1 Structural and Biochemical Properties of Hsp40/Hsp70 Chaperone System

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Abstract

Hsp70s are ubiquitous molecular chaperones that act in a myriad of cellular functions, affecting virtually all aspects in the life of proteins from synthesis to degradation. Hsp70 proteins act in the cell in cooperation with a large set of dedicated co-chaperones consisting of J-domain proteins and nucleotide exchange factors that regulate the Hsp70 chaperone cycle. Recent studies have made significant progress towards obtaining a better understanding of the mechanisms through which Hsp70s and their co-chaperones operate, providing insights into structural, kinetic, and functional features of the various members of this network. In this chapter we describe the emerging working principles of the Hsp70 machine and its co-chaperones, and highlight how mechanistic aspects of this network are tied to distinct protein folding functions.

Keywords

Hsp70 chaperones · J-domain proteins · Nucleotide exchange factors · Protein folding · Heat shock proteins · Quality control · DnaK

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1.1 Hsp70 Chaperone System

In order to survive, organisms must be able to maintain cellular homeostasis in a constantly changing environment. Molecular chaperones are essential to this effort, as they provide a "buffer" that helps protect cellular proteins from the damaging effects of extreme conditions, such as sudden increase in temperature, oxidative stress, exposure to heavy metals, hypoxia, and metabolic dysfunction (Balchin et al. 2016; Craig 2018; Fernandez-Fernandez et al. 2017; Nillegoda et al. 2018; Mogk et al. 2018).

In fact, molecular chaperones were initially defined as heat shock proteins (HSPs), as their protein levels in almost all organisms were highly increased in response to elevated temperatures (Lindquist 1986). As more has been discovered regarding molecular chaperones, however, this group has more broadly been defined as consisting of any protein that assists the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but which is not a component of these assembled structures when they perform their normal biological functions.

Hsp70 proteins are a textbook case of this behavior and belong to a ubiquitous and abundant family of molecular chaperones that regulates protein quality control and homeostasis in a stunningly wide array of cellular processes (Balchin et al. 2016; Craig 2018; Fernandez-Fernandez et al. 2017; Nillegoda et al. 2018; Mogk et al.

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2018). Members of this family play key roles in functions such as folding of newly synthesized proteins, stabilization and refolding of misfolded proteins, solubilization of protein aggregates, protein trafficking, and proteolytic degradation of unstable proteins. The many housekeeping and stress-associated protein-folding activities in which Hsp70s participate not only underscore the critical importance of these chaperones for the maintenance of protein homeostasis, but also link them to numerous pathophysiological conditions in humans (Qu et al. 2015), including neurodegenerative diseases, cancer, and organismal aging (Walther et al. 2015).

Not surprisingly, then, Hsp70s are amongst the most abundant chaperones in the cell, accounting for as much as 0.5–2% of the total cellular protein mass (Finka et al. 2015). In humans, there are at least 13 distinct genes, located on several different chromosomes, that encode for Hsp70 isoforms (Kampinga and Craig 2010), with several of these being distinct from the canonical, cytosolic Hsp70s by differences not only in their localization (Table 1.1), but also

Table 1.1 The Hsp70 family of molecular chaperones

Hsp70 chaperones		Cellular localization	Properties	Alternative names
Human	HSPA1A	Cytosol, nucleus, and nucleoli upon heat shock	Strongly stress inducible	HSP70-1, Hsp72
	HSPA1B	Cytosol, nucleus, and nucleoli upon heat shock	Strongly stress inducible	HSP70-2, Hsp70-2
	HSPA1L	Mostly cytosol under basal conditions. Nucleus but not nucleoli upon heat shock	Constitutively expressed	HSP70-hom, Hsp70-1L, Hsp70-1t, Hum70t
	HSPA2	Nucleus	Constitutively expressed	Hsp70.2
	HSPA5	Endoplasmic reticulum (ER)	Constitutively expressed	Grp78, BiP, Mif-2
	HSPA6	Cytosol and nucleus	Stress inducible, no basal expression	HSP70B’
	HSPA8	Cytosol, nucleus, and cell membrane	Constitutively expressed. Moderately induced by stress	Hsc70, Hsc71, Hsp71, Hsp73
	HSPA9	Mitochondria	Constitutively expressed	Grp75, HspA9B, MOT, MOT2, mtHsp70, mortalin
	HSPA12A	Intracellular	Constitutively expressed	
	HSPA12B	Intracellular	Constitutively expressed	
	HSPA14	Cytosol	Ribosomes associated Hsp70 Stress inducible	Hsp70L1
Yeast	Ssa1	Cytosol and nucleus	Constitutively expressed	
	Ssa2	Cytosol and nucleus	Constitutively expressed	
	Ssa3	Cytosol and nucleus	Stress inducible	
	Ssa4	Cytosol and nucleus	Stress inducible	
	Ssb1	Cytosol and nucleus	Ribosome targeting Hsp70	
	Ssb2	Cytosol and nucleus	Ribosome targeting Hsp70	
	Kar2	Endoplasmic reticulum (ER)	Induced by ER stressors	Grp78, Bip
	Ssc1	Mitochondria	Constitutively expressed Involved in pre-protein import into the mitochondrial matrix	mtHSP70, Ens1
	Ssq1	Mitochondria	Involved in iron-sulfur cluster biogenesis	Ssh1, Ssc2
	Ssc3	Mitochondria		Ecm10
	Ssz1	Cytosol	Ribosomes associated Hsp70	Pdr13
Bacteria	DnaK	Cytosol	Stress inducible	
	HscA	Cytosol	Involved in iron-sulfur cluster biogenesis	
	HscC	Cytosol		ybeW

in their substrate recognition and allosteric control. Levels of Hsp70s are tightly regulated according to cellular needs (e.g. growth or tissue-specific functions), and Hsp70 family members exist in most cell compartments (cytoplasm, nucleus, ER, mitochondria, chloroplasts). Some members even associate directly with target sites such as ribosomal tunnel exits and membrane translocons where nascent and translocating substrates emerge (Craig 2018; Zhang et al. 2017). Hsp70 chaperones do not perform their tasks alone, though, but rather rely on cooperation with an extensive network of co-chaperones from the J-domain protein family (DnaJs; Hsp40s (Kampinga and Craig 2010)), as well as nucleotide exchange factors (NEF) that regulate their activity (Abrams et al. 2014; Bracher and Verghese 2015). Hsp70s also cooperate with other protein quality control systems, including small heat shock proteins; refolding chaperones and chaperonins, such as Hsp90 and GroEL/TriC; and Hsp100 disaggregation machineries, allowing further functional diversification (Balchin et al. 2016; Rosenzweig et al. 2019).

1.1.1 Hsp70 Domain Structure and Functional Cycle

Structurally, Hsp70s consist of an N-terminal nucleotide-binding domain (NBD) with ATPase activity, and a substrate-binding domain (SBD) that can be further divided into a 15 kDa polypeptide binding cleft (SBDβ), and a 10 kDa α-helical lid (SBDα) (Mayer and Bukau 2005) (Fig. 1.1a). The NBD has an actin-like fold consisting of four subdomains (IA, IB, IIA, IIB) arranged in two lobes separated by a deep cleft (Fig. 1.1b), with ATP binding being coordinated by all four subdomains (Flaherty et al. 1990). SBDβ is composed of an eight-stranded β-sandwich containing the substrate binding cavity and its central hydrophobic pocket (Morshauser et al. 1995; Zhu et al. 1996; Kityk et al. 2015), which typically interacts with short stretches enriched in aliphatic side chains (Morshauser et al. 1995; Zhu et al. 1996; Mayer and Kityk 2015) (Fig. 1.1c). NBD and SBD are connected by a highly conserved flexible linker that is essential for the allosteric mechanism coupling nucleotide and polypeptide binding (Vogel et al. 2006; Alderson et al. 2014; Zhuravleva and Gierasch 2011).

The nucleotide binding state of Hsp70 dictates the chaperone's substrate-binding affinities. In the ATP-bound state, the Hsp70 NBD and SBD are docked to each other, and the helical lid is in an open state, allowing the rapid binding and release of substrates from the binding cleft (Fig. 1.1d). Upon ATP hydrolysis, however, this docked conformation of Hsp70 changes dramatically – the SBD dissociates from the NBD and the α-helical lid completely or partially covers the polypeptide-binding cavity (Marcinowski et al. 2011; Schlecht et al. 2011), preventing substrate dissociation and resulting in an up to 100 fold increase in affinity for substrates.

ATP hydrolysis therefore acts as a switch between two conformational states and is key to the Hsp70 chaperone cycle. Because the intrinsic ATPase activity of Hsp70 is very low (approximately 1 ATP molecule per 6–40 min), Hsp70s do not generally act alone, and instead rely on co-chaperones from the J-protein family (J-domain proteins; Hsp40s) to stimulate their ATPase activity (Kampinga and Craig 2010) (Fig. 1.1e). Surprisingly, while J-proteins are strictly required for Hsp70 function, they themselves only modestly stimulate Hsp70 ATPase activity (Kityk et al. 2018). Similarly, interaction with substrates was also observed to only elicit a slight increase in Hsp70 ATP hydrolysis (Kityk et al. 2018). Synergistic binding to both substrates and J-proteins, however, stimulates Hsp70 ATP hydrolysis rates several thousand-fold, converting Hsp70 to the high affinity ADP state and providing an efficient mechanism for trapping client polypeptides (Kityk et al. 2018; Mayer 2013) (Fig. 1.1e).

As most Hsp70s also bind the resulting ADP with high affinity, a nucleotide exchange factor (NEF) is required to stimulate ADP release, thereby allowing ATP to rebind. Then, upon this ATP binding, Hsp70 will undergo conformational changes that facilitate the release of the substrate, thereby allowing a new interaction cycle to begin (Mayer 2013) (Fig. 1.1e). Hsp70s

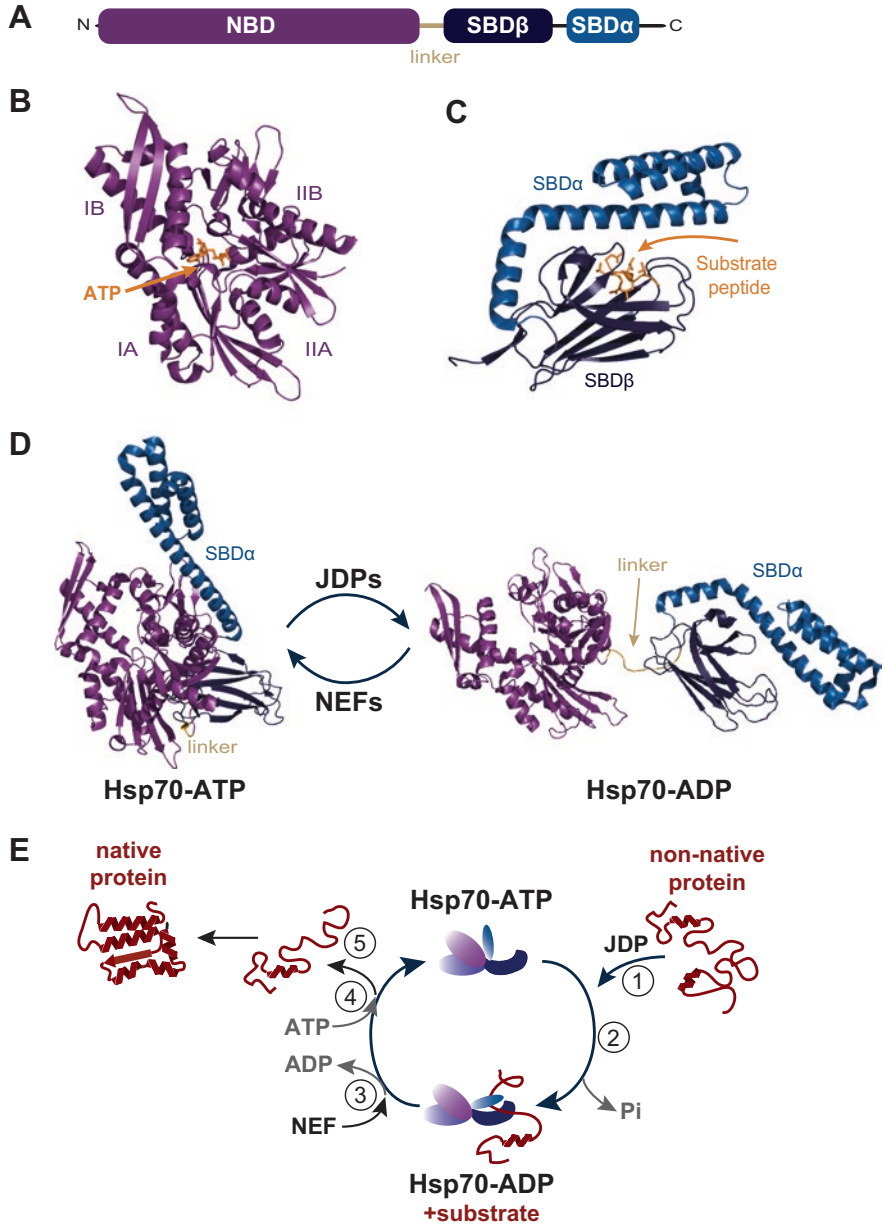

Fig. 1.1 Functional cycle and structure of Hsp70 chaperones
(**a**) Schematic representation of the domain structure of Hsp70. (NBD) is shown in purple; SBDβ, dark blue; SBDα, light blue; and the flexible linker connecting the NBD to SBD is colored light orange. (**b**) Cartoon representation of Hsp70 structure in the ATP bound state with subdomains IA, IIA, IB, and IIB denoted (PDB ID 4B9Q (Kityk et al. 2012)). (**c**) Structure of Hsp70 substrate binding domain in the high-substrate-affinity state, in complex with a peptide (PDB ID 1DKX (Zhu et al. 1996)); SBDβ is colored dark blue; SBDα, light blue; peptide, orange). (**d**) The conformational changes of Hsp70 upon ATP hydrolysis. Left – Hsp70 in the ATP state (PDB ID 4B9Q (Kityk et al. 2012)), Right – Hsp70 in the ADP-bound state (PDB ID 1KHO (Bertelsen et al. 2009)). Domains are colored as in B. (**e**) Schematic of the allosteric mechanism of Hsp70 molecular chaperones, showing steps that are facilitated by J domain and nucleotide exchange factor (NEF) co-chaperones. The Hsp70 functional cycle involves the following steps: (1) JDP-mediated delivery of substrate to ATP-bound Hsp70 (2) JDP-mediated hydrolysis of ATP to ADP, resulting in conformational changes of the Hsp70 and transition to the high-substrate-affinity ADP state (3). NEF-induced ADP dissociation (4) binding of ATP, which converts the Hsp70 to the low-substrate affinity state, leading to substrate release (5). Released substrate either folds to native state or, alternatively, re-enters the Hsp70 cycle

are thought to undergo multiple such consecutive bind and release cycles with their client proteins, in which the kinetics are custom-tailored to the needs of the particular client protein through interaction with co-chaperones (Clerico et al. 2015; Mashaghi et al. 2016).

1.1.2 Substrate Recognition and Remodeling by the Hsp70 Chaperones

Hsp70 chaperones are able to interact with an amazingly wide range of client types, including nascent (unfolded) polypeptides emerging from the ribosome, folding intermediates, natively folded proteins (e.g. the heat shock transcription factor, clathrin-coated vesicles, and replication initiation protein), misfolded proteins, and even protein aggregates and amyloid fibers (Rosenzweig et al. 2019; Clerico et al. 2019). How these chaperones recognize such a diversity of protein conformations has therefore been a matter of great interest.

Part of the answer comes from the early peptide library studies of the Hsp70 bacterial homolog, DnaK. These showed that the chaperone has a preference for 5-residue hydrophobic cores enriched in aliphatic amino acids, and flanked by positively charged regions on both sides (Rudiger et al. 1997). Such Hsp70-binding motifs are found in most proteins, but are usually buried in the hydrophobic core of the proteins when they are in their native folded state. These motifs are exposed, however, during protein synthesis, or as a result of heat stress or oxidative damage that cause protein denaturation and misfolding.

The first structural description of an Hsp70-substrate complex revealed that the model peptide (NRLLLTG) is bound to the DnaK SBD in an extended conformation (Zhu et al. 1996). This occurred along a hydrophobic cleft in the SBD, with the side-chain of the peptide's central leucine residue projecting into a small hydrophobic binding pocket (Fig. 1.1c). In addition, over a stretch of five consecutive residues the peptide backbone was further enclosed by the SBDβ cleft and stabilized through a network of hydrogen bonds. While this peptide-binding configuration is evolutionarily conserved (Morshauser et al. 1999; Pellecchia et al. 2000; Stevens et al. 2003; Cupp-Vickery et al. 2004; Jiang et al. 2005), variability has been observed with respect to the register and orientation of peptides bound to Hsp70s (Clerico et al. 2015; Zahn et al. 2013; Tapley et al. 2005), indicating that substrate binding exhibits some degree of plasticity and promiscuity.

In addition to promiscuity in substrate recognition, further heterogeneity may originate from differences in the Hsp70s themselves. While the structure of the Hsp70 SBD is largely conserved (Morshauser et al. 1999; Pellecchia et al. 2000; Stevens et al. 2003; Cupp-Vickery et al. 2004; Jiang et al. 2005), it becomes more and more evident that Hsp70s originating from different organisms, and even Hsp70s from the same organisms but from different compartments, differ in their substrate recognition preferences (Fourie et al. 1994) (see Table 1.1 for the list of Hsp70 chaperones in different organisms). For example, while cytosolic Hsp70s preferably bind leucine-enriched peptide stretches, the ER homolog, BiP, rather engages motifs containing aromatic residues (Gragerov and Gottesman 1994). A striking difference was also reported in the substrate specificity of the *E. coli* HscA and the *S. cerevisiae* mitochondrial Hsp70 Ssq1, which are involved in the biogenesis of Fe-S cluster proteins. Both chaperones, instead of leucines, preferentially recognize peptide sequences enriched in prolines (Dutkiewicz et al. 2004; Hoff et al. 2002). HscC, also in *E. coli*, further differs in substrate specificity from both HscA, and their third counterpart, DnaK (Kluck et al. 2002), and similarly, differences in substrate specificity were reported for human Hsp70 (HSPA1A) and Hsc70 (HSPA8) (Taipale et al. 2014; Mok et al. 2018). Beyond binding preferences, the kinetics of substrate interaction also vary greatly between different Hsp70s, with the eukaryotic Hsp70 chaperones displaying faster binding and release rates compared to their prokaryotic homologs (Marcinowski et al. 2013). In all, variations in binding specificities and kinetics are not only widespread between the members of the Hsp70

chaperone family, but are most likely drivers, at least in part, of Hsp70 functional diversity.

Perhaps one of the best established Hsp70 functions is interaction with nascent proteins as they exit the ribosome. There, the proposed role of Hsp70s is to delay folding of an emerging protein domain until all the sequence elements required for folding are accessible, thereby preventing the formation of non-native interactions and protecting the nascent chain from misfolding and aggregation (Balchin et al. 2016; Preissler and Deuerling 2012; Frydman 2001; Kramer et al. 2009). Some Hsp70s perform these holding functions by transient association with the ribosome, employing specialized targeting machinery, with the archetype being the yeast Hsp70, Ssb (Ssb1 and Ssb2). This chaperone is targeted to the exit of the ribosomal tunnel by the ribosome-associated complex (RAC), which is composed of the J-like protein, zuotin and the non-canonical yeast Hsp70, Ssz1 (Zhang et al. 2017; Lee et al. 2016). Ssb interacts with a large spectrum of emerging polypeptides (Willmund et al. 2013; Doring et al. 2017) by transient association with exposed hydrophobic stretches typically found in the core of folded protein domains (Doring et al. 2017). Nascent proteins can bind Ssb or even the canonical yeast Hsp70s, Ssa1–4, repeatedly during chain elongation, preventing misfolding of the mature protein (Meyer et al. 2007).

An additional example of Hsp70 interaction with unfolded proteins is during translocation of newly synthesized proteins into mitochondria, chloroplasts and the ER membranes (Craig 2018). Hsp70 binding to cytosolic precursors of these proteins keeps them in an unfolded state until a targeting machinery engages the precursors for delivery to the translocon (Craig 2018). At the *trans* side of the membranes, these polypeptide chains are then engaged by compartment specific, translocon-associated Hsp70s that help to pull the unfolded protein into the organelle and facilitate folding. Interestingly, the necessary pulling forces for this transmembranal translocation are not a product of chemical hydrolysis, but rather as a result of the low intrinsic entropy state created by the limited conformational space of the Hsp70 molecules bound next to the translocation pore. Inward movement of the translocating polypeptide, in turn, increases the available conformational space, thereby increasing entropy and generating an entropic pulling force (Craig 2018; Goloubinoff and De Los Rios 2007). Thus, through mere localized binding, Hsp70 can produce enough force (10–20pN) (Goloubinoff and De Los Rios 2007) to drive protein translocation.

While many Hsp70 clients are unfolded proteins, the chaperone is also known to interact with non-native/misfolded and, in some cases, even native, fully-folded proteins, provided their binding sites are accessible to the Hsp70 SBD. What happens to these clients, however, once they are in complex with Hsp70?

One of the earliest studies, using circular dichroism and fluorescence, of unstable proteins bound to the bacterial Hsp70 homolog, DnaK, indicated that bound substrate proteins are significantly unfolded (Palleros et al. 1994). This observation was further supported by single-molecule fluorescence spectroscopy studies of rhodanese in complex with DnaK, detecting a largely expanded unfolded conformation of the client protein while bound to the chaperone (Kellner et al. 2014).

More recently, NMR spectroscopy studies of Hsp70 with various, single-domain clients demonstrated that clients associated with Hsp70 exist in a conformationally heterogeneous, but primarily unfolded ensemble (Lee et al. 2015; Sekhar et al. 2015, 2016; Rosenzweig et al. 2017). Interestingly, in these clients, certain local structural propensities of the folded state were maintained both when the proteins were free in solution, and when they were bound to the Hsp70 chaperone (Sekhar et al. 2015). Further investigation suggested that DnaK specifically disrupted tertiary, long range contacts, while enabling local structure formation (Sekhar et al. 2016). Subsequent studies further showed that Hsp70s selectively bind to client conformations that, even transiently, expose hydrophobic binding motifs, thereby reshaping the folding energy landscape of the client and increasing the probability of achieving their proper fold (Sekhar et al. 2018).

As Hsp70s generally bind regions in their clients that are either stably or transiently exposed (Sekhar et al. 2018), one possibility is that when native clients are bound by Hsp70s, it is in linker or loop regions, while the rest of the protein remains folded. Such a mode of binding was described for a natural Hsp70 substrate, a well-folded clathrin triskelion (a trimer of clathrin heavy- and light-chain dimer), with Hsp70 binding to the QLMLT motif present in the clathrin C-terminal unstructured tails (Bocking et al. 2011). There, Hsp70 conformationally selects clathrin states that are incompatible with the assembled triskelions, thereby disassembling the clathrin cages and uncoating the budding vesicles during endocytosis (Fotin et al. 2004; Rapoport et al. 2008). Another example is DnaK, which binds to the unstructured loop region of E. coli heat shock transcription factor σ32 (Chakraborty et al. 2014; Rodriguez et al. 2008), that presumably renders the transcription factor amenable for degradation. The situation in both these cases is somewhat similar to the *in vitro* observed Hsp70 dimerization, where the SBD of one Hsp70 binds the flexible hydrophobic linker of the other, while leaving that chaperone intact (Chang et al. 2008).

Moreover, certain protein substrates may retain their tertiary structure even when Hsp70s bind to structured regions. Using disulfide cross-linking and measurement of mobility of spin labels, it was shown that the α-helical lid of the SBD does not necessarily fully close around protein substrates as it would around a peptide, potentially allowing for binding of more structured proteins (Schlecht et al. 2011). Similarly, single molecule FRET measurements between the lid and the SBD showed that while the lid is closed upon Hsp70 binding to a short, 10-residue peptide, complex formation with molten globule-like cellular substrate proteins results in a far lesser extent of lid closure (Banerjee et al. 2016).

While the function of Hsp70 binding to native proteins is still under investigation, it is clear that for unfolded and misfolded clients, the Hsp70-induced disruption of intramolecular contacts serves as an efficient way of rescuing proteins from kinetic traps that would otherwise lead to further misfolding and/or aggregation. Moreover, the resulting extended Hsp70-bound proteins may then pose a beneficial starting point for either spontaneous folding or presentation to downstream chaperone machineries.

1.2 Regulation of Hsp70 Function by Co-chaperones

Much of our mechanistic understanding of Hsp70 function comes from studies using the E. coli orthologs, which include a single Hsp70 (DnaK), a J-domain protein (DnaJ), and an NEF (GrpE). While the main players of the eukaryotic system are conserved, the diversity of the system has been greatly expanded over the course of evolution. As a result, the human genome currently contains more than 13 Hsp70s, 13 NEFs, and close to 50 J-domain proteins (JDPs) (Kampinga and Craig 2010), and this increase in potential partners has generated an enormous number of possible combinations. One reason for this expansion is localization of chaperones in specialized compartments, such as the cytoplasm, nucleus, ER, or mitochondria. Another potential explanation, however, could be the necessity for functional diversity, with this potentially being made possible via different combinations of Hsp70 (each with their own substrate specificities, levels of expression, and post-translational modifications), JDPs, and NEFs.

For instance, through specialized members of the JDP family, Hsp70s can associate with target sites such as ribosomal tunnel exits and membrane translocons, where nascent and translocating substrates emerge. Another example is the mammalian J-protein, auxilin, that is exclusively dedicated to helping Hsp70 dissociate clathrin triskelions. As other JDPs are unable to compensate for loss of auxilin, this suggests that auxilin, and potentially other co-chaperones, evolved to recruit Hsp70s into very specific functions.

The JDP-Hsp70 system, however, does not just act in isolation and often collaborates with other chaperone systems – thereby adding to its many possible roles. In folding or refolding pathways, for example, Hsp70s can hand off substantially unfolded proteins to Hsp90 or Hsp60

chaperonins for final stages of folding or activation. In other cases, Hsp70 systems have even been reported to pass client proteins to other Hsp70 systems in different compartments, thereby helping newly synthesized polypeptides reach their final cellular destination.

To help better understand this complex system of chaperones and their many possible interactions, in the following sections we will describe the key co-chaperones of the core Hsp70 machinery, and its interface with other, downstream chaperone systems.

1.2.1 J-Domain Proteins

J-domain proteins (JDPs, also known as DnaJs or Hsp40 proteins) are essential components of the Hsp70 chaperone system, as they both regulate Hsp70 chaperones by stimulating ATP hydrolysis and play an important role in initial substrate recognition and remodeling (Tiwari et al. 2013). JDPs are conserved in evolution and are defined by the presence of a J-domain, an approximately 70 residue highly conserved region containing four α-helices (Fig. 1.2a). The linker region between helices 2 and 3 is especially well conserved and contains the histidine-proline-aspartic acid (HPD) motif vital for the stimulation of Hsp70 ATP hydrolysis (Tsai and Douglas 1996) (Fig. 1.2a). J-domain proteins have historically been divided into three classes, based on the similarity of their domain arrangement to that of the bacterial DnaJ. Class A J-domain proteins (e.g. bacterial DnaJ, yeast Ydj1, and human DnaJA1–4) share four domains with the bacterial DnaJ: the N-terminal J-domain; a glycine-phenylalanine-rich linker segment of unclear function; two β-sandwich C-terminal domains, CTDI and II, that contain the substrate binding sites; and a zinc-finger like region (ZFLR) incorporated into CTDI (Fig. 1.2b, c). Class B J-domain proteins (e.g. the yeast Sis1, and human DnaJB1,4,5) share the J-domain, GF-rich linker and a C-terminal substrate binding domain (Cheetham and Caplan 1998). Class C J-domain proteins are the most heterogeneous group and share only the J-domain with DnaJ, with additional domains in these JDPs mainly serving to localize Hsp70s to specific organelles or transfer their substrates to downstream chaperones (Kampinga and Craig 2010) (Fig. 1.2b).

The three JDP classes also differ in their client specificities (Kampinga and Craig 2010), with class A and B JDPs, despite many similarities in their mode of substrate binding, recognizing distinct features in amorphous protein aggregates. Human DnaJA2, for example, preferentially binds to and assists solubilization of small aggregates (ca. 200–700 kDa), whereas DnaJB1 prefers larger species (ca. 700–5000 kDa) (Nillegoda et al. 2015, 2017). In contrast, class C J-domain proteins are generally considered to specifically interact with only a limited number of protein substrates or not to interact directly with proteins at all (Kampinga and Craig 2010). Even within the same family, the binding kinetics of different J-domain proteins with their substrates can vary greatly, from very transient to rather stable, with some JDPs having holding capabilities in their own right (e.g. DnaJB6 and DnaJB8, which are capable of efficiently blocking amyloid formation and the aggregation of misfolded proteins) (Szabo et al. 1996; Linke et al. 2003; Hageman et al. 2010; Kakkar et al. 2016). In all, there are approximately 50 different members of the J-domain protein family encoded in the human genome, ranging in size from 10 to 520 kDa (Kampinga and Craig 2010). This variety in size, stemming from a multitude of different domain structures, is also reflected in the functional diversity of the different JDPs.

Functionally, interaction of purified J-domain with Hsp70 chaperones has been shown to be sufficient for the enhancement of Hsp70 ATPase activity, and crucial missing insight into the molecular mechanism of J-domain action was recently provided by the solved X-ray structure of the *E. coli* DnaJ J-domain in complex with the ATP-bound open conformation of DnaK (Kityk et al. 2018). There, the J-domain binds at the interface between NBD and SBDβ, on top of the interdomain linker, forming polar contacts with these two domains and hydrophobic contacts with SBDβ and the linker (Fig. 1.2d). This mode of binding also explains the selective interaction

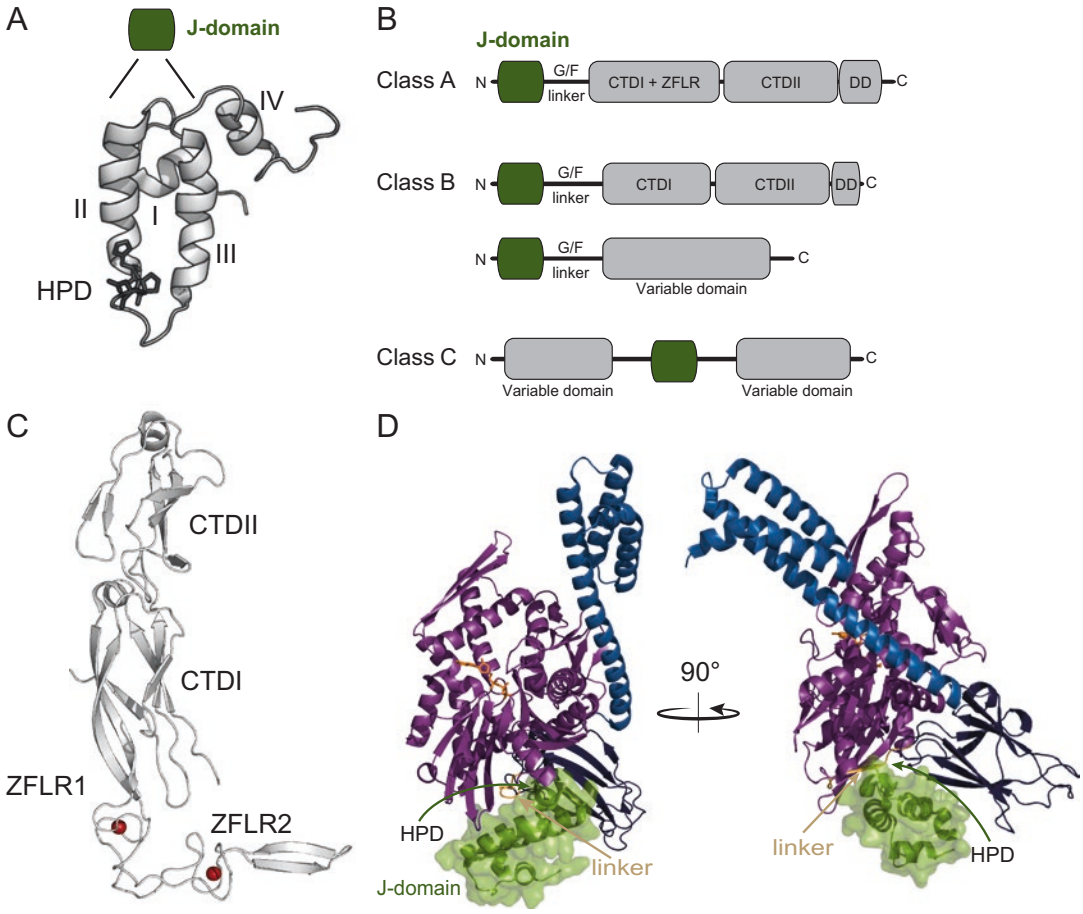

Fig. 1.2 Structure of J-domain proteins and their interaction with Hsp70s. (**a**) Cartoon representation of the J-domain structure of E. coli DnaJ (PDB ID 1XBL (Pellecchia et al. 1996)), highlighting the position of the conserved HPD motif. (**b**) General domain organization of class A (**top**), B (**middle**) and C (**bottom**) JDPs. The different domains are marked as follows: J, J-domain; G/F, Gly-Phe rich region; ZFLR, zinc-finger like region; CTD, C-terminal domain; DD, dimerization domain. (**c**) Cartoon representation of the Zn-binding and the C-terminal domains of S. cerevisiae Type I JDP, Ydj1 (PDB ID 1NLT (Li and Sha 2003)). (**d**) Structure of the J-domain of *E. coli* DnaJ in complex with *E. coli* DnaK, when the latter is in the ATP-bound open conformation (PDB ID 5NRO (Kityk et al. 2018)). The J-domain is shown in surface representation to highlight the contacts between residues of the J-domain and those of DnaK NBD (purple), SBDβ (dark blue), and the conserved linker (light orange)

of JDPs only with ATP-bound Hsp70s, as such an NBD-SBDβ interface is only formed in the ATP-bound conformation of Hsp70, and is broken upon ATP-hydrolysis. This structure also highlighted the fundamental importance of the conserved J-domain HPD motif in JDP activity, showing that the HPD interacts with key residues of the allosteric network in both the Hsp70 linker and NBD crevice, thereby arresting the NBD lobes and catalytic residues in a position optimal for ATP hydrolysis. The J-domain was further found to contact residues in the Hsp70 SBDβ that are connected to the signal pathway from the substrate to the NBD, making this transmission from the SBD to the NBD more efficient. In this manner, the J-domain couples two signals (Kityk et al. 2018) to trigger both ATP hydrolysis, and generate the ultrahigh affinity of the Hsp70 ADP-

bound equilibrium state (De Los Rios and Barducci 2014), leading to efficient trapping of substrates.

Moreover, residues in the J-domain that interact with DnaK, along with the corresponding residues in DnaK that interact with the J-domain, have been found to be highly conserved in evolution, suggesting that this mechanism is operational in all pairings of Hsp70s and J-domain proteins. Interestingly, the conserved J-domain sequences in different JDP paralogs which are thought to interact with the same Hsp70s, can deviate by several residues. This then raises the possibility that modulation within the J-domain sequences may establish a hierarchy of preferences between different JDPs and Hsp70s.

1.2.2 Nucleotide Exchange Factors

Although JDPs are considered the prime drivers of Hsp70 functional diversity, NEFs play an important role as well. The main function of NEFs is to help facilitate the exchange of ADP to ATP, which is done through a direct interaction with the Hsp70 NBD. By mediating the opening of the Hsp70 nucleotide binding cleft, NEFs facilitate the release of ADP, which in turn allows the rebinding of ATP and the subsequent release of substrates. Unlike the JDPs, which share a common J-domain, four evolutionarily unrelated families of NEFs have been identified with no sequence similarity among them. Although all NEFs interact with the Hsp70 NBD, each such family uses different mechanisms to open the Hsp70 nucleotide binding cleft for release of ADP.

In prokaryotes, mitochondria, and chloroplasts, nucleotide exchange in Hsp70s is regulated by GrpE, a homodimeric protein which consists of an N-terminal, unusually long α-helical dimerization domain, and a C-terminal β-sheet domain. The GrpE dimer interacts with a single Hsp70 molecule, inserting its β-sheet domain to literally drive a wedge into the nucleotide binding domain of Hsp70 (Harrison et al. 1997) (Fig. 1.3a, left). This complex formation induces a 14° rotation of subdomain IIB, resulting in an opening of the nucleotide binding cleft incompatible with nucleotide binding (Fig. 1.3a, right). As this interaction also induces asymmetry in the GrpE dimer, only one Hsp70 molecule can be bound at any given time.

The eukaryotic cytosol does not contain GrpE homologs, but rather nucleotide exchange is performed by three main classes of human NEFs: HspBP1, BAG proteins, and Hsp110s. While the classes are structurally distinct, with little or no homology between them, mechanistically, all seem to capture the open conformation of the Hsp70 NBD.

HspBP1/Sil1, which is found in the eukaryotic cytosol (HspBP1) and ER (Sil1), is composed entirely of four alpha-helical Armadillo repeats. The superhelical Sil1 protein wraps around subdomain IIB of the Hsp70 NBD (Fig. 1.3b, left) and rotates this subdomain around one of its helices (helix 7) (Yan et al. 2011) (Fig. 1.3b, right). Similarly to the role of GrpE in prokaryotes, the HspBP1-type NEFs seem to support the canonical chaperone actions of Hsp70 machines, from stress-related protein refolding to ER-associated degradation (ERAD) (Travers et al. 2000). HspBP1 also functionally resembles GrpE, in that both were shown to prevent unproductive rebinding of the released substrate (Gowda et al. 2018; Rosam et al. 2018), which occurs as dedicated structural elements within the NEFs that mimic motifs recognized by Hsp70s (e.g. RD of Fes1/HspBP1 (Gowda et al. 2018; Rosam et al. 2018) and possibly the N-terminal helical extension in GrpE (Harrison et al. 1997; Wu et al. 2012)) occupy the substrate binding sites on the chaperone (Fig. 1.3c).

BAG proteins form the second class of Hsp70 nucleotide exchange factors, and contain a conserved 110–124 residue long three-helix bundle BAG (Bcl2-associated athanogene) domain (Takayama et al. 1999) that binds to the subdomains IB and (mainly) IIB of Hsp70 NBD (Fig. 1.3d, left). This interaction of the BAG domain with Hsp70 locks the NBD in a conformation very similar to DnaK in complex with GrpE, with subdomain IIB tilted outward by 14°

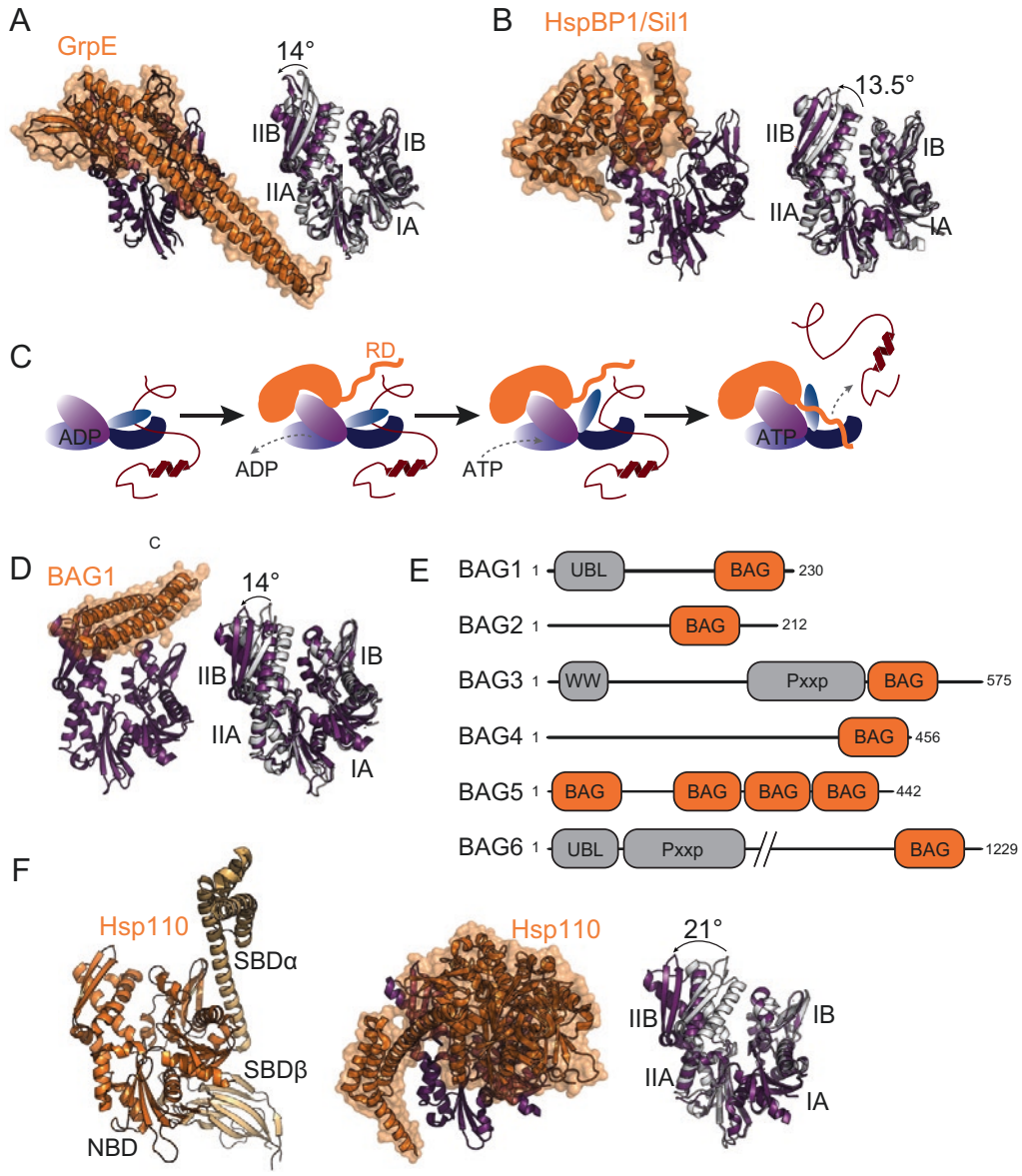

Fig. 1.3 Structures and mechanisms of nucleotide exchange factors. Exemplary structures of the four NEF families in complex with their respective Hsp70s, and the changes in the nucleotide binding domains of those Hsp70s following NEF binding. Crystal structures of (**a**) **Left:** GrpE in complex with DnaK-NBD (1DKG (Harrison et al. 1997)), (**a**) **Right:** Changes in DnaK NBD structure following GrpE binding (purple) overlaid on structure of unbound DnaK-NBD (white) to highlight the relative motion of the NBD I and II lobes upon NEF binding. (**b**) Same representations as in (A) for HspBP1 and Hsp70-NBD (3QML (Yan et al. 2011; Shomura et al. 2005)). (**c**) HspBP1 favors substrate release by preventing rebinding of the substrate after nucleotide exchange. From left to right: The Armadillo domain binds to subdomain IIB show to open the nucleotide binding cleft; after ATP binding, opening of the substrate binding cleft, and substrate dissociation; the N-terminal unstructured segment (RD, orange) of HspBP1 binds into the substrate binding pocket preventing rebinding of the substrate. (**d**) Structure of Bag1 in complex with Hcs70-NBD (1HX1 (Sondermann et al. 2001)), layout and colors are as in (A). (**e**) Domain organization of human BAG family proteins. All six reported BAG proteins contain a BAG domain at their C-terminus (orange). Some BAG proteins contain other domains, including the ubiquitin-like (UBL) domain, WW domain, and proline-rich regions (PXXP). Numbers to the right of the linear peptide sequence indicate lengths of the proteins. (*f*) Structural organization of Hsp110 NEF showing the homologies to Hsp70 NBD, SBD and Lid domains (left). Structure of Hsp110 (Sse1p; orange) in complex with Hsp70 (purple) (3D2F (Polier et al. 2008)) (middle) and an overlay of Hsp70 NBD structure with (purple) and without Hsp110 (white) to highlight the relative motion of the NBD I and II lobes upon Hsp110 binding (right)

(Sondermann et al. 2001) (Fig. 1.3d, right). In addition to this conserved domain, Bag domain proteins also contain a number of additional interaction domains, through which these NEFs can (1) be localized to specific subcellular structures, (2) ensure precise timing and targeting of nucleotide exchange, and (3) allow for timed transfer of Hsp70 substrates to other complexes (Fig. 1.3e). Such a mechanism, for example, can be found for BAG1, which contains an integral ubiquitin-like (UBL) domain that can serve as a proteasomal targeting signal, promoting the transfer of Hsp70-bound client proteins to the proteasome for degradation (Luders et al. 2000). Another NEF, BAG3, triggers the recruitment of the autophagic ubiquitin adaptor p62, and thus facilitates Hsp70-assisted substrate degradation through the autophagosome-lysosome pathway.

Hsp110 proteins, members of the third class of human NEFs, were initially grouped as Hsp70 family members because of similarities in sequence. Like Hsp70s, they consist of an N-terminal nucleotide binding domain (NBD) that is connected to a peptide-binding domain (in this case, a nine-stranded β-sandwich) by a flexible linker and an alpha-helical lid (SBDα) (Liu and Hendrickson 2007) (Fig. 1.3f, left). Hsp110s catalyze nucleotide exchange by a head-to-head interaction of their NBD with the NBD of Hsp70s, attaching to the side of subdomain IIB, while anchoring to the remainder of the NBD (Polier et al. 2008; Schuermann et al. 2008) (Fig. 1.3f, middle) and tilting subdomain IIB outward in a manner similar to GrpE (Fig. 1.3f, right). Intriguingly, some Hsp110s, similarly to Hsp70s, can directly bind unfolded proteins and prevent their aggregation. Moreover, Hsp110s are essential components in the human disaggregation machinery and cannot be replaced by any other class of NEF (Nillegoda et al. 2015). While Hsp110s display both structure and sequence similarity to canonical Hsp70s, and have even shown some level of ATPase activity, they cannot, however, employ a nucleotide-dependent, peptide-binding release cycle (Brodsky et al. 1999; Garcia et al. 2017).

1.2.2.1 Nucleotide Cycle Regulation Beyond JDPs and NEFs

Several factors have been identified, which, although not universal and thus not part of the core Hsp70 machinery, affect Hsp70 ATP hydrolysis and ADP release. The best studied factor, Hip (also known as p48), was identified over a decade ago as a protein that preferentially binds to and stabilizes the ADP-bound state of Hsp70 (Hohfeld et al. 1995). Hip competes with the BAG1 NEF for binding to Hsp70s NBD, thereby slowing down the nucleotide release and extending the time for which client proteins remain bound (Li et al. 2013). This prolonged substrate residence on Hsp70, mediated by Hip, may serve to prevent aggregation, as increased levels of Hip have been shown to reduce pathologic protein aggregation associated with Parkinson's disease (Roodveldt et al. 2009). Furthermore, through its tetratricopeptide repeat (TPR) domain, Hip can also coordinate between Hsp70s and additional cellular chaperones and factors. Thus, Hip protein may serve as an additional layer in the regulation of protein quality control, on top of the multiple isoforms of J proteins and different types of NEFs in the cell.

1.3 Hsp70s Interaction with Other Cellular Chaperone Systems

The Hsp70 chaperone machinery often doesn't act alone, but rather works in concert with other chaperone machines, such as the cytosolic AAA+ disaggregases, Hsp90 chaperones, and small heat shock proteins. This wide array of possible interactions generates a multitude of possible outcomes, where the mutually exclusive binding of Hsp70 to a specific co-chaperone essentially dictates the fate of its substrate.

One such example is the interaction of Hsp70, through its intrinsically disordered C-terminal EEVD amino acids, to TPR-domain co-chaperones, Hop and CHIP. Hop co-chaperone (Hsp70/Hsp90 organizing protein) can simulta-

neously bind Hsp70 (through its TRP1 domain) and Hsp90 (through the TPRA2 domain), thus serving as a bridge and facilitating the efficient transfer of substrates between these two chaperone systems (Scheufler et al. 2000). CHIP (carboxyl terminus of Hsc70 interacting protein) also interacts with the Hsp70 EEVD tail through its N-terminal TPR domain. However, unlike the folding function associated with Hop, the CHIP-Hsp70 interaction facilitates ubiquitination of Hsp70-bound substrates, thereby targeting these clients for proteasomal degradation (McDonough and Patterson 2003). Thus, when Hop and CHIP, through their TPR domains, compete for binding to the Hsp70 C-terminal tail, for the substrate it is truly a matter of life (folding) or death (degradation).

The conserved EEVD motif in the Hsp70 CTD has further been proposed to interact with additional proteins, including a receptor on the mitochondrial outer membrane (Li et al. 2009), and class B JDPs (Yu et al. 2015), hinting at further, yet to be discovered functions. Although the EEVD motif is present in all cytosolic eukaryotic Hsp70s, it is not found in the mitochondrial, or ER-resident Hsp70 isoforms, or in the prokaryotic DnaK. This raises many interesting questions about how and why the domain, and its associated functional versatility, evolved.

1.3.1 Hsp70 Chaperones in Protein Disaggregation

Another remarkable example of Hsp70 cooperating with, or rather being hijacked by, other cellular chaperones, is the protein disaggregation system. The seminal work on the yeast Hsp104 disaggregase from the Lindquist laboratory showed that Hsp104 chaperone, working in collaboration with the Hsp70 system, can disassemble aggregated proteins both *in vivo* (Parsell et al. 1994) and *in vitro* (Glover and Lindquist 1998). The Hsp104 hexamer is the main engine that couples ATP hydrolysis to generate the mechanical force needed for solubilizing protein aggregates, but it does not exhibit disaggregation activity on its own and strictly requires the cooperation of Hsp70 chaperone systems (Glover and Lindquist 1998; Mogk et al. 1999, 2015). The disaggregation reaction is, in fact, initiated by Hsp70 binding to the surface of protein aggregates, and only then followed by subsequent recruitment of the Hsp104 disaggregase (Winkler et al. 2012; Acebron et al. 2009). While Hsp70s can promiscuously bind to different kinds of protein aggregates, their disaggregation potential is very limited. In this sense, Hsp104s represent specialized Hsp70 partner chaperones that expand the capabilities of the Hsp70 machinery to include protein disaggregation. In addition to targeting Hsp104s to protein aggregates, Hsp70 activity is also required for activation of the substrate-unraveling function of the hexamer (Carroni et al. 2014; Rosenzweig et al. 2013). There, a direct interaction between Hsp70 NBD and the Hsp104 coiled-coil middle domains (CCDs) releases the repression of Hsp104 threading activity, which is caused by head-to-tail interactions of neighboring CCDs in the Hsp104 hexameric ring (Carroni et al. 2014; Oguchi et al. 2012; Heuck et al. 2016). Through this protective, Hsp70-regulated mechanism, nonspecific activation of Hsp104 is prevented, along with any deleterious and uncontrolled unfolding of cellular proteins (Oguchi et al. 2012; Lipinska et al. 2013). And while the Hsp70-Hsp104 interaction occurs at the same site in the Hsp70 NBD as NEF binding, the outcomes are entirely different – serving as a prime example of the versatility of the Hsp70 system, and how, through interaction with different cellular machineries, it can be tuned to very different cellular functions.

1.4 Conclusion and Perspectives

As illustrated in this text, the Hsp70 chaperone system plays a key part in a myriad of cellular functions, and considerable progress has been made in recent years in elucidating the structural and mechanistic basis for the chaperone functions and their interaction with co-chaperones. Despite this, though, many questions still remain. It is still unclear, for example, what is the role of the SBDα lid domain in the Hsp70-substrate

interaction. Further questions include how JDP co-chaperones target their Hsp70 partner proteins to substrates, as well as the extent of substrate remodeling performed by the JDPs themselves. Moreover, with such a wide array of possible Hsp70 functions, perhaps the most important unresolved issues regarding this mechanism are what factors govern the pathway decisions which direct Hsp70 substrates to sequestration, refolding, or degradation by the ubiquitin proteasome system and autophagy; and critically, how various stress and disease states impact these vital decisions.

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