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COMMENTARY

The Hsp90 Complex—A Super-Chaperone Machine as a Novel Drug Target

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ABSTRACT. Cells respond to sudden changes in the environmental temperature with increased synthesis of a distinct number of heat shock proteins (Hsps). Analysis of the function of these proteins in recent years has shown that all the major classes of conserved Hsps are molecular chaperones involved in assisting cellular protein folding and preventing irreversible side-reactions, such as unspecific aggregation. In addition to their function under stress conditions, molecular chaperones also play a critical role under physiological conditions. Hsp90 is one of the most abundant chaperones in the cytosol of eukaryotic cells. It is part of the cell's powerful network of chaperones to fight the deleterious consequences of protein unfolding caused by nonphysiological conditions. In the absence of stress, however, Hsp90 is an obligate component of fundamental cellular processes such as hormone signaling and cell cycle control. In this context, several key regulatory proteins, such as steroid receptors, cell cycle kinases, and p53, have been identified as substrates of Hsp90. Recently, Hsp90 was shown to be the unique target for geldanamycin, a potent new anti-tumor drug that blocks cell proliferation. Interestingly, under physiological conditions, Hsp90 seems to perform its chaperone function in a complex with a set of partner proteins, suggesting that the Hsp90 complex is a multi-chaperone machine specialized in guiding the maturation of conformationally labile proteins. The regulation of key signaling molecules of the cell by the Hsp90 machinery is a stimulating new concept emerging from these studies, and Hsp90 has become a promising new drug target. BIOCHEM PHARMACOL **56**;6:675–682, 1998. © 1998 Elsevier Science Inc.

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Hsp90† is one of the most abundant proteins in the eukaryotic cell. It constitutes up to 1–2% of the cellular protein under physiological conditions, and its expression is increased several-fold in response to stress. Up to now, members of the Hsp90 family were found in the cytosol, the endoplasmic reticulum, and chloroplasts [1]. Although isoforms of Hsp90 have interchangeable functions [2], the respective genes are differentially regulated in eukaryotes. In most eukaryotic cells, one of the two cytosolic members is expressed constitutively at a high level at physiological temperatures and is induced only 2–3 times by heat shock. The second Hsp90 gene is expressed at a low basal level at normal temperatures, but expression is enhanced strongly under restrictive growth conditions like heat treatment [2, 3].

ROLE OF HSP90 IN THE CONFORMATIONAL REGULATION OF PROTEINS *IN VIVO*

The first indication of the cellular function of Hsp90s in higher eukaryotes was the surprising discovery that a major protein coprecipitating with steroid hormone receptors in the absence of hormone was identical to Hsp90 [4, 5]. These complexes were stable because they survived immunoprecipitation. Later on, it was shown that the addition of steroid hormone causes the apparent dissociation of the complex and the dimerization of the receptor, which is required for DNA binding and transcriptional activation [5]. The aporeceptor complexes of GR, MR, PR, AR, ER, and DR receptors are inactive as transcriptional regulators. Here, association with Hsp90 can be considered as an intermediate step in the folding process [6]. Unfortunately, the precise function of Hsp90 in this context remains enigmatic. It is reasonable to speculate that regulation of receptor conformation by Hsp90 adds an additional level of control to these signaling cascades.

Independently, Hsp90 was identified to be associated with v-src kinases, which have the potential to transform cells, thus causing unregulated proliferation. Again, as for steroid receptors, stable complexes could be isolated by immunoprecipitation [7, 8]. The interaction with Hsp90 seemed to be an integral part of the maturation process, and once the v-src kinase became attached to the plasma membrane via myristoylation, Hsp90 dissociated. Up to now, various tyrosine and serine/threonine kinases could be detected in complexes with Hsp90 *in vitro* and *in vivo* [9]. It was shown that Hsp90 is necessary for the correct folding of kinases such as Raf kinase [10] and eIF-2α kinase [11].

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† *Abbreviations:* FKBP, FK506 binding protein; GA, geldanamycin; GR, glucocorticoid receptor; Hsp, heat shock protein; MHC, major histocompatibility complex; PR, progesterone receptor; RNP, ribonucleoprotein; TPR, tetratricopeptide repeat; and v-src, viral-src pp60 kinase.

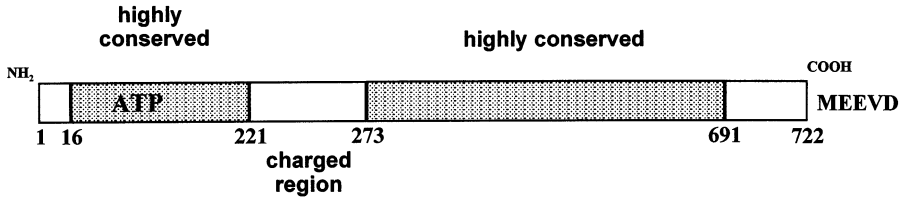

FIG. 1. Primary structure of Hsp90. The shaded boxes indicate highly conserved regions of Hsp90 as analyzed by comparison of all known Hsp90 sequences [1]. The blank boxes represent regions of low homology. The highly charged region differs in length between 0 (bacteria) and 52 (human) amino acids, while the C-terminal pentapeptid MEEVD is conserved throughout most members of the Hsp90 family detected thus far. The numbers of the amino acids are given for alpha Hsp90 from *Homo sapiens*.

Interestingly, not only the cytosolic member of the Hsp90 family, but also its homolog in the endoplasmic reticulum, Grp94, forms complexes with kinases like p185-erbB2 [12]. In general, Grp94 seems to be involved in the folding of secreted proteins [13, 14]. It remains to be seen whether a similar set of Hsp90 partner proteins (see below) exists in the endoplasmic reticulum as in the cytosol.

Additional substrates that require Hsp90 for activity *in vivo* are reverse transcriptase and p53. Surprisingly, viruses take host Hsp90 and its partner proteins aboard, which seems to be required, e.g. for the activation of viral reverse transcriptase [15, 16]. Hsp90 interacts with reverse transcriptase to facilitate the formation of an RNP complex between the polymerase and an RNA ligand. This RNP complex is required early in replication for viral assembly and initiation of DNA synthesis through a protein-priming mechanism [15]. Further investigations reported the additional involvement of p23 and an ATP-dependent step for RNP formation. It could be shown that a complex of Hsp90 and p23 is incorporated into the viral nucleocapsids in a polymerase-dependent reaction [16]. The Hsp90/p23 complex and potentially other components are thought to maintain reverse transcriptase in a conformation competent for RNA packaging and DNA synthesis.

p53 is a tumor suppressor protein that affects cell cycle arrest and apoptosis in the case of DNA damage and was found to be another Hsp90 substrate. p53 is one of the most commonly mutated proteins found in tumor cells [17]. Furthermore, wild-type p53 is highly flexible and, therefore, can achieve different conformations. The vast majority of p53 mutations result in proteins of altered conformation, together with loss of DNA-binding activity and prolonged half-life. It could be shown that p53 folds correctly in *in vitro* translation systems such as rabbit reticulocyte lysates or wheat germ extracts without involvement of Hsp90 [18, 19]. Some point mutants of p53, however, are temperature-sensitive for conformational changes, and the p53 protein can be found in the wild-type or in mutant conformations. Interestingly, the number of molecules in the mutant conformation is increased in the presence of Hsp90 and p23. Therefore, it is thought that Hsp90 is required for the shift of balance between various mutant p53 conformations [19].

Beside its role in protein folding, Hsp90 seems to play an important role in the degradation of substrates by the ubiquitin-proteasome pathway [20, 21]. In addition to proteins, peptides seem to be efficiently bound to Hsp90. Grp94, in particular, was identified as a peptide binding protein [22–24] involved in the presentation of tumor-specific antigens [25]. In this context, it has been suggested that tumor immunogenicity is based on a variety of peptides that are noncovalently associated with Grp94 [26]. Hsp90 proteins together with Hsp70 seem to present their peptides to the MHC class I molecules. The involvement of Hsp90 and Hsp70 in antigen presentation also implies that in an organism experiencing the stress of infection the MHC non-restricted presentation of non-self antigens becomes more dominant [23] and increases the efficiency of immune surveillance. Peptide-loaded chaperones may prime cytotoxic lymphocytes via the peptide-presenting macrophage MHC class I molecules even after lysis of the original infected or malignant cell, which extends the cytotoxic response and therefore makes it more efficient [27]. This may offer a new strategy for anti-tumor vaccination [23].

DISSECTION OF Hsp90 FUNCTION

Proteolysis experiments and sequence alignments suggested that Hsp90 is composed of several domains including two highly conserved regions common to all members of the Hsp90 family, separated by a highly charged region of varying length [1] (Fig. 1). The function of the charged region remains elusive, since its deletion in yeast Hsp90 did not result in a detectable phenotype *in vivo* [28]. The structure of the N-terminal domain of Hsp90 has been solved by x-ray crystallography [29–31]. This domain consists of nine helices and an anti-parallel β-sheet of eight strands that fold into an α-β sandwich. Interestingly, this domain could be crystallized in the presence of ATP or ADP, but both nucleotides did not lead to significant structural changes in comparison to the nucleotide-free protein [29, 31]. Unlike Hsp90 or other ATP-binding chaperones, where bound nucleotide adopts a fully extended conformation [32], the nucleotide in the ADP/Hsp90 complex has a much more compacted structure (Fig. 2). The overall tertiary fold of the N-terminal domain of Hsp90 [30, 31] has a remarkable and unexpected similarity to the N-terminal ATP-binding fragment of the bacterial type II topoisomerase, DNA gyrase B protein [33]. Consistent with the overall structural homology between N-

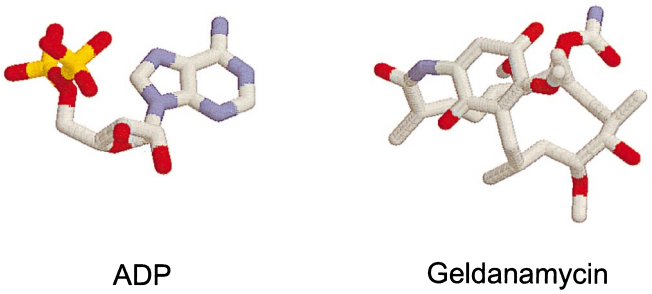

FIG. 2. Structures of ADP and geldanamycin (GA) in the Hsp90-bound conformation. Both structures are shown in the orientation in which they are bound to Hsp90. Purple sticks indicate nitrogen, red sticks oxygen, and orange sticks phosphorus. Although of different chemical nature, the ansamycin antibiotic GA mimics the stereochemistry of Hsp90-bound ADP and acts as a competitive inhibitor.

terminal domains of Hsp90 and DNA gyrase, the conformation of both ADP/ATP-binding sites is remarkably similar, and many of the residues interacting with the nucleotides seem to be conserved [34]. Whether these proteins have comparable origins in evolution and whether they share common functional features like ATPase activity is far from being understood.

Progress in understanding the functional properties of Hsp90 has been achieved by *in vitro* experiments, which demonstrated the general chaperone activity of Hsp90 in protein folding under physiological and heat shock conditions [35–38]. In these experiments, Hsp90 functioned as an ATP-independent chaperone [37–39]. Dissecting Hsp90 into domains allowed more detailed investigations of the structure–function relationship of Hsp90. Surprisingly, both the N- and C-terminal domains were found to be active in the trademark function of molecular chaperones, the suppression of aggregation of non-native proteins [40]. These results strongly suggest that Hsp90 contains two independent chaperone sites. In the full-length protein, these sites seem to contribute independently to the chaperone activity [40].

Further analysis showed that the two chaperone sites differ in substrate specificity and nucleotide dependence [40]. The N-terminal chaperone site binds ATP with a K_D in the high micromolar range [31, 41], which raised the question about the involvement of ATP in the functional cycle of Hsp90. For a long time, no ATP-dependence could be detected except for the binding of p23 to Hsp90 (see below). However, new findings suggest that ATP-binding to the N-terminal domain alters the substrate affinity of this chaperone site [40]. Surprisingly, the low ATPase activity of this domain does not seem to be involved in the substrate cycle. It remains to be seen whether partner proteins, as in the case of Hsp70, may regulate the ATPase of Hsp90. The substrates bound to the N-terminal chaperone site have to be in an unfolded conformation. These include proteins and polypeptides with 13–30 amino acid lengths [40].

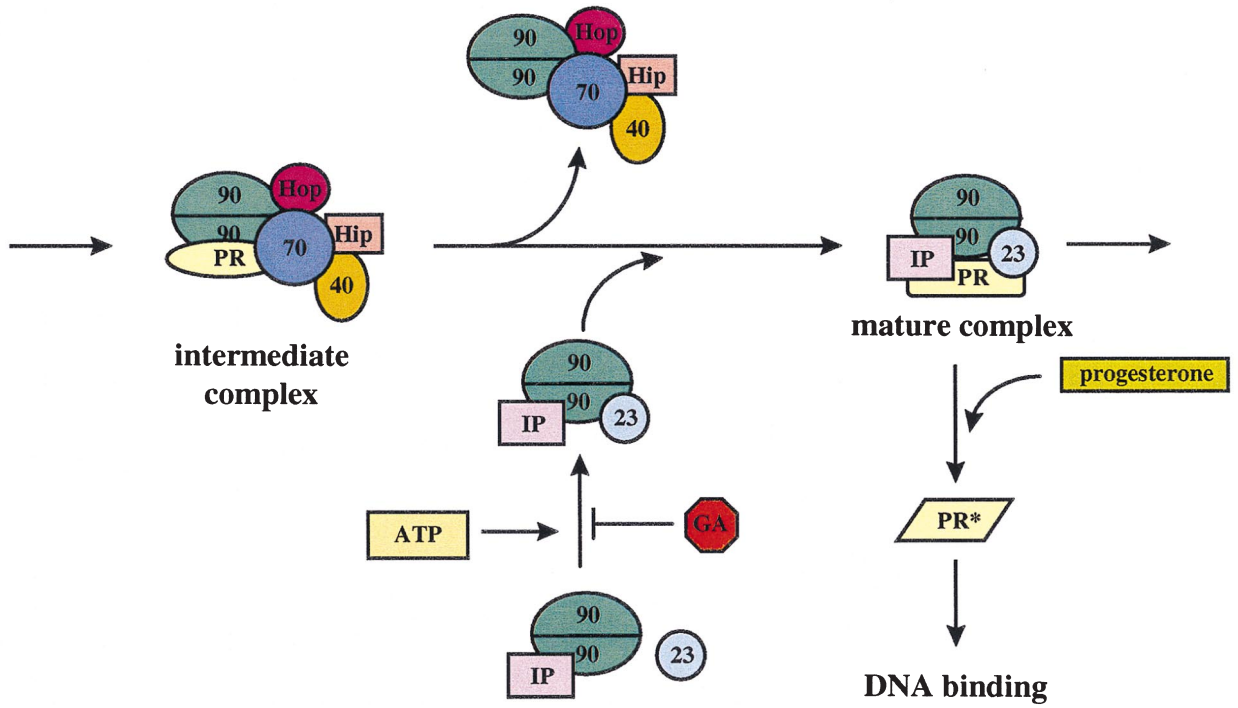

FIG. 3. Basic steps of the Hsp90-progesterone receptor cycle. After initial steps (not shown), the progesterone receptor (PR) forms an intermediate complex, which is highly dynamic and involves Hsp90 (90), Hsp70 (70), Hip, and Hop. Whether Hsp40 (40) participates in this complex is discussed controversially. After dissociation of the intermediate complex, the mature complex consisting of one of the immunophilins (IP), p23 (23), and Hsp90 is formed. This step requires ATP at the stage of the p23–Hsp90 interaction and is blocked in the presence of GA. After dissociation of the mature complex, PR dimerizes in the presence of hormone and binds DNA. In the absence of hormone, PR is able to reenter the chaperone-cycle at earlier stages, which is not shown in this model.

Partially structured molecules such as thermally unfolding citrate synthase could not be protected against aggregation by this domain.

In contrast, the C-terminal chaperone site binds promiscuously to completely unfolded or partially folded proteins and peptides of variable lengths. Because the ATP-independent C-terminal chaperone site contributes, to a large extent, to the chaperone activity of Hsp90 using *in vitro* folding assays, the previously observed ATP-independent chaperoning of protein folding can now be understood and reconciled with the finding of an ATP-binding site [36, 40]. It is tempting to speculate that the partner proteins may have a regulatory role in the substrate binding properties of Hsp90 as most of them are binding to the TPR-recognition site of Hsp90, which is located in the C-terminal domain [42].

In yeast, Hsp90 is an essential protein that is required in even higher concentrations for growth of cells at elevated temperatures [2]. Progress in understanding its general functional principles *in vivo* has been achieved by reconstituting steroid receptor activation and src kinase maturation heterologously in yeast [8, 43]. This allowed investigators to employ the powerful tools of yeast genetics to manipulate the interaction between Hsp90 and natural substrates. In this context, broadly distributed point mutations of Hsp90 affecting the function of target proteins like GR and v-src kinase could be detected, indicating the involvement of whole Hsp90 in the maturation of these substrates [43–45]. For hormone receptors, Hsp90 is continuously required to maintain an activated state capable of hormone binding [43, 45]. On the other hand, the function of Hsp90 in the maturation of kinases involves separable roles in protein folding and membrane attachment of the kinase, which leads to activation [43]. Recent results suggest that in yeast Hsp90 may not be essential for the *de novo* folding of most proteins, but that it is required for a specific subset of proteins having difficulties reaching their native conformations [46]. Under stress conditions, Hsp90 also does not seem to generally protect proteins from thermal inactivation but rather to enhance the rate at which heat-damaged proteins are reactivated, as shown with model substrate proteins like luciferase [46, 47]. The question of whether Hsp90 is a promiscuous chaperone [37] or a dedicated folding helper for a special set of proteins [46] remains to be solved.

Hsp90 AS THE CENTRAL PLATFORM OF DISTINCT MULTI-CHAPERONE COMPLEXES

At least for some non-native proteins such as steroid receptors and kinases, Hsp90 functions in concert with a well-defined set of cofactors (Table 1 and Fig. 3), which are essential to drive the cycle of Hsp90–substrate interaction. The existence of these heteroprotein complexes could be demonstrated even in the absence of specific substrate proteins [5], leading to the assumption that they may represent "super chaperone complexes" [37].

TABLE 1. Hsp90 partner proteins

General name	Other names	Function
Hsp70		Chaperone
Hip	p48	Hsp70 cofactor
Hsp40	Hdj1, Ydj1	Hsp70 cofactor
Hop	p60, Sti1	Assembly factor for Hsp70 and Hsp90
p23		Chaperone
Cdc37	p50	Kinase-specific cofactor
FKBP51	p54, FKBP54	Prolylisomerase, chaperone
FKBP52	p56, p59, Hsp56, HBI	Prolylisomerase, chaperone
Cyp40		Prolylisomerase, chaperone
PP5		Protein-serine phosphatase

In the case of steroid receptor activation, a number of different complexes composed of Hsp90 and certain partner proteins have been identified through which the receptor has to move in an ordered fashion to allow hormone binding (Fig. 3). While the participation of different partner proteins at different stages of the activation cycle has been established by immunoprecipitations from cell lysates [5, 48], the function of the individual proteins remains a mystery. The main compounds of these complexes seem to be the same for different substrates. However, in the case of kinases, the partner protein CDC37/p50 seems to be substrate-specific as it is not found in complex with steroid receptors [5]. These observations lead to the conclusion that Hsp90 may be the central platform for the assembly of distinct chaperone complexes with similar helpers for different clients.

Steroid hormone receptor assembly is a highly ordered process involving at least six proteins in addition to Hsp90 (Table 1 and Fig. 3). The so-called intermediate complex that binds to receptors contains Hsp90 and Hsp70 (Fig. 3), a well-examined chaperone involved in general folding processes in the cell [49]. Hip and probably Hsp40 also appear in the early complexes, either being recruited directly in the complex or entering after association with Hsp70 [48, 50]. Hip ["Hsp70-interacting protein"] participates in the regulation of Hsp70. One Hip oligomer binds to the ATPase domains of at least two Hsp70 molecules. While ATP hydrolysis remains the rate-limiting step in the ATPase cycle, Hip stabilizes the ADP state of Hsp70, which has a high affinity for substrate protein [50]. Binding of Hsp40 to Hsp70 is thought to stimulate ATP hydrolysis and to accelerate the release of ATP from Hsp70 [51]. Together with Hip, Hsp40 is required to regulate the ATPase and chaperone cycle of Hsp70. A 60-kDa stress-related protein, which was found to link Hsp70 to Hsp90 and was therefore named Hop (Hsp90–Hsp70 organizing protein), could also be shown to cooperate with Hsp90 in the intermediate complex [48]. Investigations on chaperone function *in vitro* indicated the ATP-dependence of this complex without detailed information on the energy-dependent step [52]. The intermediate complex dissociates

rapidly and is displaced by the mature complexes (Fig. 3). In addition to Hsp90, these complexes contain a small protein called p23 and one of three large immunophilins (FKBP51, FKBP52, or Cyp40) (Table 1 and Fig. 3). Immunophilins are peptidylprolyl isomerases that are inhibited by specific immunosuppressants that can be subdivided into two classes according to their ability to bind either cyclosporin A (cyclophilins) or FK506 and rapamycin (FKBPs). At physiological conditions, the interaction of the steroid receptor with the Hsp90 complexes is dynamic. The receptor seems to be released, followed by hormone-induced activation. In the absence of hormone, the receptor will reenter the assembly pathway. Thus, there is a dynamic turnover of receptor complexes in the chaperone cycle [48].

Recently, purified partner proteins of these complexes were analyzed *in vitro* with a view to checking whether they were also able to interact with non-native proteins in a chaperone-like manner. Both the immunophilins and p23 could be shown to selectively recognize, bind, and stabilize non-native proteins, establishing them as molecular chaperones, while Hop showed no detectable chaperone function in the assays used [53, 54]. Thus, the Hsp90 super-chaperone complex consists of several chaperone components that are able to interact directly with non-native protein. Further investigations of these complexes showed that the immunophilins [55], a protein phosphatase (PP5) with up-to-now unknown function [56], p23 [57], and Hop [58, 59] seem to bind directly to Hsp90. Hop, the immunophilins, and PP5 could be shown to bind to Hsp90 via their TPR domains [42, 55, 56, 60, 61]. In the case of Hsp70, the "organizing protein" Hop is essential for complex formation with Hsp90 [59]. The p23/Hsp90 interaction, in particular, seems to be the critical step in the formation of the mature hormone receptor complex (Fig. 3). p23 binds to Hsp90 only in the presence of ATP, magnesium, and elevated temperatures [57]. Because AMPPNP, a nonhydrolyzable ATP analog, can substitute for ATP in the association of Hsp90 and p23, ATP hydrolysis does not seem to be required for this association [57]. Binding of p23 to Hsp90 involves thus far unknown sites on Hsp90 excluding the TPR-recognition domain involved in the binding of immunophilins and Hop [42]. While the chaperone function of single Hsp90 partner proteins is established *in vitro*, nothing is known about their function and physiological importance in complex with Hsp90.

In addition to experiments in cell lysates of higher eukaryotes, insights in the *in vivo* functions of the Hsp90 partner proteins came from studies on yeast, which contains a set of Hsp90 cochaperones similar to those of higher eukaryotes [62]. To analyze the general role of the Hsp90 partner proteins in the super-chaperone cycle, their function was tested *in vivo*. Mutations in Ydj1, a member of the Hsp40 family, exerted strong and specific effects on various Hsp90 substrates like v-src kinase [63, 64]. A strain containing a Ydj1 point mutation showed reduced accumulation and activity of the v-src kinase similar to the situation found in the presence of Hsp90 mutants [63]. These data underlined the functional interplay of the Hsp90 and the Hsp70/Hsp40 machinery *in vivo*; however, the precise interaction is far from being understood. Further investigations showed that Sti1, the yeast homolog of Hop, and yeast immunophilins are of general importance in the maturation of different Hsp90 target proteins like v-src kinase and GR [65, 66]. This supported the view that Hsp90 folds structurally very different target proteins by a similar mechanism, together with a comparable set of cofactors.

Hsp90 AS THE TARGET OF THE NOVEL ANTI-TUMOR DRUG GELDANAMYCIN

Recently, Hsp90 surfaced as the target for a potent new anti-tumor drug. Screens of natural compounds for substances inhibiting the proliferation of tumor cells identified the benzochinone ansamycin GA as a promising candidate [67, 68]. GA from *Streptomyces hygroscopicus var. geldanus* and some natural analogs from various *Nocardia* species inhibited growth of a large number of cancer cell lines when administered in the sub-micromolar range [69]. Initially, GA was supposed to be an inhibitor for cell cycle kinases [70], since they were found to be inactive in the presence of GA. Later on, it turned out that the effects on the kinase activity are indirect and that the only pharmacologically specific drug target is Hsp90 [71, 72]. Incubation of cell lines with GA led to the rapid dissociation of Raf-1–Hsp90 complexes, concomitant with a markedly decreased half-life of the Raf-1 protein. Apparently, GA induced continued disruption of the Raf-1–Hsp90 complex, resulting in the loss of Raf-1 protein from the cell, although Raf-1 synthesis was actually increased [73, 74]. Prevention of Raf-1–Hsp90 complex formation by GA interfered with trafficking of newly synthesized Raf-1 from the cytosol to the plasma membrane, indicating the essential association with Hsp90 for both Raf-1 protein stability and its proper localization in the cell [73, 74]. As for the kinase, it could also be shown that GA abolishes the effects of Hsp90 on the conformation of p53 mutants [18]. Since general viability was not affected by GA, it is tempting to speculate that GA may be an inhibitor of specific Hsp90 functions. This notion was subsequently confirmed, as it was demonstrated that GA does not completely inactivate Hsp90 functions but rather blocks a distinct step in the sophisticated and dynamic cycling of partner and/or substrate proteins [5, 75]. This step was found to be the ATP-dependent binding of the partner protein p23 to Hsp90 [5, 76] (Fig. 3). GA treatment leads to an arrest of the receptor cycle at a stage in which the substrate protein is complexed with Hsp90, Hsp70, and p60 [76]. Whether the effect of GA as an anti-tumor drug on Hsp90 function *in vivo* is exclusively due to interfering with the binding of p23 [5] or whether GA additionally exerts direct effects on the interaction of Hsp90 with other molecules remains to be seen.

In vitro results with isolated Hsp90 domains led to the assumption that GA, similarly to ATP, alters the affinity of

the N-terminal chaperone site of Hsp90 for non-native proteins, while the C-terminal site was not influenced by the drug [40]. The elucidation of the structure of the N-terminal domain in the presence of GA revealed that GA binds to the ATP-binding site of Hsp90 [31]. GA consists of an ansa ring closed by an embedded benzoquinone with a pendant carbamate group approximately halfway around the ansa ring (Fig. 2). Comparison of the structures of the Hsp90 N-terminal domain in the presence of GA or ATP indicates that GA mimicks ADP/ATP, using the interactions offered by the nucleotide binding site of Hsp90 [31]. Almost all of the hydrophobic interactions between GA and Hsp90 [30] have precise equivalents in the interactions between Hsp90 and ADP/ATP [31]. The specificity of GA-binding to Hsp90 is highlighted by the observation that GA is not binding to Hsp70 [72], where the bound nucleotide adopts a different conformation.

The finding that GA binds to the ATP pocket of Hsp90 has shed some light on the structural basis of its inhibition of Hsp90. However, since the exact role of ATP in the chaperone cycle of Hsp90 is far from clear, a definite mechanism cannot be proposed at the moment.

CONCLUSION

The first identifications of certain classes of proteins as natural substrates of Hsp90 suggested that, in contrast to the other promiscuous chaperones, Hsp90 is a specific or dedicated chaperone [46]. However, the basis for this potential specificity remains enigmatic since the structures of kinases and steroid receptors do not share obvious common structural elements. Furthermore, this specificity would not explain the necessity of increased Hsp90 expression (up to 15-fold) under stress conditions. Rather, stress-induced overexpression of Hsp90 is in agreement with the *in vitro* results suggesting that Hsp90 acts as a general molecular chaperone [39].

The chaperone cycle of Hsp90 and its partner proteins is one of the most complicated and sophisticated known. While the participation of partner proteins at certain points of the cycle is well-established, their function in the cycle remains enigmatic. Their importance for the folding of specific proteins that are involved in cell cycle control together with the specific inhibition of Hsp90 by a natural compound makes Hsp90 a potent new drug target.

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