was from Affinity Bioreagents (Golden, CO, USA). γ-Phosphate-linked ATP–Sepharose was prepared according to [24]. All the other chemicals used were from Sigma Chemicals Co. Fluka AG.

Purification of Hsp90

Hsp90 was purified from rat liver using consecutive chromatography steps on ButylSepharose 4B, DEAE–Sepharose Fast Flow, Econo-Pac HTP and mono-Q FPLC as described previously [25]. The purity of the final Hsp90 preparations was >95% as judged by silver staining of SDS polyacrylamide gels [26]. Protein concentrations were determined according to Bradford [27].

Oxidative nucleotide affinity cleavage

Affinity cleavage was performed as described by Alonso and Rubio [28], according to the details given in Sőti *et al.* [20]. Briefly, 2 μg purified rat liver Hsp90 was preincubated in the absence or presence of 36 mM GA for 1 h on ice in 20 mM Hepes, 50 mM KCl pH 7.4. Different nucleotides or analogs were added at a final concentration of 1 mM, if not otherwise indicated, and after an additional incubation of 15 min at 37 °C affinity cleavage was induced by the addition of 0.5 mM $FeCl_3$ and 30 mM ascorbate and completed by an additional incubation of 30 min at 37 °C. Hsp90 fragmentation was assessed by sequential immunoblotting with anti-(C-terminal) and anti-(N-terminal) Igs.

Quantification of nucleotide binding

Quantitative determinations were performed as described earlier [20]. Blots were analyzed by densitometry of the most prominent fragments. The N-terminally cleaved 70-kDa fragment (C70) was taken as a representative of N-terminal nucleotide binding, the C-terminally cleaved 46-kDa fragment (N46) represented the C-terminal nucleotide binding, respectively.

ATP–Sepharose binding

Between 3 and 5 μg rat Hsp90 was preincubated on ice for 1 h in 200 μL of a buffer consisting of 20 mM Hepes, 50 mM KCl, 6 mM $MgCl_2$, 0.01% NP40 pH 7.5. In the case of ATP competition, samples contained an ATP regeneration system (10 mM creatine phosphate and 20 $U \cdot mL^{-1}$ creatine kinase). Finally, 25 μL ATP–Sepharose was added and tubes were incubated at 37 °C for 30 min with frequent agitation, then the resin was pelleted, washed three or four times with the above buffer and analyzed by SDS/PAGE.

Results

γ-Phosphate-linked ATP–Sepharose binds Hsp90 via both its N- and C-terminal ATP-binding sites

In our previous experiments, we analyzed the N-, and C-terminal nucleotide binding sites of Hsp90 using two independent techniques. The oxidative nucleotide affinity cleavage was successfully applied to Hsp90 in our previous work [20]. γ-Phosphate-linked ATP–Sepharose binding has been used as the first biochemical assay for the unambiguous identification of Hsp90 as an ATP-binding protein by Grenert *et al.* [9]. Though C-terminal fragments of Hsp90 also bound to the resin [19], and we demonstrated that Hsp90 was able to bind in the presence of a saturating concentration of the N-terminal inhibitor, GA [20], others could not detect binding under these circumstances [29]. We were intrigued by this apparent contradiction, and made an additional attempt to resolve the discrepancy.

Using the affinity cleavage the hydroxyl radicals generated by the oxidation of iron tethered to the polyphosphate moiety of ATP resulted in two major cleavage products in Hsp90: a 70-kDa major Hsp90 fragment (C70) at the N-terminal binding site, and a 46-kDa major fragment (N46) at the C-terminal Hsp90 nucleotide binding site ([20] and Fig. 1A, lane 3). The C-terminal site became accessible only if the N-terminal site was occupied and not cleaved—in our case with the N-terminal specific inhibitor GA (Fig. 1A, lane 4) [13,19]. Performing the cleavage reaction on Hsp90 bound to the γ-phosphate-linked ATP–Sepharose resin showed that Hsp90 is bound to the ATP–Sepharose

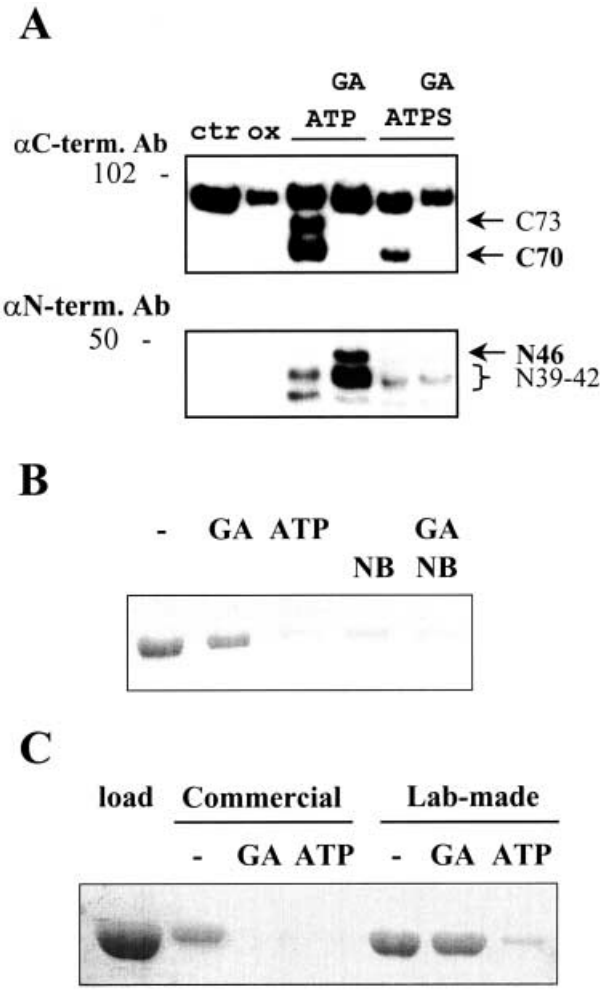

Fig. 1. γ-Phosphate-linked ATP–Sepharose binds Hsp90 via both its N- and C-terminal ATP-binding sites. (A) Affinity cleavage on γ-phosphate-linked ATP–Sepharose. Ctr, Untreated protein; ox, protein incubated with redox system. In lanes 5 and 6 (ATPS), 25 μL γ-phosphate-linked ATP–Sepharose was added instead of ATP. C70 and N46 denote the major N- and C-terminal ADP/ATP-fragments, respectively. Similarly, C73 and N39-42 indicate the major N- and C-terminal ATP fragments, respectively. (B) Novobiocin inhibits γ-phosphate-linked ATP–Sepharose binding. Hsp90 was preincubated in the absence or presence of 36 μM geldanamycin (GA) and/or 10 mM novobiocin (NB). (C) Different γ-phosphate-linked ATP-Sepharose resins interact differently with the C-terminal nucleotide binding domain of Hsp90. Binding of Hsp90 to the commercially available and 'lab-made' ATP-Sepharose resins was analyzed as described in Materials and Methods. Figures are representatives of three independent experiments.

through both nucleotide binding domains (lane 5; C70 and N46), and in the presence of GA, only the C-terminal site is cleaved (lane 6; N46). Unbound Hsp90 in the supernatant did not undergo any ATP-dependent cleavage (data not shown). Fig. 1A also shows that the fragments characteristic of the γ-phosphate (C73 and N39-42) appear neither at the N- nor the C-terminal site, respectively. Instead, the 39-kDa fragment present at the C-terminal site is produced by the diphosphate moiety of ADP [20]. The reason for this may be that the ATP-bound resin may impose a steric hindrance to the binding of the terminal phosphate, therefore Hsp90 adopts an 'ADP-conformation' [20] on the resin. This may explain how the C-terminal binding site could escape attention, where the affinity towards ATP is higher than to ADP [19,20].

Independent evidence for the involvement of both ATP-binding sites in Hsp90/ATP–Sepharose interactions comes from the application of different Hsp90 inhibitors (Fig. 1B). While binding of Hsp90 was not prevented by the N-terminal-specific GA (lane 3) or radicicol (data not shown), novobiocin inhibited binding completely (lanes 5 and 6). This experiment gave further evidence that Hsp90 is also bound to the ATP–Sepharose *via* its C-terminal nucleotide binding site, and confirmed our previous observation that novobiocin, which binds to the C terminus of Hsp90 [19] allosterically inhibits the N-terminal binding site [20]. It has to be noted, that using several lots of commercially available ATP–Sepharose the C-terminal binding was not always detected, especially when the assay was conducted under more stringent conditions (e.g. three washes, data not shown).

Comparative analysis of the nucleotide specificity of Hsp90 nucleotide binding sites

After demonstrating that these techniques may be used to study the biochemistry of the nucleotide binding domains, we performed a comparative analysis of the nucleotide specificity of Hsp90 nucleotide binding sites. Fig. 2A shows that the N-terminal nucleotide binding site prefers adenine nucleotides (ATP and dATP). Binding of CTP was slightly permitted, while GTP and UTP did not bind to this site. We observed no significant binding of dGTP and dUTP, or UDP-glucose to the N-terminal binding site (data not shown). The C-terminal domain allowed binding of all kinds of nucleotides tested. We would like to note that the anti-(C-terminal) Ig K3725B, and the anti-(N-terminal) Ig PA3-012 used in most of our experiments were both Hsp90β-specific antibodies. However, analysis of silver stained gels, as well as the repetition of few selected experiments with the K41218 anti-(N-terminal) Ig, which recognizes both Hsp90 isoforms, revealed no significant differences between the nucleotide-binding specificities of Hsp90α and Hsp90β (data not shown).

As an additional proof, we analyzed the competition of these nucleotides with ATP–Sepharose binding, in the absence (N- and C-terminal binding), and in the presence (only C-terminal binding) of GA. Fig. 2B shows that these experiments yielded similar results. ATP and CTP competed with both sites, while GTP and UTP exhibited a C-terminal preference (Fig. 2B). These experiments provided evidence for the applicability of the nucleotide affinity cleavage technique to study the specificity of the nucleotide binding sites. Since GTP is a C-terminal-specific nucleotide, we further analyzed the properties of Hsp90 nucleotide binding sites using affinity cleavage with different ATP- and GTP-derivatives.

Interactions of nonhydrolyzable nucleotides with Hsp90

In agreement with the specificity profile of the previous experiments, the N-terminal domain bound the

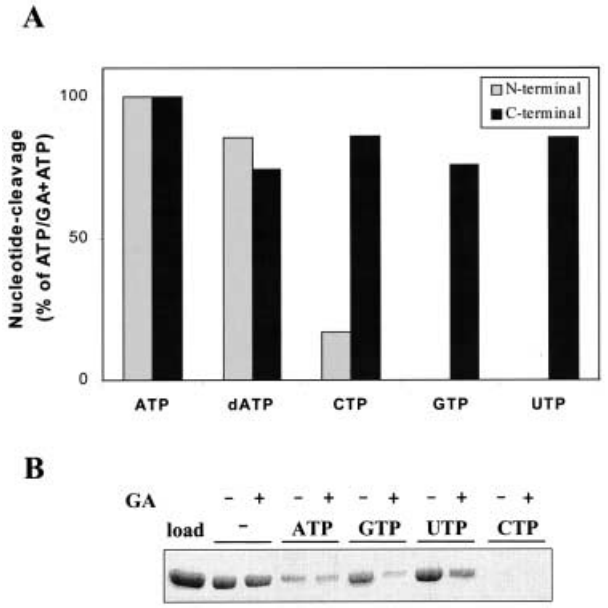

Fig. 2. The Hsp90 N- and C-terminal nucleotide-binding sites display divergent nucleotide specificities. (A) Affinity cleavage assay. Hsp90 was affinity-cleaved in the presence of various nucleotides at a concentration of 1 mM. Nucleotide binding was determined in the absence (N-terminal) or in the presence (C-terminal) of 36 μM GA. Blots were analyzed by densitometry of the N-terminally cleaved 70-kDa (C70) or the C-terminally cleaved 46-kDa (N46) major fragments for N- and C-terminal nucleotide binding, respectively. Data were normalized to the cleavage-efficiency of ATP and GA + ATP in N- and C-terminal nucleotide binding, respectively, and are the means of two independent experiments. (B) ATP–Sepharose competition. Hsp90 was preincubated with 20 mM nucleotides as indicated, then ATP–Sepharose binding was tested. Note that the ATP–Sepharose has a ligand density of 10–15 μmol·mL^{-1}. The figure represents one of two experiments with similar results.

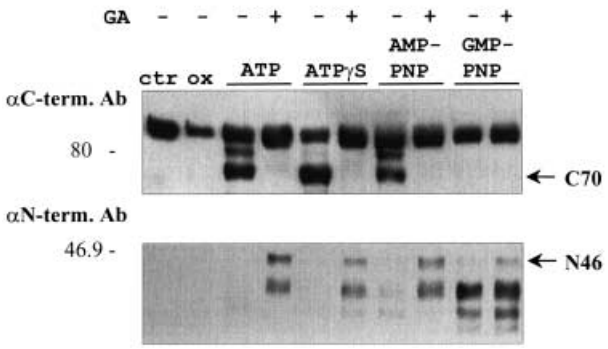

Fig. 3. Interactions of nonhydrolyzable nucleotides with Hsp90. Hsp90 was preincubated in the absence or presence of 36 μM GA, affinity-cleaved using 2 mM ATP, 1 mM ATPγS, adenyl-5′-yl-imidodiphosphate (AMP-PNP) or guanyl-5′-yl-imidodiphosphate (GMP-PNP) and cleavage products were assessed. Western blots are representative of three independent experiments.

poorly hydrolyzable ATP analog, adenosine 5′-[γ-thio]-triphosphate (ATPγS), and the unhydrolyzable adenyl-5′-yl-imidodiphosphate (AMP-PNP), but not guanyl-5′-yl-imidodiphosphate (GMP-PNP, Fig. 3). Binding of both ATPγS and AMP-PNP could be prevented by GA. Binding of these nucleotides to Hsp90 is in agreement with several previous reports (reviewed in [3]). ATPγS usually contains enough ADP to saturate the N-terminal nucleotide binding site, which has a 10- to 20-fold lower affinity to ATP than to ADP [9,13]. ATPγS produced an N-terminal fragmentation resembling that of ADP (see the absence of the C73 γ-phosphate binding fragment in lane 5) [20], but the application of an ATP regeneration system restored the usual ATP cleavage pattern (data not shown).

The C-terminal domain bound each nonhydrolyzable nucleotides tested. GMP-PNP produced a strong fragmentation at the C-terminal domain, seen in blots developed with either anti-(N-terminal) or anti-(C-terminal) Ig (Fig. 3 and data not shown). Interestingly, GMP-PNP could interact with the middle-C-terminal domain in the absence of GA (Fig. 3).

Differently substituted nucleotide analogs bind better to the C-terminal than to the N-terminal domain of Hsp90

It has been reported that Hsp90 cannot bind strongly to adenine-modified nucleotide analogs, but interacts with ribose-modified ATP with an affinity comparable to that of unmodified ATP [6]. Therefore we studied the interaction of differently substituted nucleotides with Hsp90. N^6-etheno-ATP, and the 2′,3′-trinitrophenyl ATP derivative, TNP-ATP displayed a much weaker binding to the Hsp90 N terminus than ATP (Fig. 4). GA competed with the N-terminal binding of both nucleotides. In agreement with no binding of GTP and GMP-PNP to the N terminus (Figs 2 and 3) N-terminal binding of TNP-GTP was not detected (Fig. 4). The C-terminal domain bound each of these nucleotide analogs. TNP-nucleotide binding was possible without GA, though the characteristic N46 band was stronger in the presence of GA. Similarly to GNP-PNP, TNP-nucleotides produced stronger fragmentation at the C-terminal domain, seen in blots developed with either anti-N- or anti-C-terminal Igs (Fig. 4 and data not shown).

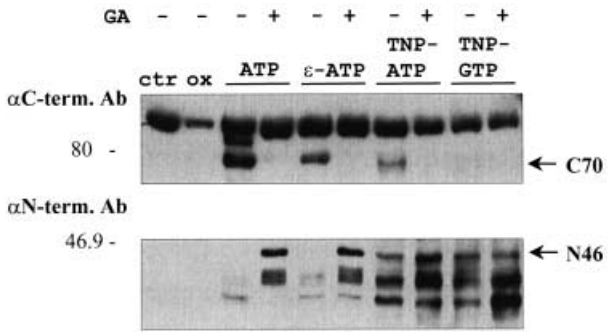

Fig. 4. Differently substituted nucleotide analogs bind better to the C-terminal than to the N-terminal domain of Hsp90. After a preincubation in the absence or presence of 36 mM GA, Hsp90 was affinity-cleaved using 1 mM of ATP, N^6-etheno-ATP (ε-ATP), 2′,3′-O-(2,4,6-trinitrophenyl)-ATP or 2′,3′-*O*-(2,4,6-trinitrophenyl)-GTP (TNP-ATP and TNP-GTP, respectively) and cleavage products were analyzed. Western blots are representative of three independent experiments.

Binding of TNP-nucleotides was also confirmed by fluorescence measurements, but the small increase in quantum yield made detailed analysis impossible (data not shown).

Nicotinamide-adenine dinucleotides bind to the N-terminal, but not to the C-terminal domain of Hsp90

After an earlier prediction of Callebaut *et al.* [30] Garnier *et al.* [21] also proposed the C-terminal ATP binding site to be a Rossmann fold. Following these suggestions we became interested to measure if nicotinamide adenine dinucleotides bind to Hsp90. Here we could utilize the diphosphate structure as a good chelator of Fe^{2+} ions allowing an oxidative cleavage reaction similar to that with nucleoside triphosphates or nucleoside diphosphates. To our surprise, it was the N-terminal domain of Hsp90, which bound both NAD^+ and NADH + H^+. GA competed with both nucleotides efficiently (Fig. 5). Similar to our results with the ribose-substituted nucleotide analog, TNP-ATP, the esterification of ribose-2′-OH both in $NADP^+$ and NADPH strongly inhibited their binding (Fig. 5). On the contrary, none of the nicotinamide adenine dinucleotides displayed a significant interaction with the C-terminal nucleotide binding site. ATP + GA, as a positive control, induced the appearance of the N46 in the presence of all nucleotides (Fig. 5).

Binding of alarmones to Hsp90

Diadenosine polyphosphates and diguanosine polyphosphates serve as alarmones both in prokaryotic and eukaryotic organisms [31]. Moreover, their interaction with the Hsp70 homologue molecular chaperone, DnaK, has been shown [32,33]. We were interested whether these alarmones bind to Hsp90. Fig. 6 shows that indeed, all of the diadenosine polyphosphates bound to the N-terminal site of Hsp90 at 1 mM, and binding could be inhibited by GA. However, none of the diadenosine polyphosphates tested displayed a significant binding to the C-terminal site of Hsp90, and they did not bind to the N-terminal site at a final concentration of 2 μM. Half-maximal binding of diadenosine polyphosphate (AP_4A) to the N-terminal domain occurred above 200 μM (which is the highest physiological concentration; Fig. 6 and data not shown). Interestingly,

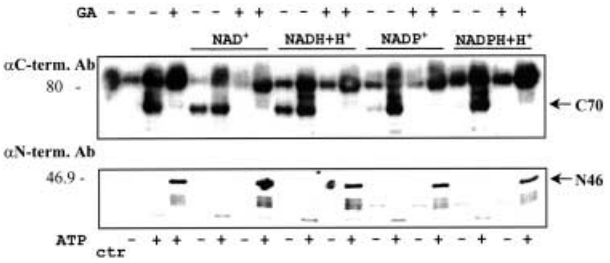

Fig. 5. Nicotinamide adenine dinucleotides bind to the N-terminal, but not to the C-terminal domain of Hsp90. After a preincubation in the absence or presence of 36 μM GA, Hsp90 was affinity-cleaved in the presence of ATP, and/or different nicotinamide adenine dinucleotides at final concentrations of 1 mM, as indicated. Western blots are representative of two independent experiments.

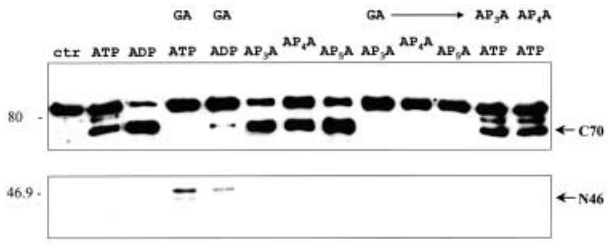

Fig. 6. Binding of diadenosine polyphosphates to Hsp90. After a preincubation in the absence or presence of 36 μM GA, or 1 mM diadenosine polyphosphates as indicated, Hsp90 was affinity-cleaved in the presence of ATP, ADP or diadenosine polyphosphates at a final concentration of 1 mM. Western blots are representative of three independent experiments.

alarmones induced a stronger cleavage than ATP (Fig. 6), which is not due to their higher binding efficiency to Hsp90 as the characteristic alarmone cleavage pattern could be 'diminished' (i.e. competed) by the addition of equimolar ATP (compare the second vs. the last two lanes of Fig. 6). The results show that the cleavage efficiency of the β-phosphate-linked Fe^{2+} is weaker with ATP than with ADP and ADP-like compounds such as alarmones. ATP may induce a different conformation of Hsp90 than ADP or alarmones, probably because Hsp90 should adopt a thermodynamically less favored conformation to capture the ATP-γ-phosphate. Diguanosine polyphosphate (GP_4G) displayed a very weak binding, which was exclusive to the C terminal domain (data not shown). Based on our data Hsp90 does not seem to be a specific alarmone-binding protein *in vitro*.

Binding of noniron-chelating nucleotide analogs and pyrophosphate to Hsp90

We were interested whether a common structural element of the many nucleotide polyphosphates tested, pyrophosphate, is able to induce a specific cleavage pattern of Hsp90 in our oxidative cleavage assay. Indeed, pyrophosphate bound weakly to the N- and much stronger to the C-terminal

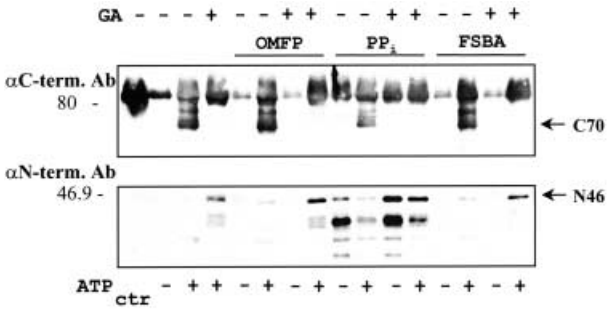

Fig. 7. Binding of noniron-chelating nucleotide analogs and pyrophosphate to Hsp90. After a preincubation in the absence or presence of 36 μM GA, Hsp90 was affinity-cleaved in the presence of 1 mM ATP, and/or 0.1 mM *o*-methylfluorescein-phosphate (OMFP), 1 mM sodium-pyrophosphate (PP_i) and 1 mM fluorosulfonyl-benzoyl-adenosine (FSBA), as indicated. Western blots are representative of two independent experiments.

domains in the absence of GA (Fig. 7). Binding to the N-terminal domain was inhibited by GA. Pyrophosphate cleavage was much less specific than that of the nucleotides, since pyrophosphate induced a strong, GA-independent fragmentation of both the C-terminal and the middle domain of Hsp90 (Fig. 7 and data not shown). ATP inhibited the pyrophosphate-induced C-terminal cleavage (Fig. 7).

o-Methylfluorescein phosphate (OMFP) was a good substrate of the Hsp90-associated ATPase in our previous experiments and competed well with ATP in the 'regular' assays of the Hsp90-associated ATPase [34]. Fluorosulfonyl-benzoyl-adenosine (FSBA) has been used to label and identify ATP-binding sites [35,36] and also weakly labeled Hsp90 (data not shown). Therefore we wanted to know if the hydrolyzable 'ATP-analog' OMFP as well as FSBA [35,36], interact with the oxidative affinity cleavage assay despite the fact that they do not efficiently chelate iron. Nevertheless, in our experiments they displayed a weak binding to the N-terminal domain (Fig. 7). Neither OMFP, nor FSBA could compete with ATP at their maximal concentration of 0.1 and 1 mM, respectively. However, they opened the C-terminal nucleotide-binding domain in the absence of GA, and induced ATP-binding and the appearance of the specific N46 fragment (Fig. 7). Fluorescein isothiocyanate behaved similarly to OMFP and FSBA (data not shown).

Discussion

Nucleotide affinity cleavage as a tool to characterize the specificity of nucleotide binding domains

Using the well characterized N-terminal nucleotide binding site and the ATP–Sepharose assay we could demonstrate for the first time that nucleotide affinity cleavage is a useful technique to study the biochemical properties of nucleotide binding domains. It may be especially important in case of: (a) multiple nucleotide binding sites, because they can be distinguished; (b) low affinity interactions; and (c) 'stringent' site structure, where, e.g. fluorophore or other substitution is not well tolerated. Though it has not yet been shown, the Fe(II)–ATP complex may display a different binding affinity, or even the orientation (therefore the cleavage) of the iron-polyphosphate moiety might differ form that of the biologically predominant magnesium–ATP. Furthermore, the susceptibility of neighboring peptide bonds may differ from protein to protein, resulting in different cleavage efficiency. Further studies are needed to investigate the general applicability of this technique in nucleotide-binding proteins.

Nucleotide binding to the N-terminal domain of Hsp90

The N-terminal nucleotide binding site of Hsp90 is fairly specific. It binds ATP and 2′-deoxy-ATP with similar efficiency (Fig. 8). On the contrary, it does not show a significant interaction with GTP, pirimidine nucleotides, and nucleotides in which the ribose-2′-OH position has been substituted (TNP, ribose-attached resin; phosphate in NADP). The integrity of the adenine ring is also important for binding, since Hsp90 does not bind to C8-linked ATP-resins under stringent conditions ([6,13]; Cs. Sőti and P. Csermely, unpublished observations), and a substitution at the 6-adenine position (e.g. etheno-ATP) disrupts binding as well.

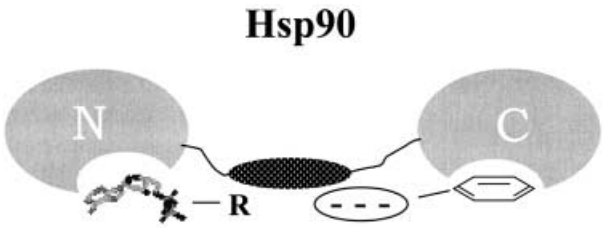

Fig. 8. Nucleotide specificity of the N- and C-terminal nucleotide binding sites of Hsp90. N-terminal domain (N) requires adenine nucleotides with an intact adenine ring; the stick model is the structure of the kinked ADP in the Hsp90 crystal, the phosphates pointing out of the domain; R stands for phosphates (ATP) or other moieties as in NAD or adenosine alarmones. γ-Phosphate is anchored in the middle domain (black). C-terminal domain (C) needs a larger hydrophobic moiety (labeled by the aromatic ring) connected to charged residues (phosphates, like pyrophosphate; labeled by negative charges). The large hydrophobic domain allows the binding of a variety of purine and pirimidine nucleotides. The charged residues bind to a region close to the N-terminal γ-phosphate binding motif.

The Hsp90 N-terminal nucleotide binding site binds NAD and adenosine polyphosphate alarmones. It is worth noting that NAD binding of Hsp90 may interfere with some ATPase measurements based on coupled assays at low ATP concentrations [12]. However, Hsp90 does not show a NADPH : quinone oxidoreductase activity [37], and its alarmone binding efficiency is fairly low. Alarmone binding gives another evidence that the γ-phosphate should point out of the nucleotide binding cleft, and the bulky second adenine should protrude far from the domain reinforcing the notions made by the γ-phosphate-linked ATP–Sepharose [9,20].

Nucleotide binding to the C-terminal domain of Hsp90

Nucleotide binding to the C-terminal nucleotide binding site is fairly unspecific. This site binds both purine and pirimidine nucleotides, when the N-terminal site is already occupied (Fig. 8). UTP and GTP are C-terminal-specific nucleotides. Based on the demonstration that autophosphorylation of Hsp90 is insensitive to high concentrations of GA, but inhibited by novobiocin, a recent report [38] suggested that the C-terminal ATP-binding site may be responsible for Hsp90 autophosphorylation. In light of these data our earlier finding that Hsp90 autophosphorylation can be achieved by GTP [5] gives an additional support for the C-terminal specificity of GTP.

Our experiments showed that the C-terminal site also interacts with ribose-modified nucleotides with affinities comparable to unsubstituted ATP, which may shed new light on earlier findings [6]. The C-terminal site (unlike the N-terminal site) does not interact with nicotinamide adenine dinucleotides and alarmones. This is in contrast with the predictions of Garnier *et al.* [21], who proposed the C terminus as a NAD-binding site.

Binding to the C-terminal site demands both charged groups and a large, hydrophobic moiety (e.g. ATP can inhibit pyrophosphate binding). The negligible alarmone binding suggests that the C-terminal site is more restricted with respect to the phosphate positioning, since another nucleoside weakens the affinity. On the other hand, our previous assumptions [20] indicated that the γ-phosphate binding site is beyond the C-terminal domain. It is still an interesting open question how much the C-terminal nucleotide binding site overlaps with the C-terminal dimerization domain and with the C-terminal binding sites for substrates and for Hsp90-interacting cochaperones.

As an important finding of our present studies, some nucleotide analogs, such as TNP-nucleotides and pyrophosphate bind to the C-terminal nucleotide binding site without the requirement for previous occupancy of the N-terminal site. The structural means by which these nucleotide analogs release the N-terminal site-mediated block of C-terminal binding need to be clarified in further experiments.

As another interesting outcome, experiments shown in Fig. 7 provide additional evidence for the domain–domain interactions of Hsp90: N-terminal ATP-binding and cleavage inhibit pyrophosphate-dependent cleavage of the C-terminal domain (Fig. 7, lane 10 bottom panel). On the other hand, noniron binding N-terminal ATP agonists unlock the C-terminal domain and permit ATP binding and fragmentation (Fig. 7, lanes 6 and 14, bottom panel).

In conclusion, the present studies provide the first systematic and detailed characterization of the nucleotide binding specificity of the N- and C-terminal nucleotide binding sites of the 90-kDa molecular chaperone, Hsp90. Our data also provide additional evidence for the domain–domain interactions of Hsp90 and help the design of new Hsp90 inhibitors, which would be highly useful both in uncovering the physiological function and mechanism of Hsp90 action and also in clinical practice.

Acknowledgements

We thank G. Nardai (Semmelweis University, Department of Medical Chemistry, Budapest, Hungary) for his help in the purification of Hsp90. We thank K. Mihály (Semmelweis University, Department of Medical Chemistry, Budapest, Hungary) for technical assistance. The advice of G. Vereb (Department of Medical Chemistry, Debrecen University, Hungary) is gratefully acknowledged. Our special thanks to T. Nemoto (Department of Oral Biochemistry, Nagasaki University, Nagasaki, Japan) H. Iwanari and H. Yamashita (Institute of Immunology Ltd, Tokyo, Japan) for providing us with the K3725B anti-Hsp90 antibody. This work was supported by research grants from the Hungarian Science Foundation (OTKA-T37357), from the Hungarian Ministry of Social Welfare (ETT-21/00) and from the International Centre for Genetic Engineering and Biotechnology (ICGEB, CRP/HUN 99-02).

References

1. Csermely, P., Schnaider, T., Sőti, Cs, Prohászka, Z. & Nardai, G. (1998) The 90 kDa molecular chaperones: structure, function and clinical applications. A comprehensive review. *Pharmacol. Therapeutics* **79**, 129–168.
2. Richter, K. & Buchner, J. (2001) Hsp90: chaperoning signal transduction. *J. Cell Physiol.* **188**, 281–290.
3. Pearl, L.H. & Prodromou, C. (2002) Structure, function and mechanism of the Hsp90 molecular chaperone. *Adv. Prot. Chem.* **59**, 157–186.
4. Pratt, W.B. & Toft, D.O. (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70/based chaperone machinery. *Exp. Biol. Med.* **228**, 111–133.
5. Csermely, P. & Kahn, C.R. (1991) The 90 kDa heat shock protein (hsp-90) possesses an ATP-binding site and autophosphorylating activity. *J. Biol. Chem.* **266**, 4943–4950.
6. Scheibel, T., Neuhofen, S., Weikl, T., Mayr, C., Reinstein, J., Vogel, P.D. & Buchner, J. (1997) ATP-binding properties of human hsp90. *J. Biol. Chem.* **272**, 18608–18613.
7. Csermely, P., Kajtár, J., Hollósi, M., Jalsovszky, G., Holly, S., Kahn, C.R., Gergely, P. Jr, Sőti, Cs, Mihály, K. & Somogyi, J. (1993) ATP induces a conformational change of the 90 kDa heat shock protein (hsp-90). *J. Biol. Chem.* **268**, 1901–1907.
8. Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E.S., Litwack, G. & Toft, D.O. (1997) Nucleotides and two functional states of hsp90. *J. Biol. Chem.* **272**, 8007–8012.
9. Grenert, J.P., Sullivan, W.P., Adden, P., Haystead, T.A.J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H.J., Schulte, T.W., Sausville, E., Neckers, L.M. & Toft, D.O. (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ADP/ATP switch domain that regulates hsp90 conformation. *J. Biol. Chem.* **272**, 23843–23850.
10. Grenert, J.P., Johnson, B.D. & Toft, D.O. (1999) The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J. Biol. Chem.* **274**, 17525–17533.
11. Obermann, W.M., Sondermann, H., Russo, A.A., Pavletich, N.P. & Hartl, F.U. (1998) *In vivo* function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J. Cell. Biol.* **143**, 901–910.
12. Panaretou, B., Prodromou, C., Roe, M., O'Brien, R., Ladbury, J.E., Piper, W. & Pearl, L.H. (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J.* **17**, 4829–4836.
13. Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**, 65–75.
14. Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U. & Pavletich, N.P. (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* **89**, 239–250.
15. Soga, S., Kozawa, T., Narumi, H., Akinaga, S., Irie, K., Matsumoto, K., Sharma, S.V., Nakano, H., Mizukami, T. & Hara, M. (1998) Radicicol leads to selective depletion of Raf kinase and disrupts K-Ras-activated aberrant signaling pathway. *J. Biol. Chem.* **273**, 822–828.
16. Schulte, T.W., Akinaga, S., Murakata, T., Agatsuma, T., Sugimoto, S., Nakano, H., Lee, Y.S., Simen, B.B., Argon, Y., Felts, S., Toft, D.O., Neckers, L.M. & Sharma, S.V. (1999) Interaction of radicicol with members of the heat shock protein 90 family of molecular chaperones. *Mol. Endocrinol.* **13**, 1435–1448.
17. Whitesell, L., Mimnaugh, E.D., De Costa, B., Myers, C.E. & Neckers, L.M. (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl Acad. Sci. USA* **91**, 8324–8328.
18. Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouathek, O., Danishefsky, S., Rosen, N. & Hartl, F.U. (1996) Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc. Natl Acad. Sci. USA* **93**, 14536–14541.
19. Marcu, M.G., Chadli, A., Bouhouche, I., Catelli, M. & Neckers, L.M. (2000) The heat shock protein 90 antagonist novobiocin

interacts with a previously unrecognized ATP-binding domain in the carboxy terminus of the chaperone. *J. Biol. Chem.* **275**, 37181–37186.
20. Sőti, Cs, Rácz, A. & Csermely, P. (2002) A nucleotide-dependent molecular switch controls ATP binding at the C-terminal domain of Hsp90: N-terminal nucleotide binding unmasks a C-terminal binding pocket. *J. Biol. Chem.* **277**, 7066–7075.
21. Garnier, C., Lafitte, D., Tsvetkov, P.O., Barbier, P., Leclerc-Devin, J., Millot, J.-M., Briand, C., Makarov, A.A., Catelli, M.G. & Peyrot, V. (2002) Binding of ATP to Heat Shock Protein 90. Evidence for an ATP-binding site in the C-terminal domain. *J. Biol. Chem.* **277**, 12208–12214.
22. Freitag, D.G., Ouimet, P.M., Girvitz, T.L. & Kapoor, M. (1997) Heat shock protein 80 of *Neurospora crassa*, a cytosolic molecular chaperone of the stress 90 family, interacts directly with heat shock protein 70. *Biochemistry* **36**, 10221–10229.
23. Nemoto, T., Sato, N., Iwanari, H., Yamashita, H. & Takashi, T. (1997) Domain structures and immunogenic regions of the 90-kDa heat-shock protein (hsp90) – Probing with a library of anti-hsp90 monoclonal antibodies and limited proteolysis. *J. Biol. Chem.* **272**, 26179–26187.
24. Haystead, C.M.M., Gregory, P., Sturgill, T.W. & Haystead, T.A.J. (1993) γ-phosphate-linked ATP-Sepharose for the affinity purification of protein kinases. Rapid purification to homogeneity of skeletal muscle mitogen-activated protein kinase kinase. *Eur. J. Biochem.* **214**, 459–467.
25. Sőti, Cs, Radics, L., Yahara, I. & Csermely, P. (1998) Interaction of vanadate oligomers and permolybdate with the 90-kDa heat-shock protein, Hsp90. *Eur. J. Biochem.* **255**, 611–617.
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
27. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **72**, 248–254.
28. Alonso, E. & Rubio, V. (1995) Affinity cleavage of carbamoyl-phosphate synthetase I localizes regions of the enzyme interacting with the molecule of ATP that phosphorylates carbamate. *Eur. J. Biochem.* **229**, 377–384.
29. Felts, S.J., Owen, B.A.L., Nguyen, P., Trepel, J., Donner, D.B. & Toft, D.O. (2000) The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. *J. Biol. Chem.* **275**, 3305–3312.
30. Callebaut, I., Catelli, M.G., Portetelle, D., Meng, X., Cadepond, F., Burny, A., Baulieu, E.-E. & Mornon, J.-P. (1994) Redox mechanism for the chaperone activity of heat shock proteins HSPs 60, 70 and 90 as suggested by hydrophobic cluster analysis: hypothesis. *C. R. Acad. Sci. Paris* **317**, 721–729.
31. McLennan, A.G. (2000) Dinucleoside polyphosphates-friend or foe? *Pharmacol. Ther.* **87**, 73–89.
32. Johnstone, D.B. & Farr, S.B. (1991) AppppA binds to several proteins in *Escherichia coli*, including the heat shock and oxidative stress proteins DnaK, GroEL, E89, C45 and C40. *EMBO J.* **10**, 3897–3904.
33. Bochner, B.R., Zylicz, M. & Georgopoulos, C. (1986) *Escherichia coli* DnaK protein possesses a 5′-nucleotidase activity that is inhibited by AppppA. *J. Bacteriol.* **168**, 931–935.
34. Nardai, G., Schnaider, T., Sőti, Cs, Ryan, M.T., Hoj, P.B., Somogyi, J. & Csermely, P. (1996) Characterization of the 90 kDa heat shock protein (HSP90)-associated ATP/GTP-ase. *J. Biosci.* **21**, 179–190.
35. Kim, H., Lee, L. & Evans, D.R. (1991) Identification of the ATP binding sites of the carbamyl phosphate synthetase domain of the syrian hamster multifunctional protein CAD by affinity labeling with 5′-[p-(fluorosulfonyl) benzoyl]adenosine. *Biochemistry* **30**, 10322–10329.
36. Vereb, G., Balla, A., Gergely, P., Wymann, M.P., Gulkan, H., Suer, S. & Heilmeyer, L.M. (2001) The ATP-binding site of brain phosphatidylinositol 4-kinase PI4K230 as revealed by 5′-p-fluorosulfonylbenzoyladenosine. *Int. J. Biochem. Cell Biol.* **33**, 249–259.
37. Nardai, G., Sass, B., Eber, J. & Orosz, Gy. & Csermely, P. (2000) Reactive cysteines of the 90 kDa heat shock protein, Hsp90. *Arch. Biochem. Biophys.* **384**, 59–67.
38. Langer, T., Schlatter, H. & Fasold, H. (2002) Evidence that the novobiocin-sensitive ATP-binding site of the heat shock protein 90 (Hsp90) is necessary for its autophosphorylation. *Cell Biol. Int.* **26**, 653–657.