Review

CellPress

Hsp90 interaction with clients

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The conserved Hsp90 chaperone is an ATP-controlled machine that assists the folding and controls the stability of select proteins. Emerging data explain how Hsp90 achieves client specificity and its role in the cellular chaperone cascade. Interestingly, Hsp90 has an extended substrate binding interface that crosses domain boundaries, exhibiting specificity for proteins with hydrophobic residues spread over a large area regardless of whether they are disordered, partly folded, or even folded. This specificity principle ensures that clients preferentially bind to Hsp70 early on in the folding path, but downstream folding intermediates bind Hsp90. Discussed here, the emerging model is that the Hsp90 ATPase does not modulate client affinity but instead controls substrate influx from Hsp70.

Hsp90 as a major chaperone: what does it do, and how?

Protein folding is a fundamental process that is essential for life. Proteins fold by embarking on folding pathways in which the protein adopts a 3D structure by nucleating around a hydrophobic core [1]. In the cell, this vital process is guarded by the proteostasis network, which controls protein fate at all stages and thereby prevents toxic side reactions (see Glossary) [2–4]. Of particular importance is the shielding of hydrophobic residues, which are temporally exposed during initial folding but also upon damaging of existing proteins [2–4]. Uncontrolled exposure of hydrophobic stretches leads to protein aggregation and has fatal consequences. Therefore, the proteostasis network controls protein fate at any time, which includes supporting initial protein folding, repairing damaged proteins, and initiating degradation on demand. Major players in the proteostasis network are conserved families of molecular chaperones [2–4]. Key chaperones in the cytoplasm are the ATP-driven Hsp70 and Hsp90 chaperone families [5–8]. Originally their genes were discovered to be upregulated upon heat stress, thus they were named heat shock proteins (Hsps) [9]. By now, however, we know that most family members are rather constitutively expressed and involved in maintaining proteostasis at any time.

Molecular chaperone families differ in architecture and mechanism, but also in their substrate pool [5–7]. There is no obligate, stringently required order of chaperone action that would be essential for all proteins, and in particular small single-domain proteins may fold without any chaperone assistance. However, specificity defines some order in chaperone action. Hsp70s recognise short and highly hydrophobic stretches, which often are integral components of the hydrophobic core of the protein [10,11]. Hsp70s thus act early on the folding path, and they have the potential to interact with most proteins in the cell when they are unfolded. Hsp90 typically interacts with intermediates at later folding stages than Hsp70 [8]. Proteins requiring Hsp90 assistance are a select pool of proteins, highly enriched in signalling proteins or factors destabilised in protein folding-related diseases [8,12]. These Hsp90 substrates are often referred,

Glossary

Aha1: the Hsp90 co-chaperone that stimulates the ATP hydrolysis rate. Aha1 binds to Hsp90-N and Hsp90-M and competes with p23.
Cdc37: a kinase-specific substrate targeting factor of Hsp90. The human homologue is known as p50.
Cdk4: cyclin-dependent kinase 4 requires Hsp90 to reach the active state, like many other kinases.
CHIP: an E3 ubiquitin ligase, specifically interacts with both Hsp70 and Hsp90. It specifically targets the TPR motifs at the C terminus of the chaperone by its TPR domain and ubiquitylates chaperone-bound clients.
Client: Hsp90 substrates are also known as clients. In this review, we use both terms synonymously.
EM: single-particle electron microscopy provides structural information on protein complexes by constructing 3D models of biomolecules from 2D electron micrographs. In cryo-EM, particles are studied at cryogenic temperatures in their native states, whereas negative staining techniques involve the use of heavy metal salts, which interact with the electron beam and produce phase contrast.
Hsp90: 'Hsp' stands for heat shock protein, '90' for an apparent molecular weight of 90 kDa. The predominant homologue in the human cytosol is the constitutive Hsp90β, while Hsp90α is heat inducible. Thus, Hsp90β is not a heat shock protein, and it is 83 kDa.
NMR: nuclear magnetic resonance provides a dynamic picture of molecules and protein complexes in solution, based on signals of nuclei that have a particular label.
p23: the Hsp90 co-chaperone that slows down ATP hydrolysis. p23 binds to both N-terminal domains and Hsp90-M and competes with Aha1. p23 binding induces N-terminal dimerisation of Hsp90.
Proteostasis network: 'proteostasis' is an acronym for protein homeostasis. It involves all maintenance processes from protein synthesis to protein degradation. In particular, molecular chaperones and proteases control proteostasis; together they form the proteostasis network.
SAXS: small angle X-ray scattering, a solution method that provides a distance distribution curve between the atoms of a molecule. The distance distribution allows one to calculate structural models in solutions if further data are available, e.g., from crystallographic or NMR studies.
TPR motif: a tetratricopeptide motif is a specific recognition site at the C terminus of a protein. Recognition typically involves the carboxy group of the last residue, thus several TPR binding proteins need to compete for the same site.

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Keywords: molecular chaperones; protein folding; heat shock proteins; protein–protein interactions; Alzheimer disease; intrinsically disordered proteins.

0968-0004/
 http://dx.doi.org/10.1016/j.tibs.2014.12.002

somewhat anthropomorphically, as 'clients' [12]. The function of Hsp90 in the folding path of its clients, however, remained largely elusive because it was unclear how Hsp90 recognised its clients [13]. Recent progress now offers answers.

Here, we provide a synthesis of the concepts of Hsp90-mediated chaperoning based on the recent advances in understanding how it recognises clients. We describe the nature of the Hsp90 substrate binding site, discuss regulation of Hsp90–substrate interaction, and walk step-by-step along the Hsp90 interaction path with client proteins.

The nature of the Hsp90 substrate binding site

The current understanding of the nature of the Hsp90 substrate binding site is based on our structural understanding of Hsp90 as obtained by a range of biophysical techniques, and interaction studies with a range of natural clients, in particular kinases, steroid receptors, and the disordered protein Tau. We will elucidate the shape of the Hsp90 dimer and compare the regions to which the various studies mapped substrate binding.

Hsp90 is a challenging target for structural analysis

The Hsp90 family is conserved from bacteria to man, implying that it is part of a fundamental process in biology [12]. Hsp90 chaperones consist of three domains, the N-terminal, middle, and C-terminal domains (Hsp90-N, -M, and -C) (Figure 1A). A four-helix bundle in Hsp90-C ensures that Hsp90 is a homo-dimer under physiological conditions [14]. The domain interfaces between Hsp90-N and Hsp90-M and between Hsp90-M and Hsp90-C are dynamic, resulting in an ensemble of conformations in which the tips of the N-terminal domains of the Hsp90 dimer span an approximately 250-Å range according to small angle X-ray scattering (SAXS) experiments (Figure 1B) [14–16]. Remarkably, this elongated surface does not have any pocket suitable to enclose protein substrates. Because Hsp90 lacked an obvious location for client binding, controversial discussions around the mechanism of Hsp90 function raged for years [13].

The difficulty in identifying the client binding site is linked to the technically challenging nature of the Hsp90 system for structural biology methods. Earlier structural work with isolated domains proposed substrate binding sites in each domain, but a decisive structural picture remained elusive [14,17,18]. When finally full-length structures of Hsp90 homologues appeared, they did not show an obvious binding site as they lacked a bound client [16,19–21]. Crystal structures of Hsp90–substrate complexes do not exist to date, as the dynamic nature of such a complex has so far frustrated any crystallisation attempts. Hsp90, however, is also a challenging object to study by other structural biology methods; the size of Hsp90–substrate complexes (around 200 kDa) makes them large for nuclear

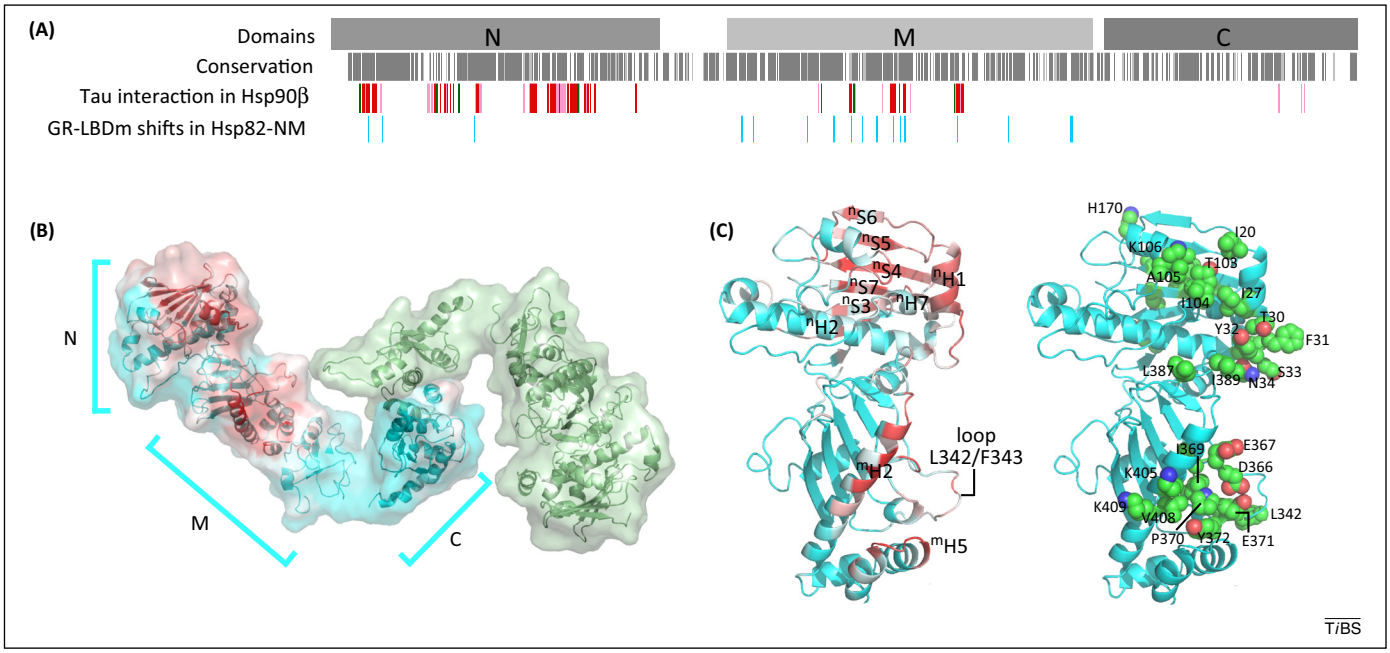

Figure 1. Substrate binding in Hsp90. **(A)** The first row shows the domain boundaries of Hsp90-N, -M, -C, indicated in shades of grey. The second row shows that human Hsp90β and yeast Hsp82 are highly conserved (dark grey, identical; light grey, conserved). The third row indicates the Tau binding site in human Hsp90β (Tau contact residues, increasing red indicates increasing closeness to Tau; isoleucine shifts in NMR experiments, dark green [15]). The fourth row shows residues shifting upon binding of a stabilised ligand binding domain of glucocorticoid receptor (GR-LBDm) to the yeast Hsp82–NM fragment (cyan), which was key to mapping the complex [32]. **(B)** The Tau binding site on Hsp90 dimer. The structural model of Hsp90 bound to Tau obtained by nuclear magnetic resonance (NMR) and small angle X-ray scattering (SAXS) is shown as ribbon and surface (80% transparent) representation. Shown is the Hsp90 dimer (Tau-binding Hsp90 protomer, cyan; other Hsp90 protomer, green; domains are indicated [15]; Tau binding on the Hsp90 surface is indicated in red). **(C)** A zoomed-in view of the Tau binding site. On the left, Hsp90 is shown in ribbon and coloured as in (B). On the right, residues involved in Tau interaction are depicted as spheres. Hsp90 is shown in cyan, interacting residues are coloured by atom (green, carbon; blue, nitrogen; red, oxygen). In Hsp90-N, hydrophobic contacts are spread over the β-sheet nS2–nS7, the helices nH1, nH7, and nH8, and the loop segments G102–K106 and T30–K35, where a small hydrophobic patch is formed on the β-sheet nS2–nS7 by the methyl groups from Thr84 (Cα, Cγ2), Lys179 (Cγ), Val143 (Cγ1, Cγ2), Ile181 (Cδ1, Cα, Cγ2), and Ile145 (Cδ1). Three aromatic residues, Tyr215, His170, and His183, are scattered around the hydrophobic patch as well as several charged residues with no distinct charge pattern. Closer to the N–M interface, residues Leu23, Leu26, Phe31, and Ile104 provide exposed hydrophobic sites that are involved in an interaction with Leu387 and Ile389 on the other Hsp90 protomer when Hsp90 is in a closed state, yet are fully exposed in an open dimer. In Hsp90-M, the Tau binding interface consists of the helices mH1, mH2, mH5, mH4, and mH9 and the loop N terminus of mH2. Pro370, Val408, Lys405, and Lys409 provide methyl groups to a small hydrophobic site. The binding site is on one side bordered by the long helix mH2 and on the other side by the loops around Leu342/Phe343 and Asp366-Tyr372.

Box 1. NMR spectroscopy

NMR spectroscopy is based on the quantum mechanical properties of the atomic nuclei, which depend on the local environment of the atoms of interest. Thus, NMR experiments provide information on how these atoms are chemically linked as well as their relative distance to each other in space and their motion relative to each other. NMR spectroscopic studies of proteins involve isotope-labelled proteins at specific nuclei (i.e., ^{1}H, ^{13}C, and ^{15}N), which make various parts of the protein structure accessible to NMR experiments. Different types of NMR experiments monitor specific types of connections between select nuclei in the proteins [23].

The most widespread method in protein NMR studies involves labelling of proteins with the isotope ^{15}N to monitor backbone amide groups. In these experiments, each amino acid in the protein corresponds to a specific peak in the NMR spectrum, resulting in a fingerprint of the protein. Any change in the environment of an amino acid shifts its corresponding peak to a new position in the NMR spectrum. Thus, one can study protein–protein and protein–ligand interactions by monitoring the peaks shifting upon complex formation with non-labelled and, therefore, NMR-invisible interaction partners [23,82]. Yet, in addition to direct binding of the interaction partner, conformational changes upon complex formation can lead to the shifting of backbone amide peaks.

A certain set of NMR experiments can be employed to reveal the identity of the peaks in the NMR spectra. The application of conventional NMR methods is limited to proteins up to 50 kDa, as large proteins have broad NMR peaks with lower intensity. In the case of the 170-kDa Hsp90 dimer, these methods are not suitable, but they have been instrumental to assign the NMR domain signals in domain fragments [24,40].

Recent advances in NMR spectroscopy allow the study of large protein complexes by employing novel methodologies. One of them involves the specific labelling of the side chain methyl groups of aliphatic amino acids, in particular of isoleucine [38,39]. This method made NMR studies accessible to large protein complexes such as the Hsp90 full-length protein and its complexes with nucleotides, co-chaperones, and clients [15,24,40]. Isoleucine labelling results in fewer signals than backbone labelling of all residues, but it avoids typical artefacts derived from working with domain fragments only.

magnetic resonance (NMR) spectroscopy (Box 1) and yet small for single-particle electron microscopy (EM) studies (Box 2) [22–24].

Not only Hsp90 itself but also its substrate pool is challenging for structural studies. Hsp90 appears to chaperone a subset of the proteome, chiefly to signal transduction compounds [8,12,25]. The most prominent client groups are kinases and transcription factors, and most functional insights derive from studying those interactions [8,25,26]. Hsp90 also interacts with other disease-related proteins such as the tumour suppressor p53, cystic fibrosis transmembrane conductance regulator (CFTR), and the microtubule-binding protein Tau [27–29]. Many members of this substrate pool are notoriously difficult to work with at the high concentration required for structural biology methods.

Kinase binds to the N-terminal and middle domains

Pioneering work by the Pearl and Saibil groups revealed the first low-resolution impression of the Hsp90 dimer bound to a substrate protein, cyclin-dependent kinase 4 (Cdk4) [30]. Cdk4 is one of the many kinases that require Hsp90 for expression due to their low intrinsic stabilities [31]. Negative staining EM data of Hsp90 dimers bound to Cdk4 and a kinase-specific substrate-targeting factor, Cdc37, provided a structural model of the complex at 20 Å resolution. Cdk4 binds to the N-terminal and middle domains of Hsp90. The kinase domain consists of two lobes, which are both largely folded in this complex. The overall orientation of two lobes of the kinase to each other is distorted when bound to Hsp90. Although the negative stain EM model lacks structural detail due to low resolution, until recently this was the only structural model of Hsp90 bound to a natural folding intermediate in a maturing state.

More detailed insights into the substrate binding site has recently come from structural models of complexes of human Hsp90 bound to Tau and of yeast Hsp90 bound to the ligand-binding domain of glucocorticoid receptor [15,32].

The substrate binding interface in the Hsp90–Tau complex

The Hsp90–Tau complex was the first complex for which both the chaperone and the client had been characterised by a high-resolution method, NMR spectroscopy [15]. Tau is a natural substrate of Hsp90 [27,33]. Tau binding to microtubules stabilises them, thereby controlling their growth [34]. Aggregation of Tau leads to insoluble tangles, which trigger Alzheimer disease and Parkinson disease [34–36]. Therefore, Tau clearance in neurons is vital. Hsp90 participates in controlling Tau levels [27,33]. Hsp90–Tau complexes are targeted by the E3 ubiquitin-protein ligase CHIP, which leads to ubiquitylation and subsequent degradation of Tau [37]. *In vitro*, Hsp90 and Tau are able to bind to each other in the absence of nucleotide or any other cofactor [15]. They form a complex with typical affinity for a chaperone–substrate interaction (5 μM).

The combination of several biophysical techniques has now revealed a structural model for the Hsp90–Tau complex [15]. Key data were derived from NMR experiments using a novel approach that monitors isoleucine side chains to increase sensitivity and provide NMR signals for this unusually large system, allowing for the mapping of binding partners (Box 1) [24,38–40]. Complementary to the NMR data, SAXS data generated a low-resolution picture of the overall shape of the complex [15]. Combining both NMR and SAXS data with pre-existing crystal structures of Hsp90 homologues revealed a high-resolution model of the Hsp90–Tau complex in solution (Figure 1B). The solution model reflects the physiological shape of the complex.

Tau interacts with a large, extended surface ranging from the N-terminal domain down to the middle domain. The interface is 106-Å in length and covers 840 $Å^2$ [15]. The properties of the binding site differ significantly from that of other chaperones, such as the chaperonins and Hsp70s [5,10,41,42]. Hsp90 does not provide any pocket or containment. In turn, it provides a significantly larger surface area than other chaperones to interact with substrate protein. Together, this results in a different substrate recognition principle compared to the other chaperones.

Chemistry of the Tau binding site

Hsp90 also differs from other chaperones by not being able to conceal its substrate binding site, so it cannot prevent clients

Box 2. Building structural models of Hsp90 complexes

Protein crystallography is the prevailing method to obtain high resolution structures. Yet, packing molecules in crystals can lead to non-physiological protein conformations [83]. Additionally, obtaining crystals for proteins with unstructured parts is not feasible. Crystal structures of four dimeric Hsp90s are available, but none include the partially unfolded or unstructured client [16,19–21]. The combination of NMR spectroscopy (Box 1) with SAXS and/or EM and crystallographic information led to structural models of Hsp90s with clients.

SAXS

SAXS records the scattering intensity of X-rays by a macromolecule at varying angles [83]. The scattering curve, where the scattering intensity is plotted per scattering angle, is then transformed into a pairwise-distance distribution function, which is the set of all distances inside a protein complex. These data allow calculating low-resolution models providing information on the overall shape and size of macromolecules [84]. The precise placement of binding partners in protein complexes requires additional information, which may derive from NMR and crystal structures [85]. This combination led to a structural model of human Hsp90–Tau. The local high-resolution information is derived from the NMR data of the Hsp90 full-length protein. This was combined with the information on the global shape of the complex determined by SAXS measurements and the available crystal structures of domains. This led to a high-resolution model of the complex, revealing the Hsp90 client binding site [15].

Single particle EM

EM is combined with image analysis to determine protein structures from low to high resolution [22]. The electron micrographs of biological macromolecules are very noisy thus the signal-to-noise ratio is improved by image processing that involves averaging large numbers of individual 2D EM projections. To enhance the contrast of the biomolecules, samples can be embedded in a heavy metal salt solution that fills the cavities and the space around the molecules but does not penetrate the hydrophobic protein interior. As a result, negatively stained specimens show protein envelopes with higher contrast [86]. This technique provided a low-resolution model of the Hsp90–Cdk4–Cdc37 complex [13]. In the case of the complex of Hsp90 with GR-LBDm, resolution had been improved by NMR data of domain fragments and the high-resolution information from crystal structures to map the binding site [32]. SAXS data confirmed these results [32].

Cryo-EM, which involves rapid cooling of a thin layer of an aqueous solution of macromolecules, may increase resolution significantly [22]. However, to date, cryo-EM has not succeeded in elucidating the structure of Hsp90–client complexes.

to access the binding site at any time. This raises the question why this constantly exposed Hsp90 binding site may not constantly glue to a large fraction of the proteome. Clearly Hsp90 needs to ensure client specificity differently than other ATP-controlled chaperones. A key principle guiding recognition of Tau by Hsp90 is the spreading of a large number of hydrophobic contacts over a large surface, so that each contact on its own contributes only a little to the total affinity (Figures 1C and 2A–C) [15]. In total, 14 residues contribute at least one hydrophobic interaction with Tau. The binding contacts are spread over Hsp90-N and Hsp90-M, so that the binding site does not have a highly hydrophobic spot (Figure 2B). The C-terminal end of the binding surface is not precisely defined, and a potential participation of Hsp90-C cannot be excluded. Due to this spreading, Hsp90 is unlikely to effectively compete with Hsp70 for highly hydrophobic stretches exposed by early folding intermediates.

In addition to hydrophobic interactions, coulomb forces strengthen substrate interactions (Figure 2C). The Tau binding surface has an even distribution of basic and acidic residues. Overall, however, Hsp90 is negatively charged and will be attracted to basic segments.

Hsp90 binds Tau in the aggregation-prone repeat region

The complementary pieces to the Hsp90–substrate puzzle belong to Tau. Intriguingly, Hsp90 binds to the repeat region of Tau. NMR data map Hsp90 binding to an approximately 170-residue region between amino acids 210 and 380 [15]. It is this region that mediates Tau binding to microtubules, and, strikingly, the same region that leads to toxic aggregation in neurons.

Tau segments recognised by Hsp90 contain some large hydrophobic or aromatic residues (between residues 210 and 380, 1 in 4.4 residues are Leu, Ile, Val, Phe, Tyr, or Trp) [50]. The frequency of hydrophobic residues is higher than typically expected for intrinsically disordered proteins (1 in 4.9 residues) but lower than in globular proteins (1 in 3.3 residues) [43]. With the exception of two Hsp70 binding sites, most hydrophobic residues are scattered throughout the repeat region.

Charges contribute to specificity; the acidic Hsp90 prefers the positively charged microtubule-binding domain to the Tau C terminus even at high ionic strength, despite a similar degree of hydrophobicity. In summary, the Hsp90 binding region of Tau has moderate hydrophobicity and a positive net charge, complementary to the negative net charge of Hsp90.

Hsp90 also binds folded protein

Hsp90 has a physiological function in controlling Tau levels [27,37]. Hsp90 and Tau forms a complex with physiologically relevant affinity, given the high levels of both proteins in the cell [15]. Nevertheless, Tau is an unexpected client for a chaperone, as it is an intrinsically disordered protein (Box 3) [44]. Intrinsically disordered proteins function without adopting stable, regular 3D structures [45–47]. It is an intriguing question why a protein that never folds at all interacts with molecular chaperones.

The puzzle becomes even more stunning when looking at another substrate that recently entered the stage, the natively folded ligand binding domain of glucocorticoid receptor (GR-LBD). GR-LBD is a 251-amino acid domain that receives the steroid hormone. It is difficult to produce and to keep in solution; even at room temperature, it is unstable and aggregation-prone [32,48]. Folding intermediates of steroid receptors stringently depend on Hsp90 to reach a conformation that allows binding to hormone [49]. Therefore, the non-native GR-LBD became a paradigmatic substrate of Hsp90 [26]. It is remarkable, however, that also natively folded GR-LBD may still enjoy the attention of Hsp90.

Notably, GR does not need chaperones to bind to the hormone *in vitro* [32,50]. By contrast, *in vivo* hormone receptors require the successive action of Hsp70 and Hsp90 to take up the hormone [26]. This implies that

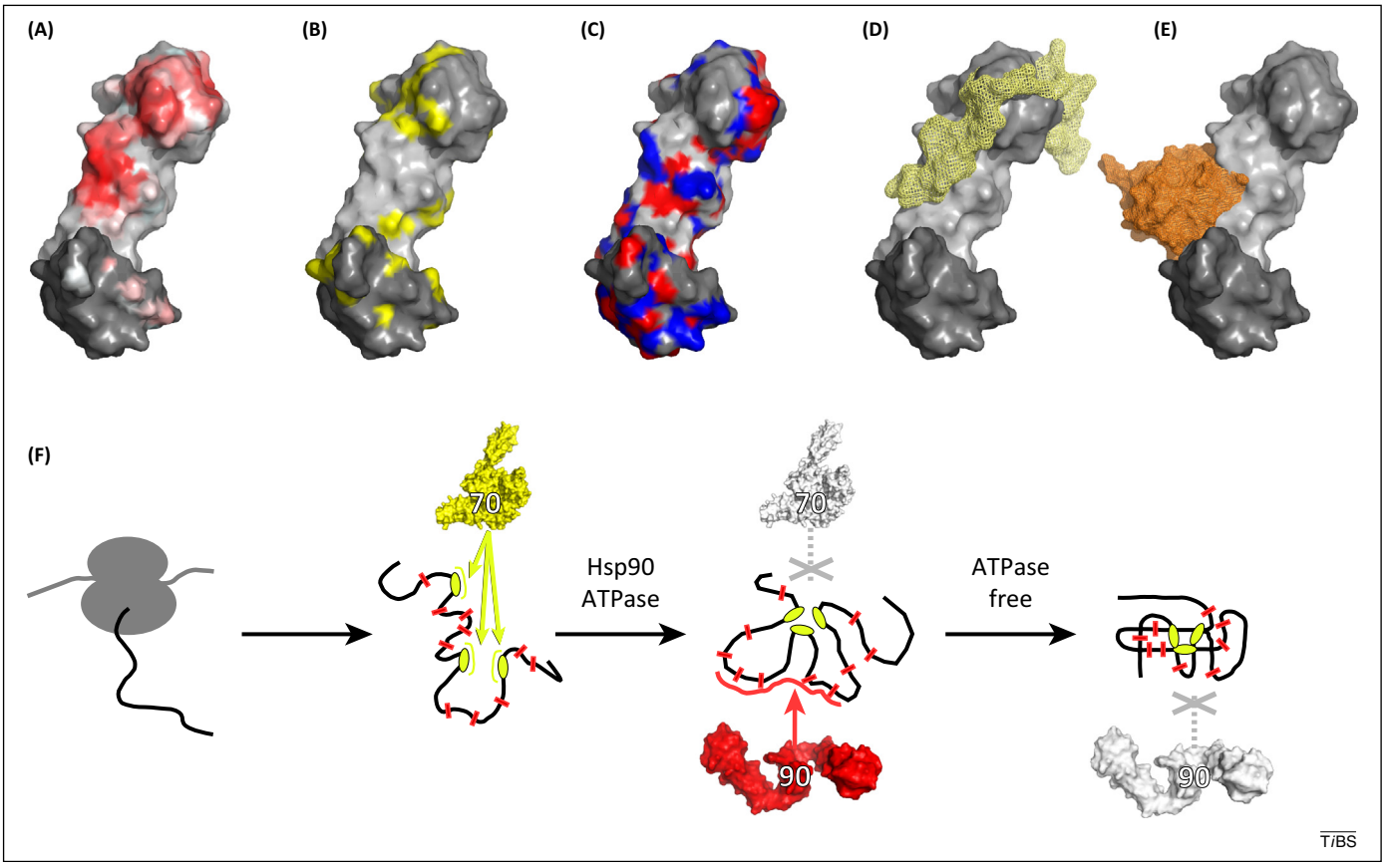

Figure 2. Specificity guides Hsp90 towards clients in late folding stages. (A–C) Tau and glucocorticoid receptor (GR) binding largely overlap in Hsp90-M. **(A)** The Tau binding site on Hsp90 (Tau binding site coloured as in Figure 1B, Hsp90-N, -M, and -C are depicted in different shades of grey in the Tau binding Hsp90 protomer). **(B)** Large hydrophobic and aromatic residues appear only scattered on the Hsp90 surface [yellow, Leu, Ile, Val, Phe, Tyr, and Trp; colouring of Hsp90 as in (A)]. **(C)** The Hsp90 binding site contains both positively (blue) and negatively (red) charged residues [colouring of Hsp90 as in (A)]. **(D)** The Hsp90-binding stretch of Tau {yellow mesh, the five best nuclear magnetic resonance (NMR)/small angle X-ray scattering (SAXS) models of Tau, disordered parts outside Hsp90-binding site are not shown; Hsp90-N, -M, and -C are depicted in different shades of grey in the Tau binding Hsp90 protomer [15]}. **(E)** GR-LBDm binding to Hsp90 (orange mesh, graphical overlay of GR-LBDm on human Hsp90 based on the orientation of the complex of GR-LBDm with yeast Hsp82 [32]; Hsp90-N, -M, and -C are depicted in different shades of grey in the Tau binding Hsp90 protomer). **(F)** Timing of chaperone action. Hsp70 (yellow [87]) binds to early intermediates due to its high affinity toward short, highly hydrophobic stretches (yellow dots). During the folding process, the Hsp70 binding sites build up the hydrophobic core. The Hsp90 ATPase is used to transfer client to Hsp90 [50]. Hsp90 (red [15]) has preference for late folding stages due to its ability to interact with hydrophobic residues scattered over a large surface (red dashes); Hsp90 may also bind to disordered or folded proteins that mimic the surface pattern typical for late folding stages. The substrate may complete folding and may not require further chaperone support. We consider it plausible that the Hsp90 ATPase is not crucial to trigger substrate release.

the chaperones keep the instable steroid receptor out of dead ends such as aggregation, degradation, or unwanted complexes with other proteins rather than actively inducing a hormone-accepting conformation.

The instable GR-LBD is also challenging to work with *in vitro*. The Buchner group stabilised GR-LBD by site-directed mutagenesis, creating a monomeric penta-point mutant (GR-LBDm), which pushes the stability window above 50 °C [32]. Surprisingly, folded GR-LBDm also binds to yeast Hsp90 with significant affinity (K_d 3 μM) [32]. NMR analysis of isotope-labelled fragments of yeast Hsp90 maps binding predominantly in Hsp90-M, and only to a minor extent in Hsp90-N and Hsp90-C (Figure 1A) [32]. The NMR data were key to obtaining structural models in combination with negative staining EM and SAXS measurements (Box 2), which revealed that GR-LBDm binding centred on Hsp90-M [32].

Box 3. Outstanding questions

- How does ATPase control substrate influx into the Hsp90 system?
- What is Hsp90 doing to its protein substrate?
- How often do chaperones bind to natively unfolded proteins, and to what effect?

GR and Tau binding sites overlap

The side-by-side comparison reveals that the binding sites for both disordered Tau and folded GR overlap in Hsp90-M (Figure 2D,E). The GR interface on Hsp90 largely overlaps with the Tau binding site with an extension towards the C terminus of Hsp90 [15,32]. The overlap of binding sites of such different clients suggests that this binding area describes a general substrate binding site. Tau shows additional interactions in Hsp90-N. GR-LBDm, however, is a folded, compact domain fragment that is smaller than Tau and other clients, therefore it could not possibly cover Hsp90-N and Hsp90-M simultaneously.

Comparison of both studies confirms that Hsp90 neither recognises a particular sequence nor a specific structural motif because Tau and GR-LBDm share neither [15,32]. For Tau, the substrate recognition principle is based on distributing hydrophobic interactions over a large surface, supported by some electrostatic interaction (Figure 1C) [15]. Given that the binding sites of both

proteins overlap significantly, the Tau recognition principle – spreading moderate hydrophobicity over a large area – may apply to GR-LBDm and possibly clients in general, including those in late folding stages. Consequently, both folded and unfolded proteins may be disguised as folding intermediates. The picture that emerges from synthesis of these data suggests that clients generally make use of the same elongated binding interface.

Substrate binding in Hsp90-C

The structural models of full-length Hsp90 with Tau, GR-LBDm, and Cdk4 do not show significant client interaction with Hsp90-C [13,15,32]. Nevertheless, some data hint at a possible role for Hsp90-C. The isolated Hsp90-C fragment shows some interaction with GR-LBDm in NMR experiments [32]. At subphysiological ionic strength and high protein concentrations, native tumour suppressor p53 showed low affinity interactions with various parts of Hsp90, including a C-terminal segment consisting of five acidic residues [51,52]. A 131-residue fragment of *Staphylococcus* nuclease (Δ131Δ) is a model substrate that also interacts with Hsp90-C in low ionic strength solutions, yet at a different site than mapped for p53 [53]. However, low ionic strength and high protein concentrations strongly favour protein–protein interactions and it remains to be seen which interactions prevail under physiological conditions.

Based on the Δ131Δ studies, point mutations in *Escherichia coli* Hsp90 have been identified that disrupt its interaction with substrates [54]. These mutants are in the Hsp90-M and Hsp90-C domains. The most dramatic mutations are in the interface between the middle and C-terminal domains [54]. This location may support the idea that dynamic interactions around the inter-domain interfaces may play a key role in controlling substrate interactions.

Together, the Hsp90 binding site is extended and spans several domains – it is therefore exposed at any time. The substrate recognition principle is based on spreading contacts over a large interaction interface. Remarkably, Hsp90 binds to both intrinsically disordered and natively folded protein substrates, with significant overlap of the binding sites in Hsp90-M. While Hsp90 binding to clients *in vitro* takes place both in the presence and absence of nucleotide and cofactors, the system is tightly regulated under physiological conditions.

Regulation of Hsp90

Hsp90 is not a passive protein scavenger; it is an ATP-controlled machine with a tightly regulated active cycle [5,55–57]. The N-terminal domain contains the pocket that binds and hydrolyses ATP [56]. The hydrolysis reaction requires a second conformational change in addition to C-terminal dimerisation. Instead of pointing in opposite directions, as in the open conformation, both N-terminal domains must connect to each other and provide a second, transient dimerisation interface [21]. Notably, this closed conformation appears only transiently in the human Hsp90–ATP ensemble; most molecules have an extended conformation such as nucleotide-free Hsp90 [58].

Under physiological conditions, Hsp90 is predominantly in an ATP-bound state. One might suspect that ATP would modulate Hsp90 interaction with substrates, however evidence suggests that it has little effect on substrate affinity. ATP-bound Hsp90 has an affinity for Tau that is identical to its affinity in the apo state (5 μM) [15]. Similarly, for the complex of yeast Hsp90 with GR-LBDm, the affinity does not differ significantly in various nucleotide states. When bound to ATP or non-hydrolysable ATP analogues, the affinity (K_d) of Hsp90 for GR-LBDm ranges from 1 to 5 μM; in the apo and ADP state, the K_d is around 3 μM [32]. We consider it unlikely that these variations in affinity stringently determine substrate binding and release.

Given that ATP does not trigger any obligatory conformational change in human Hsp90, it is consistent that the affinity for Tau does not change [15]. The nucleotide, however, changes the dynamics of the Hsp90–Tau complex. Experimentally this became evident in NMR spectra of the Hsp90–Tau–ATP complex, which showed sharpening of the signals, a typical sign of changes in protein dynamics [15].

In summary, the functional role of the ATPase cycle is poorly understood. ATP binding does not trigger substrate release, as it does for Hsp70 chaperones, and it does not stringently alter hydrophobicity, as is seen for chaperonins [5,7,10,16,32,41]. However, the ATPase activity is essential *in vivo*, and the inhibition of the Hsp90 ATPase affects folding and stability of Hsp90 clients [55,59,60]. Therefore, it is not surprising that there are tight regulatory mechanisms in place to control the ATP cycle.

Co-chaperones and adaptors control the Hsp90 machine

Control of the ATP cycle requires interaction with a network of partner proteins. Human Hsp90 is tightly controlled by a plethora of co-chaperones [61]. Some of them have a direct impact on conformational plasticity, in particular the antagonist p23 and Aha1. The ATPase cycle is stalled in the ATP state by p23, which mediates direct interaction of both N-terminal domains of the Hsp90 dimer [21,57]. ATP hydrolysis is stimulated by Aha1, which binds to the long helix mH2 in Hsp90-M, on the edge of the substrate binding site, and to the p23 contact site in Hsp90-N [62,63].

The Hsp90 dimer acts asymmetrically *in vivo* [64]. Thus, the substrate may bind on one protomer and the co-chaperones on the other one [30,65]. Co-chaperones contact surfaces of Hsp90 that show only minimal overlap with the substrate binding site [15]. Nevertheless, folded GR-LBDm and the p23 and Aha1 cannot simultaneously bind on the same side [32].

Substrate influx and efflux into the Hsp90 system is also supported by co-chaperones [12,61]. Kinases such as Cdk4 are brought in by Cdc37, which binds to the N-terminal domain [30,66]. The connector protein Hop, which binds to the tetratricopeptide repeat (TPR) motif at the very C terminus of Hsp90, facilitates transfer of clients from the Hsp70 system [67–69]. GR is the paradigm for this transfer, and potentially the same route may be used by Tau. The TPR motif is also patronised by the ubiquitin ligase CHIP, which marks some Hsp90-bound substrates, such as Tau, for degradation [37,70,71]. Notably, bacterial, mitochondrial, and endoplasmatic Hsp90s have not shown to have binding partners homologous to p23, Aha1, Hop, or

CHIP. Despite extensive tuning in the eukaryotic cytosol, the active cycle, therefore, is inherent to Hsp90 itself.

In eukaryotes, post-translational modifications (PTMs) of Hsp90 offer an additional layer for regulation. Hsp90 undergoes various PTMs including phosphorylation, acetylation, methylation, S-nitrosylation, ubiquitylation, and SUMOylation (conjugation with small ubiquitin-related modifier) at several residues scattered throughout its three domains [72–75]. It is interesting that some client kinases can phosphorylate Hsp90 including c-Src kinase and protein kinase A (PKA), potentially offering a mechanism to link maturation and regulation [76,77]. Typically, however, PTMs modulate the conformational cycle of Hsp90 and its interaction with co-chaperones, thereby fine-tuning its activity.

Reconstitution of the active cycle

Tuning of the active cycle of Hsp90 has been pioneered by Toft and coworkers using GR as substrate [26]. The Agard group recently reconstituted substrate transfer from Hsp70 to Hsp90 [50]. Hsp70 binding destabilises GR-LBD, consistent with the need for Hsp70 to embed a short hydrophobic stretch in its substrate binding pocket [10,11,78]. Consequently, Hsp70-bound GR cannot bind to the steroid hormone. Excitingly, it has now been shown that it is Hsp90 that rescues the Hsp70-bound GR out of this trap. Effective dissociation of the Hsp70–GR complex requires a functional Hsp90 ATPase cycle regulated by the co-chaperones Hop and p23 [50]. Inhibition of Hsp90 ATPase activity by drugs or by blocking ATP hydrolysis through point mutations prevents release from Hsp70 [50].

Notably, both recent studies on the GR–Hsp90 interaction did not observe substrate-induced ATPase stimulation, which has been observed previously [32,48,50]. Further mechanistic understanding could derive from structural elucidation of the complex of both chaperones bound to Hop and GR. A cryo-EM reconstruction of the complex, however, failed to provide sufficient resolution to identify GR in the complex with Hsp90, Hsp70, and Hop [50].

Both nucleotides and a plethora of cofactors play an essential role *in vivo* to control activity of the Hsp90 system. Crucial for understanding of the Hsp90 function is the link to the Hsp70 system. The complementary activities of the two major ATP-controlled chaperone machines in the cell provide an effective and versatile relay system to control protein folding in the cell.

The Hsp70/Hsp90 chaperoning cascade

The description of the substrate binding site of Hsp90 fits into a consistent picture of the action of chaperone cascades in the cell (Figure 2F):

(i) *Hsp70 acts earlier than Hsp90.* This is determined by the high affinity of Hsp70 for short, five-residue-long stretches containing typically three or more large hydrophobic or aromatic residues [10,11]. Such stretches are typically found inside the hydrophobic core of both folded proteins and late folding intermediates [15]. Thus, typically proteins need to unfold to expose Hsp70 binding sites, which are confirmed in the Hsp70–GR complex [50].

(ii) *Hsp90 does not require any specific structure of its substrates; it selects for a particular surface pattern.* Hsp90 does bind to proteins that expose some hydrophobic residues scattered over a large surface area [10]. Such a feature is provided by unfolded proteins (e.g., the p53 core domain), folding intermediates at later stages in the folding process (e.g., Cdk4 or GR-LBD), disordered proteins (e.g., Tau, α-synuclein, and Protein OS-9), and even native proteins (e.g., GR-LBDm) [30,32,79–81].

(iii) *Substrate binding properties determine that substrates will typically travel from Hsp70 to Hsp90, and not vice versa*. Hsp90 cannot compete with Hsp70 for short, highly hydrophobic stretches. In turn, Hsp90-bound GR-LBD can bind hormone, while Hsp70-bound GR-LBD cannot [50]. This is consistent with Hsp70 guarding integral elements of the hydrophobic core, which stalls folding. Hsp90 in turn binds at a later, near native or native state, which would require GR-LBD to travel backwards on the folding path to re-bind to Hsp70.

(iv) *Hsp90 substrate affinity is independent of the nucleotide state.* The affinity of Hsp90 for such divergent clients as disordered Tau, folded GR-LBD, and unfolded model substrate (Δ131Δ) does not significantly differ in different nucleotide states [15,32,53].

(v) *The Hsp90 ATPase manages substrate transfer from Hsp70 to Hsp90.* Hsp90 acts as a substrate release factor for Hsp70, and ATP hydrolysis ensures substrate influx from Hsp70 [50]. The binding principle of Hsp90 may not allow it to effectively compete with Hsp70 for substrate binding in an unregulated setting.

(vi) *Substrate release might be autonomous*. Substrate release of Hsp90 is not well understood. However, it may be that there is much less to understand than we previously thought. Hsp90 stabilises proteins in near-native states, and native states, as well as disordered proteins. In addition, steroid receptors can bind hormone while bound to Hsp90, suggesting a native or near-native conformation [50]. Substrate affinity in the low micromolar range for Tau and GR-LBDm and the fast exchange kinetics observed in NMR experiments imply that release is possible at any time [15,50]. Thus, in contrast to Hsp70, Hsp90 does not stall the folding path, and, therefore, regulation of substrate efflux may not be stringently required.

Concluding remarks

The client binding principle of Hsp90 relies on distributing hydrophobic contacts over a large surface [15]. Hsp90 does not have a binding pocket and cannot compete with Hsp70 for binding short hydrophobic stretches [15]. The Hsp90 ATPase, however, ensures that the client comes in from Hsp70 [50]. Significant overlap of the binding sites of folded GR and disordered Tau in the middle domain of Hsp90 suggests that highly diverse clients share the same binding principle (Figure 2BC).

In our view, this results in a consistent picture of the Hsp90 mechanism. ATP is required to bring in substrate

from Hsp70. We suspect that ATP-driven substrate transfer from Hsp70 to Hsp90 could be the actual essential function of the nucleotide cycle *in vivo*. Consistently, inhibition of Hsp90 by drugs blocking the ATP binding pocket disturbed interaction with Hsp90 clients, including kinases, steroid receptors and disordered proteins such as Tau [33,59,60]. Also, other client influx pathways such as Cdc37 may require ATP for the same purpose. While the Hsp90 ATPase utilizes (co-)chaperone partners to facilitate substrate transfer, the nucleotide state does not significantly modulate client affinity. After client influx, therefore, the ATPase may not be further required to manage the client.

The Hsp90 binding principle is suitable to bind to late folding stages but is not exclusive to them. Hsp90 is at the crossroads of late folding and early degradation trajectories. This makes Hsp90 a molecular switchboard to control the fate of its clients.

Acknowledgements

We thank Martina Radli, Tania Morán Luengo, and Melanie Balhuizen for comments on the manuscript. The work of the Rüdiger laboratory is funded by Marie-Curie Actions of the European Union (ITN-IDP 'ManiFold' and ITN 'WntsApp').

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