Chapter 1
Hsp70-Substrate Interactions

Roman Kityk and Matthias P. Mayer

Abstract The highly abundant and evolutionary conserved Hsp70 chaperones are central components of the cellular protein quality control system, surveilling the folding status of cellular proteins from birth at the ribosome to death through degradation. To no other chaperone families, more different functions have been assigned, and it is not surprising that Hsp70s are implicated in many developmental processes and pathological conditions. This versatility is due to the fact that Hsp70s bind tweezer-like degenerate motifs present in virtually all proteins, generally found in the hydrophobic core of the native conformation but exposed in the nascent state at the ribosome or translocation pores or upon stress-induced denaturation and aggregation. Recent years have seen much progress in understanding the molecular mechanism of this chaperone family. In this chapter, we review the current knowledge on structure, different conformational states, allostery, and regulation by co-chaperones in the context of Hsp70-substrate interaction.

Keywords Chaperones · Hsp70 · Hsp90 · Protein folding · Protein degradation · Protein-protein interactions · Quality control · Stress response

Abbreviations

ER	Endoplasmic reticulum
Hsp	Heat shock protein
JDP	J-domain protein, also called DnaJ proteins or Hsp40
NBD	Nucleotide-binding domain
NEF	Nucleotide exchange factor
SBD	Substrate-binding domain
SBDα	α-Helical lid subdomain of the SBD
SBDβ	β-Sandwich subdomain of the SBD
UPR	Unfolded protein response

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R. J. Binder, P. K. Srivastava (eds.), *Heat Shock Proteins in the Immune System*,
https://doi.org/10.1007/978-3-319-69042-1_1

1.1 Introduction

The 70 kDa heat shock proteins (Hsp70s) are conserved throughout all domains of life—from bacteria to humans. As integral elements of the chaperone network in the cell, Hsp70s perform many functions, both under stress and normal conditions [1]. Hsp70s accompany proteins from "cradle to grave" as they are often among the first proteins, outside the ribosomal exit tunnel, emerging polypeptides encounter, and they are also among the last proteins before proteolytic degradation in the proteasome or lysosome [2–9] (Fig. 1.1). Nascent polypeptide chains expose hydrophobic regions which are prone to unproductive intermolecular interactions. Thus, binding of Hsp70s to newly synthesized polypeptide chains prevents aggregation and assists in de novo folding of proteins. If required, partially folded substrates can be transferred to the Hsp60 or Hsp90 systems for maturation [11–13]. Hsp70s also assist in protein translocation across membranes into endoplasmic reticulum (ER), mitochondria, and plastids [14–16]. Thereby, Hsp70s act on both sides of the membranes: cytosolic Hsp70 escorts proteins targeted for organelles in a translocation-competent state, and organellar Hsp70s (e.g. BiP in the ER, mtHsp70 in mitochondria) bind the emerging substrates at the translocation pore and promote the transport into the lumen of the organelle [16–19]. Stress conditions and macromolecular crowding in the cell promote protein aggregation, which can be prevented or counteracted by disaggregation activity of Hsp70s in cooperation with other chaperone families [20–22].

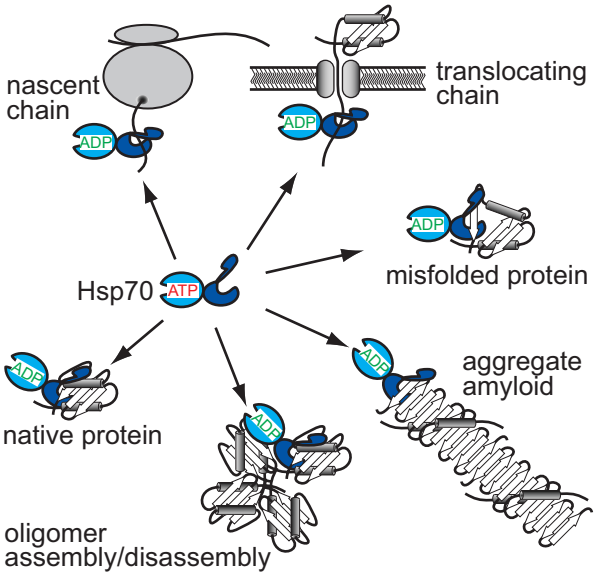

Fig. 1.1 Multiple functions of Hsp70 chaperones in the cell. The cartoon illustrates the different types of substrates and protein conformations encountered by Hsp70s, including extended conformations in de novo folding and translocation; partially folded, molten globule-like and misfolded conformations; native proteins; protein assemblies; and amorphous and amyloidic aggregates (figure from [10])

Given the wide range of tasks performed by Hsp70s in the cell, it is not surprising that a number of diseases are linked to the activity of this chaperone family, including cancer and neurodegenerative disorders to give just two examples. It was reported that Hsp70 levels in cancer cells are elevated, increasing their viability and drug resistance [23–28]. Hsp70 controls the activity of wild-type and mutant tumour suppressor p53, thereby counteracting the induction of apoptosis [29–31]. On the other side, overexpression of Hsp70 can overcome negative symptoms of neurodegenerative diseases [32–34]. Such an involvement of Hsp70 in oncogenesis and neurodegeneration processes highlights the importance of understanding the molecular mechanisms which govern the functioning of Hsp70.

1.2 Hsp70 Functional Cycle

Hsp70s consist of a 45 kDa N-terminal nucleotide-binding domain (NBD), which possesses low intrinsic ATPase activity, and a 25 kDa C-terminal substrate-binding domain (SBD), connected via a conserved hydrophobic linker. At the heart of Hsp70s' chaperone activity is the ATPase cycle, in which they oscillate between two distinct functional states (Fig. 1.2). The ATP state is characterized by low affinity and high exchange rates for polypeptide substrates, while in the ADP-bound state, Hsp70s have high affinity and low on- and off-rates for substrates [35, 36]. During their chaperone cycle, Hsp70s are aided by co-chaperones—J-domain proteins (JDPs), some of which also interact with substrates—and nucleotide exchange factors (NEFs).

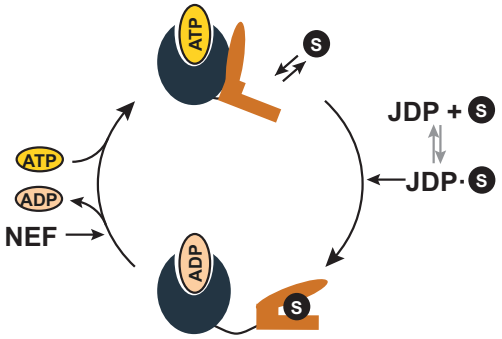

Fig. 1.2 Functional cycle of Hsp70 proteins. Hsp70s cycle between two states—the ATP-bound state with low affinity for substrates (S) and the ADP-bound state with high affinity for substrates. The cycle is controlled by internal allostery of the protein and modulated by co-chaperones. J-domain proteins (JDPs) facilitate the transfer of the substrate onto Hsp70 and couple substrate binding with ATP hydrolysis, resulting in efficient trapping of the substrate. Nucleotide exchange factors (NEFs) catalyse ADP release, accelerating rebinding of ATP and leading to a conformational change of the chaperone and release of the substrate

The chaperone cycle starts with a rapid and transient association of Hsp70·ATP with the substrate. JDPs catalyse this step by stimulating the low intrinsic ATPase activity of Hsp70s in synergism with the substrate some 100–1000-fold, resulting in trapping of the substrate [37–39]. The release of the substrate requires the exchange of ADP for ATP, as this becomes the rate-limiting step of the ATPase cycle in the presence of JDPs. NEFs accelerate ADP release [40–43], and subsequent binding of ATP displaces the NEF and converts Hsp70 into the low affinity state, leading to substrate release and the reset of the cycle.

1.3 Structural Basis for Hsp70-Substrate Interactions

The Hsp70s' capability to perform diverse tasks relies on their ability to bind in an ATP-dependent manner short degenerative peptide motifs enriched in hydrophobic and positively charged residues, which can be found on average every 30–40 residues within virtually all proteins, except for intrinsically disordered proteins [44]. Most of the structural information about the SBD-substrate interactions is based on the X-ray structure of the SBD from *E. coli* Hsp70 homolog DnaK co-crystalized with a model substrate peptide [45]. The substrate-binding domain consists of a β-sandwich subdomain (SBDβ), harbouring two β-sheets with four strands each, and an α-helical subdomain (SBDα) containing five helices—A, B, C, D, and E (Fig. 1.3b). The substrate-binding pocket is formed by the two twisted β-sheets and two sets of loops—$L_{1,2}$ and $L_{3,4}$—which form a cradle for the substrate backbone, stabilized by a second layer of loops, $L_{4,5}$ and $L_{5,6}$. Helices A and B pack against and stabilize $L_{4,5}$ and $L_{1,2}$, and the distal part of helix B forms a salt bridge and two hydrogen bonds with $L_{3,4}$ and $L_{5,6}$ and thus acts like a lid and a latch, which closes over a bound peptide substrate [46]. Whether helices C, D and, E have an additional function other than stabilizing the distal part of helix B is unclear. The last 31 residues at the C terminus were cleaved off prior to crystallization due to the high degree of flexibility in this region. In *E. coli*, the C terminus of the SBD was suggested to be involved in interactions with substrates [47]. In eukaryotic organisms, the C terminus of cytosolic Hsp70 homologs ends in the EEVD motif, enabling interactions of Hsp70s with TPR domain containing co-chaperones [48].

The crystal structure reveals that peptides bind to the SBD in an extended conformation. Five amino acids are engaged in two main types of interaction with DnaK. First, hydrogen bonds are formed between the substrate and loops $L_{1,2}$ and $L_{3,4}$, in particular involving the backbone of the substrate, explaining the preference for natural peptides made of L-amino acids over peptides made of D-amino acids [49]. The second type of interactions between peptide and DnaK are van de Waals interactions, between hydrophobic side chains of the substrate and hydrophobic residues lining the substrate-binding cavity. Additionally, the surface surrounding of the substrate-binding cleft is negatively charged, which explains why DnaK prefers substrates containing a central core of hydrophobic amino acids, flanked by positively charged residues [44].

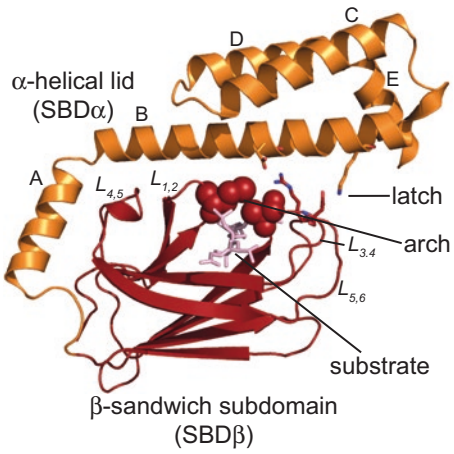

Fig. 1.3 Secondary structure representation of the SBD from *E. coli* DnaK in the substrate-bound state (PDB code, 1DKX). The SBD consists of a β-sandwich subdomain and an α-helical lid which closes over it. The lid in the closed state is stabilized by the electrostatic latch formed between helix B and loops $L_{3,4}$ and $L_{5,6}$. Peptide substrate is bound in an extended conformation and is enclosed in the substrate-binding cleft by the hydrophobic arch formed by the inner loops $L_{1,2}$ and $L_{3,4}$ and stabilized by the outer loops $L_{4,5}$ and $L_{5,6}$

Efficient substrate release from the Hsp70-SBD is achieved via ATP binding by the Hsp70-NBD. Crystal structures of a two-domain construct of DnaK·ATP provided the molecular basis for the low substrate affinity and for interdomain communication within Hsp70s [50, 51]. ATP binding induces a rotation of NBD subdomains as compared to the ADP state and in the SBD SBDβ and α-helical lid detach from each other and dock to different parts of the NBD. Thus, the lid does not cover the substrate-binding channel anymore, and the SBDβ is stabilized in a wide open conformation with $L_{1,2}$ and $L_{4,5}$ shifted towards the NBD, consistent with low affinity and high dissociation rates for the substrate in the ATP state (Fig. 1.4). In addition, the hydrophobic pocket in the SBDβ, which binds the central hydrophobic residue of the substrate in the structure of the SBD-substrate peptide complex, is diminished in width in the ATP-bound state. All of these ATP-induced structural rearrangements result in efficient substrate release. The interface between NBD and SBDβ contains an extensive H-bond network, which was shown to be the heart of the allosteric mechanism in Hsp70s [52, 53].

1.4 Mechanism of Action of Hsp70 Chaperones

Different models have been proposed to explain the effects of Hsp70s on substrate proteins. In the “kinetic partitioning” model, the chaperone does not affect the conformation of unfolded or misfolded substrates but only binds transiently to their exposed hydrophobic polypeptide stretches, preventing intermolecular

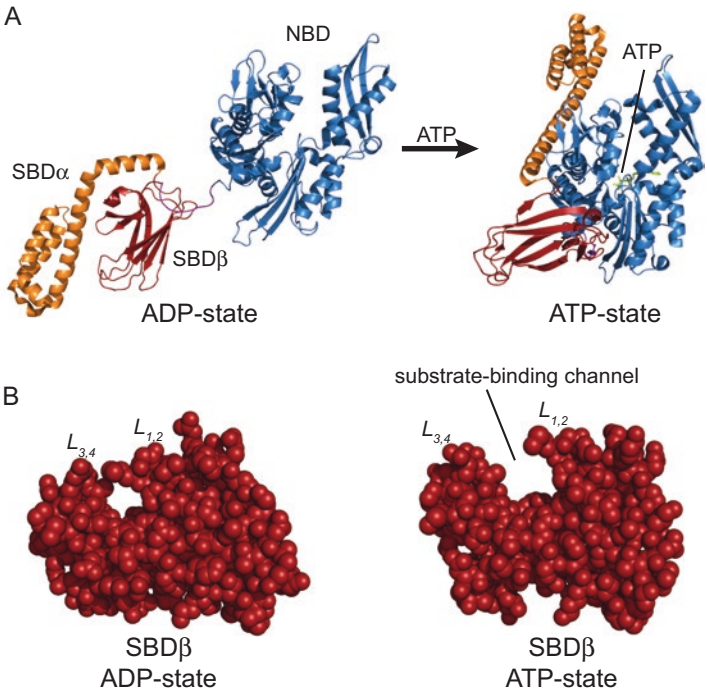

Fig. 1.4 Structural basis for the ATP-induced substrate release from the Hsp70s. (**a**) Binding of ATP leads to dramatic structural changes in DnaK. On the left—NMR structure of DnaK·ADP (PDB code, 2KHO), which demonstrates that in the ADP-bound state, NBD and SBD are in disjoined conformation; on the right, structure of DnaK·ATP (PDB code, 4B9Q), in which the SBD (both SBDα and SBDβ) is docked onto NBD and stabilized in the open conformation. (**b**) Filled space representations of the substrate-bound SBDβ (PDB code, 1DKX; represents the ADP state) and SBDβ in the DnaK·ATP structure (PDB code, 4B9Q); in the ATP state, loops $L_{1,2}$ and $L_{3,4}$ do not enclose the substrate-binding pocket leaving the substrate-binding channel widely open, which results in low affinity and high exchange rates for the substrate

homotypic association of these hydrophobic sites, thereby decreasing the pool of the free, aggregation-prone species in solution. In contrast to folding, protein aggregation is a concentration-dependent process, and thus, "kinetic partitioning" would facilitate the folding pathway by decreasing the rates of aggregation. Such a mechanism explains how Hsp70s may prevent protein aggregation and promote de novo folding of nascent polypeptides, but does not seem to be operative for refolding of proteins which are trapped in a non-native conformation. An alternative model suggests that Hsp70s can induce unfolding of misfolded substrates through binding-release cycles, allowing them to refold subsequently. Evidence was provided that the DnaK system unfolds a misfolded model substrate, a variant of firefly luciferase, prior to refolding [54]. Evidence was also provided that DnaK induces local unfolding in a native protein [55]. A different

study suggested that Hsp70s can provide the surface on which the bound substrates can sample different conformations [56].

For protein translocation across membranes, a model called "entropic pulling" was proposed [57, 58]. Briefly, as the polypeptide chain emerges from the translocon, it interacts with Hsp70 targeted by membrane-bound JDPs. Due to the excluded volume effects (because of the close proximity to membrane), the number of possible conformations of the polypeptide bound to Hsp70 is rather limited. However, as the number of translocated residues increases, the distance between Hsp70 and the membrane becomes larger, thereby increasing the conformational freedom of the translocating peptide and the total entropy of the system. This increase in entropy generates a force which drives the translocation across the membrane. Since Hsp70-binding motifs are found in proteins on average every 30–40 amino acids, the "entropic pulling" mechanism would ensure that long polypeptide chains are efficiently translocated across the membrane. A similar mechanism could be also applicable for the disaggregation function of Hsp70s in particular for the extraction of polypeptide chains from aggregates.

1.5 Interactions of Hsp70 Chaperones with Protein Substrates

In the cell, Hsp70s encounter mainly protein substrates. Model peptide substrates are useful tools for investigation of the molecular mechanism of Hsp70-substrate interactions; however, there are certain differences in Hsp70s' interaction with peptide and protein substrates. First, peptide substrates stimulate the ATPase activity of Hsp70s much less efficiently than protein substrates, and synergism with JDPs is not observed [38]. Second, biochemical data suggests that the SBD of Hsp70s adopts different conformations when interacting with peptide and protein substrates. The α-helical lid is closed completely when Hsp70s interact with peptide substrates, while it does not close completely over protein substrates [10, 59]. Thus, Hsp70s do not necessarily interact only with extended conformations of protein substrates, in contrast to what was suggested from the crystal structure of the SBD with a substrate peptide. This mode of interaction may be crucial for protein disaggregation and interaction with native proteins.

During stress conditions (e.g. heat stress), proteins can misfold and aggregate, which can have detrimental consequences for the cell. In prokaryotes, organelles of prokaryotic origin, as well as yeast and plants, Hsp70s can disassemble protein aggregates in collaboration with Hsp100s, which are toroidal hexameric AAA+ proteins. In this case, Hsp70 acts both upstream and downstream of Hsp100, helping to extract single polypeptide chains from the aggregate, and then promotes their refolding after being unfolded by Hsp100 [60–62]. In metazoans, which lack Hsp100s, protein disaggregation is performed by Hsp70 in cooperation with Hsp110 chaperones, which are relatives of Hsp70s and act as NEFs for Hsp70s [63–65]. Recently, the human Hsp70-Hsp40-Hsp110 system was demonstrated to dissolve Parkinson's-related α-synuclein amyloid fibres [66]. In this case, the Hsp70 machin-

ery is able to fragment the fibres and thereby generates new free ends, which seem to be the preferential sites for Hsp70 action in α-synuclein fibrils, resulting in acceleration of fibril depolymerization.

Hsp70s can interact not only with unfolded or misfolded polypeptides but also with natively folded or near-native proteins, performing regulatory functions in the cell. The best studied example is the interaction of DnaK with the heat shock transcription factor σ^{32} in *E. coli* [55, 67]. Based on the homology models of σ^{32} to other σ factors, it was suggested that σ^{32} exists in a compact and an extended conformation which are in equilibrium with each other, with the latter preferentially interacting with DnaK. It was suggested that the conformational change of σ^{32} is the rate-limiting step for DnaK binding and that binding of DnaK shifts the conformational equilibrium of σ^{32} towards the extended conformation. DnaK binding results in unfolding in a defined region of σ^{32}, which was proposed to facilitate the degradation of σ^{32} by the protease FtsH [55]. Upon heat shock, DnaK gets titrated away from σ^{32} by other misfolded proteins, resulting in stabilization of σ^{32} and expression of the heat shock genes, thereby providing the foundations for the regulation of the heat shock response in *E. coli*. In eukaryotes, Hsp70 is also involved in the regulation of heat shock response in the nuclear-cytoplasmic compartment, since it was found to interact with heat shock factor 1 (HSF1) during the attenuation phase of the heat shock response [68, 69]. Similarly, the ER Hsp70 homolog BiP was proposed to be the regulator of the unfolded protein response (UPR). Initiation of the UPR through two out of three known pathways involves homodimerization of ER membrane-embedded receptors—IRE1 and PERK. Although the UPR activation mechanism is still under debate, it was suggested that BiP can bind to IRE1 and PERK and prevent their homodimerization, thereby supressing the UPR [70]. Under stress conditions, the increasing amount of the unfolded proteins in the ER outcompetes IRE1 and PERK for BiP binding, allowing the UPR receptors to dimerize. Dimerization of the IRE1 results in the activation of its RNase activity, required for the noncanonical splicing of the mRNA coding for XBP1, a transcription factor which drives the expression of the genes encoding the proteins (e.g. chaperones and BiP itself) that counteract the ER stress. Dimerized PERK phosphorylates eIF2α, globally decreasing translation levels and protein influx into the ER.

Additional examples of native proteins interacting with the *E. coli* Hsp70 system are DNA replication initiator proteins like λP [71], RepA [72], and RepE [73]. Thereby, the Hsp70 system disassembles homodimers (RepA and RepE) or a heteromeric complex (λP-DnaB) to activate DNA replication. There are many more examples of Hsp70s interacting with native or near-native proteins in eukaryotic cells in addition to the above-mentioned examples. Thereby, Hsp70s often cooperate with Hsp90 chaperones. These so-called clients of the Hsp70-Hsp90 chaperone machinery include many transcription factors, e.g. steroid hormone receptors and p53, many kinases, and many other proteins important for a large variety of cellular functions [74, 75]. For some of these clients, the interaction with Hsp70 and Hsp90 seem to be restricted to folding and maturation, but others appear to require chaperone assistance throughout their entire lifespan. Hsp70 and Hsp90 are thereby

involved in coupling environmental conditions to cellular and developmental signals controlling cell homeostasis, proliferation, differentiation, and cell death.

In addition to interactions with folded proteins, Hsp70s can also disassemble protein complexes as mentioned above. A classic example for this aspect of Hsp70 function is the disassembly of clathrin from clathrin-coated endocytic vesicles by the constitutive cytosolic Hsc70 [76]. The clathrin triskelia undergo conformational fluctuations exposing Hsc70-interacting motifs. Hsc70 binding stabilizes triskelia in a conformation that induces a strain in the clathrin baskets. Binding of multiple Hsc70 molecules to triskelia increases the conformational strain to a critical level ultimately leading to the cooperative disassembly of the clathrin coat [77, 78].

Hsp70s play also an important role at different stages of viral infections, from cell membrane penetration to capsid assembly [79]. Although it is not completely understood how Hsp70s reach the cell exterior, there is evidence that some viruses require surface-exposed Hsp70s in order to enter the host cell, including rotavirus, coxsackievirus A9 (CAV-9), dengue virus, and human T cell lymphotropic virus type 1 (HTLV-1) [80–84]. Additionally, Hsp70s were shown to be involved in the disassembly of the viral coats of polyomaviruses, papillomaviruses, and reoviruses [85, 86]. Hsp70s were also demonstrated to regulate the DNA replication process of viruses. As mentioned above, the *E. coli* DnaK system releases the helicase DnaB from the complex with λP, which is required for the initiation of DNA replication of bacteriophage λ [87, 88]. There are also reports that Hsp70 system remodels replication pre-initiation complexes of eukaryotic viruses [89–92]. Lastly, the Hsp70 system plays an important role in virion assembly of some viruses, not only during folding of the capsid proteins [93–95].

1.6 Role of Co-Chaperones

Hsp70s usually do not act on their own but are supported by a number of co-chaperones. In the simplest case, the set of required co-chaperones includes a JDP and a NEF, which stimulate ATP hydrolysis and ADP-to-ATP exchange, respectively.

JDPs are proposed to be targeting factors for Hsp70s, which bind substrates themselves and transfer them to Hsp70 or are located in close proximity where Hsp70 substrates emerge like at the ribosome or translocon. Cells express a wide spectrum of JDPs, which have diverse architecture and functions and are divided into three classes, A, B, and C, according to their domain composition [96]. All of them possess a J-domain with the conserved HPD motif, critical for the interaction with Hsp70s. Interestingly, the number of different JDPs in cells is generally much higher than the number of Hsp70s. For example, humans in total possess 11 Hsp70s but 47 JDPs, or rather 53, if all splice variants are counted separately. Taking into account the diversity of JDPs, it is not surprising that besides their basic function of substrate delivery and coupling it with ATP hydrolysis, they play an important role in determining and shaping many Hsp70 functions in the cell. As mentioned above,

JDPs can also target Hsp70 to locations where its activity is required. In eukaryotes, JDPs (e.g. zuotin in yeast, MPP11 in human cells) are one of the components of the ribosome-associated complex (RAC), targeting cytosolic Hsp70s to the ribosome for early steps of de novo folding during protein synthesis [4, 97–99]. The J-domain-containing Pam16-Pam18 complex targets the mitochondrial Hsp70 to the translocon in the inner membrane of mitochondria thereby supporting protein import into the mitochondrial matrix [19, 100]. Similarly, Sec63, which is a transmembrane JDP in the endoplasmic reticulum (ER) membrane with the J-domain in the ER lumen, targets BiP to the translocon and facilitates protein transport into the ER. It is also known that JDPs are involved in ER-associated degradation (ERAD; e.g. ERdj4 and ERdj5) [101–103] or can possess ubiquitin-interacting domains, providing a link between the Hsp70 system and the protein degradation machinery in the cell [104]. Recently, JDPs of different classes were shown to synergistically activate the disaggregation function of the metazoan Hsp70 system [64]. This study revealed that JDPs from classes A and B recognize protein aggregates of different sizes, allowing efficient Hsp70 targeting to aggregates on different stages of the disaggregation process to compensate for the heterogeneity of aggregates. The JDPs from classes A and B also form transient complexes in vitro and in vivo, and this complex formation is necessary for synergistic disaggregation. If such complexes are more common, the already large number of JDPs will be potentiated through combinatorial complex formation, most likely enlarging the substrate spectrum of Hsp70s.

There are several structurally unrelated groups of NEFs. The first NEF to be discovered was GrpE from *E. coli*. In eukaryotes, however, GrpE homologs can only be found in mitochondria and chloroplasts. In other compartments, it is replaced by NEFs from the structurally unrelated BAG, HspBP1, and Hsp110/Hsp170 families [105–108]. Hsp110s and BAG proteins are particularly interesting, because they have additional roles apart from accelerating nucleotide exchange, which shape the functional landscape of the Hsp70 system in the cell.

BAG (Bcl-2-associated athanogene) proteins are very diverse, modular, cytosolic proteins that are characterized by the presence of one or several so-called BAG domains, a three-helix bundle, which is essential for interaction with Hsp70s and provides the NEF function [109]. Some Bag proteins have been proposed to divert Hsp70 substrates to degradation. Bag1 contains a ubiquitin-like domain and is, in addition, ubiquitinated by the E3 ligase and Hsp70 co-chaperone CHIP, both of which promote binding to the proteasome, suggesting a role in linking the Hsp70 system to the proteasome degradation system [110]. Bag3 facilitates the interaction of ubiquitinated and non-ubiquitinated Hsp70 substrates with the p62 (or NRB1) adaptor protein on the phagophore membrane, providing a connection of the Hsp70 system towards autophagy [111–113]. Bag6/Bat3/Scythe interacts with pro-apoptotic factors, enhancing polyubiquitination and promoting their proteasomal degradation [114–117]. BAG proteins are also involved in other functions, including prevention of protein aggregation and protein folding [109, 117].

Hsp110/Hsp170 proteins, which act as Hsp70 NEFs in the nuclear-cytoplasmic compartment (Hsp110) and in the ER (Hsp170), are Hsp70 homologs themselves, also consisting of a nucleotide-binding domain, a β-sandwich subdomain, and an

α-helical domain, which were crystalized in a conformation highly similar to the ATP-bound open conformation of Hsp70s [118–122]. Hsp110s also were shown to interact with peptide and protein substrates [66, 123, 124]. Whether they are also able to undergo similar conformational changes as Hsp70s is not clear. Hsp110 proteins of higher eukaryotes are involved in protein disaggregation and α-synuclein fibril fragmentation together with Hsp70 [63–66].

There is also an anti-NEF called Hip (Hsp70-interacting protein) which binds to the NBD of Hsp70 at a location that overlaps with the binding sites for NEFs but does not accelerate nucleotide exchange [125]. Since nucleotide exchange is rate-limiting for substrate release, NEFs and anti-NEF regulate the lifetime of the Hsp70-substrate complex, which is likely to be important for efficient folding or transfer to other chaperone systems or the degradation system.

As mentioned above, the C terminus of eukaryotic cytosolic Hsp70s possesses an EEVD motif which is the interaction site for TPR domain containing co-chaperones. One of such co-chaperones is Hop (Hsp70–Hsp90-organizing protein; Sti1 in yeast). Hop contains three TPR domains and can simultaneously interact with the GTIEEVD motif of Hsp70 and the MEEVD motif of Hsp90, resulting in a ternary complex. Thus, it is suggested that Hop couples the functional cycles of Hsp70 and Hsp90 and facilitates loading of certain partially folded intermediates from Hsp70 onto the Hsp90 machinery [126, 127]. Such a coupling of both cycles was demonstrated to be critical for the maturation of steroid hormone receptors. Folding of the ligand-binding domain of glucocorticoid receptor was shown to require a handover from the Hsp70 onto the Hsp90 system and was dependent on the presence of Hop [128, 129]. Based on the study of the interaction between Hsp90 and Alzheimer-linked protein Tau, a mechanism of the Hsp70-Hsp90 cooperation was proposed [130]. Hsp70 was suggested to bind early to nascent polypeptide chains which still expose larger hydrophobic stretches constituting high-affinity Hsp70-binding motifs. As polypeptide folding progresses, Hsp70-binding sites become buried and inaccessible for further interaction with Hsp70, leaving, however, scattered hydrophobic residues exposed. The latter are recognized by the extended substrate-binding surface of Hsp90, which facilitates the late stages of the folding process, resulting in the maturation of the substrate protein.

Another co-chaperone that interacts with Hsp70 via the C-terminal EEVD motif is CHIP (C terminus of Hsc70-interacting protein), which possesses E3 ligase activity [131–133]. According to the current view, CHIP stochastically promotes ubiquitination of substrates that are in complex with Hsp70, preferentially targeting such misfolded proteins to the proteasome which spend longer time bound to the chaperone (i.e. undergo more binding-release cycles) and presumably are difficult to fold or stuck in a non-refoldable misfolded state [134]. Thereby, substrates which can be folded/refolded within a short time frame by Hsp70 have decreased likelihood of being targeted for degradation. Taken together, the interplay between Hsp70 and the plethora of its co-chaperones is at the core of the triage decision determining the fate of its substrates: refolding to the native state, transferring to Hsp90, and targeting for degradation by the proteasome or through autophagy.

1.7 Concluding Remarks

Hsp70s interact with a large number of substrate proteins in vivo. For *E. coli* DnaK, more than 700 in vivo substrates were identified [135]. Some of these proteins are transferred onto the Hsp60 machinery for further folding steps, while the conformational maintenance of others depends on the continuous interaction with DnaK. Thus, DnaK was proposed to be a central hub of the chaperone network in the *E. coli* cell. Proteins in eukaryotic cells are on average larger as compared to prokaryotic organisms and hence demand much more attention from the protein quality control network. Thus, in the course of evolution, the Hsp70 system developed into a very sophisticated machinery, which determines the fate and regulates the activity of many proteins.

Considering the central role which Hsp70s play in the proteostasis network in the cell, it is not surprising that deregulation of the Hsp70 activity leads to pathophysiological processes, particularly cancer and neurodegeneration. Therefore, understanding the basic molecular principles how the Hsp70 machinery functions is important from a medical perspective, and the Hsp70-substrate interactions are one of the key aspects here. Although the basic principles of the Hsp70-peptide interactions are rather well understood, the knowledge on the mechanism of Hsp70-protein interactions begins only to emerge. One of the limitations in this aspect is the lack of a high-resolution structure of Hsp70-protein substrate complexes. Another open question is how the flexibility of the SBD affects substrate specificity of different Hsp70s, since different Hsp70 homologs seem to have different conformational plasticities within their substrate-binding cleft, resulting in different kinetic parameters of Hsp70-substrate interactions [136]. Hsp70s in eukaryotic organisms are targets for multiple post-translational modifications, which might provide yet another complexity level of the regulation of Hsp70-substrate interactions and should be addressed in the future.

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