CMLS, Cell. Mol. Life Sci. 59 (2002) 1640–1648
1420-682X/02/101640-09
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CMLS Cellular and Molecular Life Sciences

Heat-shock protein 90, a chaperone for folding and regulation

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Abstract. Heat-shock protein 90 (Hsp90) is an abundant and highly conserved molecular chaperone that is essential for viability in eukaryotes. Hsp90 fulfills a housekeeping function in contributing to the folding, maintenance of structural integrity and proper regulation of a subset of cytosolic proteins. A remarkable proportion of its substrates are proteins involved in cell cycle control and signal transduction. Hsp90 acts with a cohort of Hsp90 co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function. The large conformational flexibility of Hsp90 and a multitude of dynamic co-chaperone complexes contribute to generating functional diversity, and allow Hsp90 to assist a wide range of substrates.

Key words. Molecular chaperone; protein folding; p23; Hop; Cdc37; immunophilins.

Introduction

Hsp90 was originally discovered as one of those proteins whose abundance increases upon heat stress. Whereas the term 'heat-shock protein' is perfectly appropriate for other Hsps, it is really somewhat of a misnomer for Hsp90. In most, if not all, organisms and cell types, the protein is already extremely abundant prior to cellular stress and is typically induced only a few-fold. It has been estimated that Hsp90 accounts for ~1% of the total soluble cytosolic protein in unstressed cells, making it one of the most abundant proteins [1]. Progress over the last few years has revealed 'housekeeping' functions of Hsp90 in protein folding and regulation, finally beginning to provide a rationale for its abundance (see fig. 1).

High conservation, multiple isoforms

Hsp90 is highly conserved; human Hsp90 is 60 and 40% identical to the budding yeast and *Escherichia coli* homologs, respectively. Hsp90 has been found to be essential for viability in several eukaryotic organisms, including yeast [2], *Caenorhabditis elegans* [3], and *Drosophila* [4, 5]. Cells with low levels of Hsp90 or with Hsp90 mutants are hypersensitive to stress and to Hsp90-inhibitory drugs [2, 6–11]. In prokaryotes, Hsp90 may only play an auxiliary role. Whereas it is dispensable in *E. coli* at all temperatures [12], it is essential for thermotolerance in cyanobacteria [13]. In a variety of species, such as budding yeast and mammals, genetic analyses are complicated by the fact that there are two Hsp90 genes encoding very similar isoforms (97.4% identical in budding yeast). To date, no double knockout mouse strain has been reported, but mouse embryos lacking the Hsp90β isoform die at mid-term because of defective placental development [14]. Plants appear to have even more than two Hsp90 genes [15]. Why there are multiple genes for highly similar Hsp90 isoforms in any organism is unknown. While Hsp90 forms homodimers, it remains controversial to what extent mixed isoform heterodimers exist [16, 17]. Slight functional differences between isoforms and mixed heterodimers cannot be excluded [6], but it is more likely that gene duplications have facilitated the evolution of more complex regulatory patterns.

Hsp90 family

In multicellular organisms, additional members of the Hsp90 family can be found in the endoplasmic reticulum (ER), in mitochondria, and in plants also in chloroplasts. In human, the ER version Grp94 (also called gp96) and the more prokaryote-like mitochondrial Trap1 are 49 and 35% identical to the cytosolic forms, respectively. They differ from the cytosolic forms primarily by their targeting signals and the C-terminal ER-retention signal in the

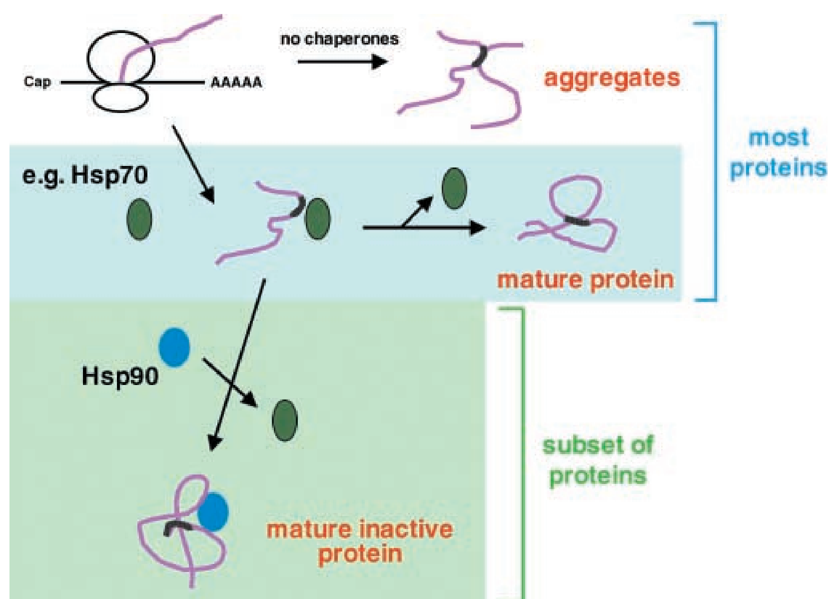

Figure 1. The general role of Hsp90 in the folding and maturation of a subset of cytosolic proteins. Other chaperones that bind hydrophobic patches of newly synthesized proteins prevent aggregation and promote folding. Hsp90 subsequently intervenes by interacting with early folding intermediates. Following protein damage, Hsp90 may also act upstream of other chaperones.

case of Grp94. Whereas the function of Trap1 remains unknown, Grp94 is now known to be dispensable for cell viability but required for innate immunity [18]. Since Trap1 and Grp94 are both absent from *Saccharomyces cerevisiae*, they may fulfill specialized functions that are dispensable in yeast. The focus of this review will be on the cytosolic members of the Hsp90 family.

Molecular chaperone activities of Hsp90 in vitro

Hsp90 promotes protein folding by preventing unfolded proteins from aggregating. Similarly, it can prevent unfolding and aggregation of folded proteins, with which it is more or less stably associated. The first hints that Hsp90 may help stabilize metastable protein domains came from studies on steroid receptor complexes in the 70s and 80s, and even preceded the formal identification of the 90-kDa component as Hsp90 (reviewed in [19]). However, with such complex systems and even more so with whole cells or crude cellular extracts, it is difficult to determine the exact biochemical functions of Hsp90. Nevertheless, through the heroic efforts of several groups the assembly of Hsp90-steroid receptor complexes has been broken down into several steps with ever-fewer components, and it continues to be a powerful paradigm [20–23]. The alternative approach to characterizing the chaperone function of Hsp90 has been to use purified components in assays with established artificial folding or aggregation substrates. Hsp90 by itself is able to prevent the aggregation of model proteins [24, 25]. The binding of Hsp90 to early unfolding intermediates [26] may contribute to its ability to collaborate with other molecular chaperones. Notably in vivo, it might typically take over newly synthesized proteins from the Hsp70 chaperone system (fig. 1). Conversely, its ability to hold denatured proteins, including large protein complexes such as β-galactosidase, in a folding-competent state for other chaperones such as Hsp70 ([27–29, see also [30]) might allow it to contribute to rescue or to dispose of damaged proteins. Surprisingly, even though Hsp90 contains an ATPase domain (see below), ATP is not needed for Hsp90 to prevent protein aggregation or for its holding function.

In contrast to the aforementioned chaperone activities, there is little evidence that Hsp90 can fold proteins by itself. Hsp90 and even a small C-terminal fragment have been reported to promote the in vitro DNA binding of bacterially produced MyoD1-E12 heterodimers [31, 32]. Whether this reflects a genuine and specific folding activity remains to be determined.

Functional domains

A complete picture of the domain organization, interaction surfaces and structure has not yet emerged. Although it was originally hoped that introducing single point mutations into the Hsp90 genes in yeast and *Drosophila* could help to map functional domains and interaction surfaces, this has not been born out except for residues involved in ATP binding and hydrolysis. What this ap-

proach has demonstrated is that the requirements of different substrates appear to be subtly different, so that single point mutants of Hsp90 can display substrate-specific defects (see for example [9, 11, 33–35]). While the molecular basis for this fascinating phenomenon remains unknown, considerable insights have been gained by cutting Hsp90 up into separate domains (fig. 2). In mapping functional domains, it must be kept in mind that Hsp90 functions with a cohort of accessory factors, collectively referred to as Hsp90 co-chaperones.

The structure of the N-terminal domain consisting of some 220 amino acids has been solved [36–38]. ADP/ATP and the Hsp90 inhibitors geldanamycin (GA) and radicicol bind the same pocket (see also [39, 40]). Compared with other molecular chaperones and kinases, it is unusual that ADP/ATP bind with the adenosine towards the bottom of the pocket and the γ-phosphate of ATP towards the surface. Hsp90 shares the three-dimensional fold of the ATP-binding region with DNA gyrase B, the histidine kinases EnvZ and CheA, and MutL (reviewed in [41]). The isolated ATP-binding domain has low intrinsic ATPase activity [42, 43]. More C-terminal sequences are necessary for commitment and even more for high-level ATP hydrolysis [43, 44]. Since the intrinsically weak ATPase activity of human Hsp90 can be dramatically stimulated by an Hsp90 substrate [45], one wonders whether those more C-terminal Hsp90 sequences might act as pseudosubstrates. In any case, ATP binding and hydrolysis have been proposed to drive the opening and closing of a molecular clamp [37, 46, 47]. The jaws of the clamp consist of the N-terminal domain, whose ATP binding promotes closure. The clamp is held together by a separate and 'constitutive' dimerization domain located within the C-terminus of the protein [46, 48–50], see also [51].

Recent reports have provided evidence for a second ATP binding domain [52, 53]. It is located in the C-terminal domain and opens up when the N-terminal site is occupied. Moreover, the drug novobiocin blocks not only ATP binding to the C-terminal site but also to the N-terminal ATPase domain [53].

Following the N-terminal ATPase domain, all eukaryotic cytosolic Hsp90 proteins have a charged domain of varying size. Although it is dispensable for viability in *S. cerevisiae* [54], it confers to Hsp90 a higher affinity for nonnative proteins [55] and is required in a luciferase refolding assay [56].

At the very C-terminus, essentially all eukaryotic Hsp90s end with the pentapeptide MEEVD. This peptide constitutes the core of the Hsp90 interaction surface for the tetratricopeptide repeats (TPRs) of Hsp90 co-chaperones [57, 58]. Interestingly, this pentapeptide is not required for viability of *S. cerevisiae* [54], nor are the TPR-containing Hsp90 co-chaperones absolutely essential for viability, with the exception of Cns1 [59–63].

The chaperone activity seems to map to more than one domain. At least two domains can prevent protein aggregation and hold substrates in a folding-competent state with distinct substrate specificities [55, 56, 64, 65]. The C-terminal domain comprising the last 100 amino acids is unique in that it can bind a short peptide [55, 64].

Role of ATP

What emerges is a view of a molecule that undergoes large conformational changes in the course of its ATPase cycle, and upon binding certain Hsp90 co-chaperones [66] and drugs [67]. Intramolecular domain interactions are highly complex and dynamic (see also [44, 68]). High temperature may expose additional surfaces, allowing the formation of higher-order oligomers [28, 69]. Despite some evidence that ATP promotes substrate release [70, 71], it is not yet clear how the extensive conformational changes during the ATPase cycle relate to the chaperone functions. As mentioned above, ATP is not required in several chaperone assays, and ATP binding mutants, although they are compromised, retain some residual luciferase refolding activity [72]. ATP binding and hydrolysis may only become important in more complex systems with an interplay of Hsp90 co-chaperones and regulators. Indeed, mutagenesis of the N-terminal domain

Figure 2. Schematic representation of the domain structure of Hsp90.

of yeast Hsp90 has demonstrated that both ATP binding and hydrolysis are essential for viability [73].

Classes of Hsp90-interacting proteins

Hsp90-interacting proteins fall into three classes: co-chaperones, regulators and substrates. This classification is somewhat arbitrary, as there are at present no clear criteria to discriminate between them. Co-chaperones assist Hsp90 in handling substrate proteins. Substrates, also referred to as client proteins, typically depend on Hsp90 for folding, complex formation and/or stability.

Co-chaperones

There are several types of Hsp90 co-chaperones based on how they interact with Hsp90 and on how they affect the Hsp90 ATPase cycle and substrate binding. Most co-chaperones contain and utilize TPRs to bind Hsp90. This is the case for Hop (Sti1 in yeast), the immunophilins FKBP51, FKBP52, and cyclophilin-40 (Cpr6 and Cpr7 in yeast), protein phosphatase 5, the Ah receptor-interacting protein AIP, yeast Cns1 and *Drosophila* Dpit47, the E3 ubiquitin ligase CHIP and the myosin-binding protein UNC-45. These interactions depend on the C-terminal MEEVD of Hsp90, but additional sequences elsewhere in Hsp90 modulate these interactions [57, 66, 74, 75]. There appears to be only one TPR acceptor site per Hsp90 dimer. As a result, the stoichiometry of co-chaperones to Hsp90 is two molecules or a dimer to one Hsp90 dimer [66, 76, 77], and the binding of TPR proteins is mutually exclusive [78–80]. This also has functional consequences. One of them is that at any given time one particular TPR protein can be dominant. For example, if CHIP is recruited to Hsp90 it leads to the ubiquitination and degradation of bound substrate [81]. How Hsp90 effects this quality control with or without assistance from co-chaperones is unknown. A notable hallmark of Hsp90 substrates is that they fail to accumulate if Hsp90 inhibitors are present during de novo synthesis (see for example [82, 83]). It remains to be established whether this is due to the block of function or to the active recruitment of CHIP or to both.

p23 (Sba1 in yeast) is a small acidic protein with chaperone activity by itself [84, 85] that interacts with the N-terminal domain of Hsp90. Since this interaction is dependent on ATP binding and dimerization [47, 86–88], it is blocked by the Hsp90 inhibitors GA and radicicol, which behave as ADP mimetics [36, 38]. The p23 interaction is also counteracted by Hop/Sti1, which prevents access of ATP to the N-terminal domain of Hsp90 [66]. Thus, Hop is an early component of Hsp90 heterocomplexes as it bridges Hsp70 and Hsp90 through its two separate TPR clusters (see for example [89]). ATP and p23 drive a rearrangement to Hsp90 heterocomplexes without Hop (and Hsp70). At that point other TPR proteins are free to interact with the TPR acceptor site of Hsp90. In fact, studies with the glucocorticoid receptor as a model substrate have shown that Hsp90 and Hsp70 are sufficient for folding, but that Hop, the Hsp70 partner Ydj1 and p23 accelerate, potentiate and stabilize folding, respectively [90], even though they are not absolutely essential in vitro [21]. Likewise, glucocorticoid receptor function is only partially affected in yeast cells lacking Sti1 (Hop) [91] or Sba1 (p23) [92], or in cells with mutant Ydj1 [93]. Evidence regarding the precise role of p23 remains confusing. While it is clearly associated with many mature Hsp90-substrate heterocomplexes (see for example [83, 94, 95]), it may join Hsp90-substrate complexes ‘merely’ to stimulate substrate release [71], bind substrates independently or even after the release from Hsp90 [95, 96].

Cdc37 and its relative Harc [97] may be substrate-specific Hsp90 co-chaperones. Cdc37 has been proposed to be a specificity factor directing Hsp90 to kinases (reviewed in [98]. The Hsp90 interaction surface for Cdc37 has not been determined, even though it has been argued that the binding of TPR proteins and Cdc37 is mutually exclusive to adjacent sites [99]. More recent evidence shows that Cdc37 and TPR proteins can be found in the same Hsp90 heterocomplex [100], and that they can even interact directly [101]. Thus, one can speculate that Cdc37 might be recruited to substrates via Hop/Sti1 and Hsp70 before Hsp90 comes in to substitute for the latter proteins.

Regulators

There is mounting evidence that Hsp90 functions are regulated by phosphorylation. Hsp90 must be phosphorylated, or rephosphorylated in vitro by casein kinase II, to stimulate the proper maturation of the heme-regulated inhibitor HRI [102]. For two other Hsp90 substrates, $pp60^{v\text{-}src}$ [103] and the reovirus protein $\sigma 1$ [104], phosphorylation of Hsp90 seems to trigger substrate release. There is genetic evidence that the protein kinase B equivalent Sch9 acts as a repressor of Hsp90 functions in yeast [105]. However, the critical phosphorylation sites, the kinases that act in intact cells and the molecular mechanisms remain unknown.

Substrates

A large proportion of the 70–80 known Hsp90 target proteins (see table 1) are involved in signal transduction and cell cycle control. While the known set might still be experimentally biased, this has led to the hypothesis (for

discussion, see [83, 106]) that a general role of the Hsp90 chaperone complex might be to assist regulatory domains. This could work in two ways: (i) the association with Hsp90 keeps substrates inactive in the absence of a stimulatory signal; the appropriate signal triggers the release of the Hsp90 complex and the completion of folding; (ii) Hsp90 is required not before but after signaling to assist in final folding, complex formation and/or maintenance. Thus, depending on the substrate Hsp90 can either be associated with the inactive or the active form of the substrate. This view does not exclude the possibility that Hsp90 might fulfill more basic functions for folding and assembly of certain substrates. Indeed, Ste11 [33] and the M15 fragment of β-galactosidase [35] appear to depend on Hsp90 for folding and/or stabilization in yeast. Similarly, src family members need Hsp90 for de novo folding while they are en route to the cell membrane [107, 108]. In addition to this basic role as a facilitator of folding, Hsp90 is a repressor for the dsRNA-activated kinase PKR. It maintains the mature form of PKR in a repressed state, and its GA-induced release is sufficient to activate PKR [83]. Steroid receptors are likely to be regulated in the same way (see above), but direct in vivo evidence for the repressive effects of Hsp90 is still missing. In the case of Apaf-1, Hsp90 prevents the oligomerization mediated by cytochrome c [109], whereas it promotes proper assembly of telomerase [110] and ribonucleoprotein complexes with the reverse transcriptase of hepatitis B virus [95]. Hsp90 and p23 even remain associated with the reverse transcriptase within the viral capsid. Finally, Hsp90 is required downstream of signaling of PDK1 to Akt to prevent the inactivation of Akt by dephosphorylation by PP2A [111].

Despite a growing list of Hsp90 substrates, substrate recognition remains poorly understood. As mentioned above, Hsp90 can bind early unfolding intermediates, and several Hsp90 domains seem to have somewhat distinct substrate binding preferences. An in vivo study with bud-

Table 1. Hsp90 interacting proteins.

Chaperones and relatives:
- Cdc37 (= p50)
- Cdc37 relative Harc
- Hsp70
- Human DnaJ homolog Hsj1b
- p23/Sba1
- proteins with tetratricopeptide motifs: Hop/Sti1, FKBP51, FKBP52 (+ high MW plant homologs), cyclophilin-40/Cpr6 and Cpr7, protein phosphatase 5, Tom70, Ah receptor interacting protein AIP, Cns1 (and its Drosophila relative Dpit47), CHIP, UNC-45/She4
- Sse1

Kinases:
- Akt (= protein kinase B)
- Bcr-Abl
- casein kinase IIα catalytic subunit
- Cdk4, Cdk6, Cdk9
- c-Mos
- death domain kinase RIP
- eEF-2 kinase
- eIF2-α kinases HRI, PKR, Gcn2
- ErbB2
- IκB kinases α and β
- insulin receptor
- KSR
- MEK
- Mik1
- MOK, MAK, MRK
- Nucleophosmin-anaplastic lymphoma kinase
- PDK-1
- Pim-1
- Plk1
- pp60v-src, c-src
- src related tyrosine kinases: yes, fps, fes, fgr, hck, and lck
- Raf-1, B-Raf, Ste11
- Wee1, Swe1

Transcription factors:
- 12(S)-HETE receptor
- *Achlya* steroid (antheridiol) receptor
- all vertebrate steroid receptors (glucocorticoid, mineralocorticoid, androgen, progesterone, and estrogen receptors)
- cytoplasmic v-erbA
- Hap1
- Heat-shock transcription factor HSF-1
- p53
- PAS family members: Dioxin receptor (= AhR), Sim and HIF-1α

Others:
- actin, tubulin, myosin
- Apaf-1
- apoB
- Atrial natriuretic peptide receptor
- calcineurin/Cna2 (catalytic subunit)
- calmodulin
- calponin
- Ctf13/Skp1 component of CBF3
- DNA polymerase α
- eNOS, nNOS
- free $\beta\gamma$ subunit of G protein
- $G\alpha_0$, $G\alpha_{12}$
- GERp95
- macromolecular aminoacyl-tRNA synthetase complex
- Macrophage scavenger receptor
- Mdm2
- nascent CFTR
- proteasome
- reovirus protein σ1
- reverse transcriptase of hepatitis B virus
- SV40 large T-antigen
- telomerase
- thrombin receptor (PAR-1)
- vaccina core protein 4a

Notes: It is difficult to define clear criteria for including proteins in this list as the experimental evidence is extremely diverse. Direct interaction has only been ascertained for a minority. For some proteins, the name of the yeast homolog has been added after a slash. References and further information are available at http://www.picard.ch/DP/Hsp90facts.pdf.

ding yeast [112] has revealed that most cellular proteins do not need Hsp90 for folding under normal physiological conditions; following stress it also enhances the rate at which some damaged proteins are reactivated. However, what fundamentally distinguishes Hsp90-dependent and -independent proteins remains to be elucidated.

On an evolutionary time scale, Hsp90's role in assisting key regulatory proteins buffers organisms against the rapid and deleterious penetrance of mutations that affect folding or stability. These mutations only become phenotypically unleashed and subjected to selective pressure when Hsp90 functions are strained (under stress) or compromised (by drugs). Thus, by virtue of its chaperone functions, Hsp90 acts as a capacitor for evolution [113].

Perspectives

The Hsp90 field has literally exploded over the last few years. Progress has been based on the development of new biochemical, genetic and pharmacological tools. Notably the advent of highly specific Hsp90 inhibitors has revolutionized both basic and applied research on Hsp90. The following are some of the challenges that lie ahead to understand the Hsp90 chaperone machine at the molecular and organismic levels.

Functional domains and the role of ATP need to be defined more clearly. The static structure of the entire molecule as well as its conformational changes driven by ATP and the binding of co-chaperones and substrates need to be elucidated. In addition to the what and how, we are only beginning to address the question why Hsp90 undergoes particular changes and participates in a symphony of interactions with co-chaperones. For most Hsp90 co-chaperones, including p23 and Cdc37, the interaction surfaces have not been determined. Even for TPR proteins, we do not know exactly how the interaction surface extends beyond the very C-terminal MEEVD. Ultimately, solving the structure of co-crystals will be very helpful.

It will be interesting to establish a more complete inventory of Hsp90 substrates. A combination of proteomics and detailed analyses of the role of Hsp90 for selected substrates might eventually tell us how Hsp90 recognizes its substrates and how it is involved in making triage decisions. Such an inventory might also help to identify the substrates that account for the requirement of Hsp90 for cellular viability. Indeed, all of the yeast Hsp90 substrates characterized to date are dispensable for vegetative growth [33, 34, 114–116].

The rational use of Hsp90 drugs to treat certain types of cancer [117, 118] will certainly benefit from a better understanding of Hsp90 functions at the level of whole cells and organisms, and of its role in the evolution of species. To extend our knowledge of the 'biology of Hsp90', it will be necessary to manipulate Hsp90 and co-chaperone genes in mammals.

Further reading

Because of space constraints, it was impossible to discuss and cite all recent developments. The author maintains a continuously updated web site (http://www.picard.ch/DP/DPhome.html) with complete datasheets and bibliography on Hsp90, p23, cyclophilins and Hop as well as a list of Hsp90-interacting proteins.

Acknowledgements. I thank Olivier Donzé and Peter Dudek for critical comments on the manuscript. Chaperone projects in my lab are supported by the Canton de Genève, the Swiss National Science Foundation and the Fondation Medic.

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