Review Article

Heat Shock Protein Network: the Mode of Action, the Role in Protein Folding and Human Pathologies

(HSP / protein folding / chaperone / aggregation / neurodegenerative disease / cancer)

ALEKSANDR MELIKOV, PETR NOVÁK

BIOCEV, Faculty of Science, Charles University, Prague, Czech Republic
BIOCEV, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic

Abstract. Protein folding is an extremely complicated process, which has been extensively tackled during the last decades. *In vivo*, a certain molecular machinery is responsible for assisting the correct folding of proteins and maintaining protein homeostasis: the members of this machinery are the heat shock proteins (HSPs), which belong among molecular chaperones. Mutations in HSPs are associated with several inherited diseases, and members of this group were also proved to be involved in neurodegenerative pathologies (e.g., Alzheimer and Parkinson diseases), cancer, viral infections, and antibiotic resistance of bacteria. Therefore, it is critical to understand the principles of HSP functioning and their exact role in human physiology and pathology. This review attempts to briefly describe the main chaperone families and the interplay between individual chaperones, as well as their general and specific functions in the context of cell physiology and human diseases.

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Corresponding author: Petr Novák, BIOCEV, Faculty of Science, Charles University, Czech Republic, Prumyslova 595, 252 50 Vestec, Czech Republic; Phone and Fax: (+420) 325 873 610; e-mail: pnovak@biomed.cas.cz

Abbreviations: ADP – adenosine diphosphate, ATP – adenosine triphosphate, ER – endoplasmic reticulum, GGA – geranylgeranylacetone, HSF1 – heat shock factor 1, HSP60/70/90/100 – heat shock protein 60/70/90/100, JDP – J-domain protein, MAPK – mitogen-activated protein kinase, MTs – microtubules, MW – molecular weight, NBD – nucleotide-binding domain, NEF – nucleotide exchange factor, NFTs – neurofibrillary tangles, PDB – protein data bank, PTM – post-translational modification, SBD – substrate-binding domain, sHSPs – small heat shock proteins, UPR – unfolded protein response.

Introduction

The biological function of a protein can be fulfilled only in the case when it forms a correct tertiary structure, i.e., when the polypeptide chain adopts the native spatial configuration by reaching one of the thermodynamic energy minima. For a given protein chain there are always multiple ways to fold into the native state (Dobson et al., 1998). Several free energy landscape models have been proposed to depict the process of folding based on biophysical observations and calculations (Dill et al., 1995). One of these models, the "rugged" landscape, considers the existence of multiple energy minima; intermediate folded states of a protein can adopt these energy minima during the folding process (Dill et al., 1995). This type of spatial energy diagram is widely accepted due to the existence of a huge number of possible short- and long-distance interactions in a protein, which cannot be all satisfied at once, therefore creating a palette of folding intermediates and partially folded protein species (Bryngelson et al., 1995; Ferreiro et al., 2007). Partially folded protein conformations are often highly prone to aggregation, which results in formation of amorphous aggregates, inactive oligomers or, in extreme cases, amyloid fibrils observed in neurodegenerative diseases (Jahn and Radford, 2005). All these protein aggregates are extremely toxic and dangerous to cells and are involved in cell aging and death (Stefani and Dobson, 2003). The critical role in preventing these aggregation events and facilitating correct folding of proteins is played by molecular chaperones.

The term "molecular chaperones" was first used to describe proteins that assist the correct folding of cellular proteins or protein complexes but are not included into the final protein assembly (Ellis, 1987). Later, this definition was updated to the following: "molecular chaperone is a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations" (Hendrick and Hartl, 1993).

A chaperone must bind to the structural motifs of an unfolded protein that are not present in its final native structure (in case of an oligomeric complex, chaperones bind and stabilize its subunits before they associate). These motifs are exposed to the environment, and therefore can be bound by chaperones. An increased proportion of partially folded or unfolded proteins is often observed in stress conditions, such as heat shock or oxidative stress. Consequently, enhanced expression of certain chaperones in hyperthermal conditions was detected, and those heat-induced chaperones and their homologues acquired the name "heat shock proteins" (HSPs) (Nover and Scharf, 1984). HSPs are not the only chaperones present in cells; in fact, numerous other proteins are engaged in the correcting or facilitating protein folding. For instance, protein disulphide isomerases (PDIs) catalyse reformation of disulphide bonds; peptidyl-prolyl *cis/trans*-isomerases are responsible for isomerization of prolyl residues in client proteins; calnexin and calreticulin trim glycan chains on proteins in endoplasmic reticulum (ER) and participate in folding quality control in this compartment (Raina and Missiakas, 1997; Göthel and Marahiel, 1999; Williams, 2006). However, this review will focus specifically on the heat shock proteins.

To classify various types of HSPs, the values of their molecular weight in kilodaltons (kDa) were used, and HSP classes such as HSP70, HSP60, HSP90 emerged. It was then discovered that some of these proteins are present not only in the cytoplasm but also in other cell compartments (Munro and Pelham, 1986; Mizzen et al., 1989). In the work of Munro and Pelham, it was proposed that HSPs act via binding hydrophobic regions of unfolded or partially folded proteins exposed to the solvent, consequently releasing them and allowing them to fold in a native manner. In Fig. 1, this process of "refolding assistance" is depicted in the context of the protein folding energy landscape discussed earlier. Before a

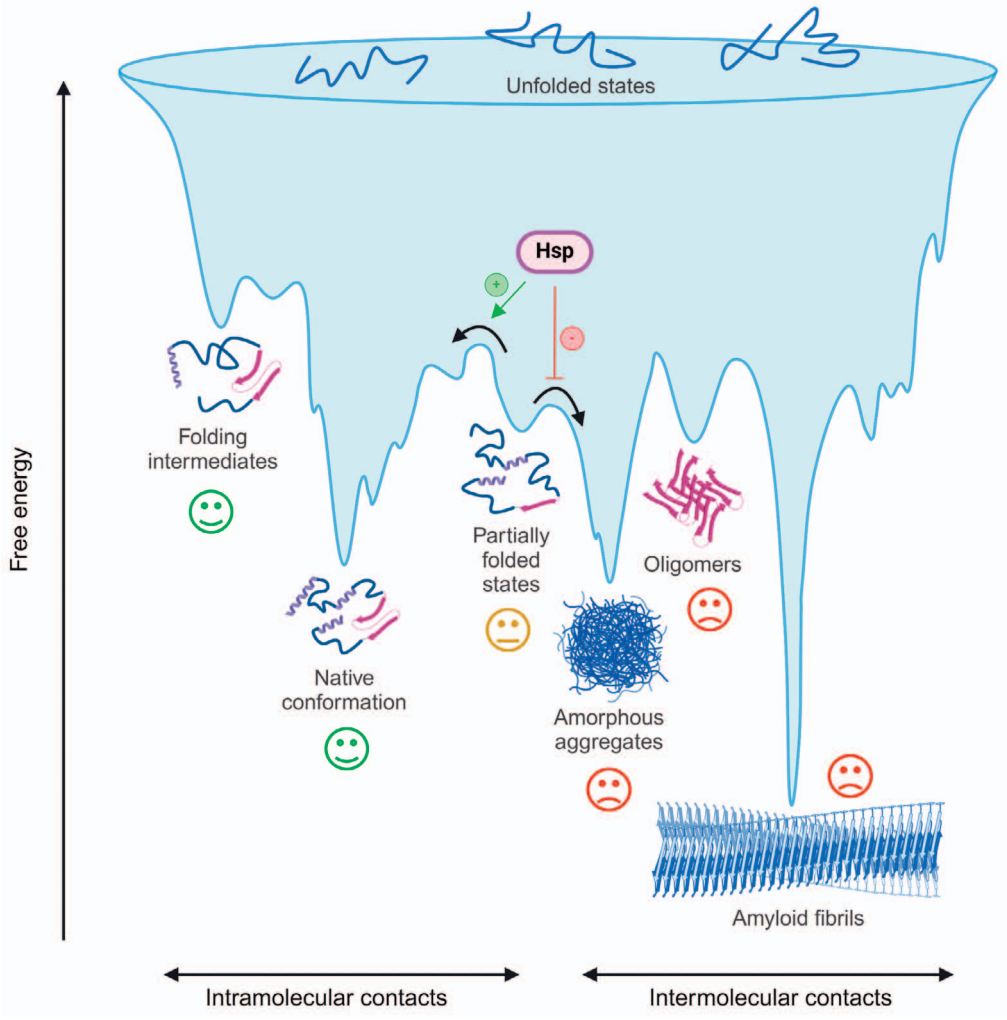

Fig. 1. The protein folding energy landscape. The high-energy unfolded states can have several scenarios: they either follow the correct folding pathway through one or several folding intermediates or become partially folded and eventually form aggregates or amyloid fibrils. Molecular chaperones act via binding of partially folded states and preventing aggregation events; then, a chaperone releases the bound substrate and allows it to refold. In addition, some chaperones (foldases) directly catalyse the folding reaction. Adapted from Hartl et al. (2011).

partially folded protein falls into an energy trap and participates in an oligomer or amorphous aggregate formation, molecular chaperones bind it to either provide enough time for refolding or prevent its aggregation. Based on these distinctions, the HSPs which catalyse the correct folding of client proteins (i.e., the transition from the unfolded state to the native state) were defined as *foldases* (for instance, HSP70, HSP60 and HSP90 families). The second type of HSPs, which bind the unfolded states and prevent or slow down their aggregation, are *holdases* and can be exemplified by small HSPs (sHSPs) (Santra et al., 2017).

During the polypeptide synthesis on ribosomes, the *de novo* folding is crucial for the correct functioning of the protein. Therefore, some HSPs are expressed constitutively: e.g., a member of the HSP70 family, HSPA5 (alias GRP78/BiP), which facilitates the translocation and the subsequent folding of newly translated secretory proteins in ER (Haas and Wabl, 1983; Gething and Sambrook, 1992). Several types of such "housekeeping" HSPs are also directly associated with ribosomes and facilitate *de novo* protein folding; they are discussed in a later chapter (see "*Ribosome-Associated Chaperones"*).

The crucial factor for the correct function of HSPs is the regulation of their working cycle: as a chaperone molecule binds to a target substrate, a mechanism for the substrate release must exist. This "switch" between the different states of a chaperone is typically provided by adenosine triphosphate (ATP). Such chaperones belong to the ATP-dependent group (represented by foldases including HSP70, HSP60 and HSP90), which can directly bind and hydrolyse ATP (Flynn et al., 1991; Todd et al., 1994; Panaretou, 1998). On the other side, there is a seemingly ATP-independent group of molecular chaperones acting without direct macroergic bond hydrolysis by the HSP molecule. Small HSPs such as bacterial trigger factor, Spy-protein and SecB can be named as examples (Mitra et al., 2022). However, sHSPs cannot release the bound protein substrates and require the ATP hydrolysis indirectly through HSP70 (see chapter "*Small HSPs*"). In addition, the functional cycle of chaperones often requires an interaction of additional protein cofactors, or co-chaperones, which present the substrate, facilitate its binding, or stimulate the ATPase activity of HSPs.

The HSP superfamily is very diverse in the sense of action mode (holdases, foldases), requirements of cofactors (ATP, co-chaperones) and structural organization. In the following paragraphs, these aspects will be discussed in more detail, as well as their role in human physiology and pathology.

Major Chaperone Classes

HSP60 Chaperonins

The HSP60 (also known as "chaperonin") family is represented by large oligomeric complexes. The name "chaperonin" was introduced to describe oligomeric complexes with the chaperone function, which were initially identified in bacteria and plants (Hemmingsen et al., 1988). Each HSP60 monomer has approximately 60 kDa (therefore HSP60). These complexes are symmetric and consist of two rings, each of which is made up of seven or eight subunits and forms a cavity capable of binding exposed hydrophobic regions of a client protein and facilitating its folding process through the ATP-driven cycle (Fig. 2). Chaperonins are ubiquitous and were identified in all three domains of life (Horwich et al., 2007). Multimeric chaperonin complexes are characterized by positive allosteric coupling of subunits within a ring, meaning that when one of the subunits within the ring binds an ATP molecule, the other ring subunits bind ATP much faster than they would do separately. However, between the two rings, there is a negative allostery – when one ring binds an unfolded protein client, the second one is blocked. That is why it is possible to distinguish between the *cis*- (extended, with a client polypeptide inside) and *trans*-ring (no client bound) (Fig. 2A). The switch between the *cis*- and *trans*-state of the ring occurs in an ATP-dependent manner: the binding of a client protein and ATP molecules to a *cis*-ring triggers the conformational change and simultaneous ATP hydrolysis. This highly coordinated allostery allows chaperonins to rapidly proceed in their working cycle (Rye et al., 1997).

All HSP60 complexes can be divided into two groups, based on their structural arrangement, as type 1 or type 2 chaperonins.

Type 1 chaperonins are present in bacterial cytosol and in mitochondria and plastids of eukaryotes; each of the rings consists of seven HSP60 monomers; therefore, the whole complex is a homo-14-mer. Type 1 chaperonins require a co-chaperone, HSP10. The most studied representative is the bacterial GroEL-GroES complex (Fig. 2B; Xu et al., 1997). The human HSP60 and HSP10 orthologues (i.e., HSPD1 and HSPE1, respectively) are present in mitochondrial matrix and strongly resemble their bacterial counterparts. The HSPD1-HSPE1 system fulfils the general chaperonin function in assisting folding and preventing aggregation in the mitochondrial matrix. Interestingly, mutations in the genes encoding HSPD1 and HSPE1 cause hereditary paraplegia (neurodegenerative disease affecting lower limb mobility) and diabetes-induced renal tubular dysfunction (Hansen et al., 2002; Aluksanasuwan et al., 2017).

Type 2 chaperonins were identified in eukaryotic and archaeal cytosol, their complexes are hetero-16-mers (two octameric rings of eight different subunits) and they do not require HSP10 as a co-chaperonin. In humans, the major representative of this group is the CCT complex (also known as TRiC, or TRiC/CCT); its subunits are encoded by genes *CCT1–CCT8*. In Figure 2C, the structural model of the human TRiC/CCT complex in the open conformation (ATP-bound) is depicted. When eight ATP molecules are bound, each monomer on the one side (*cis*-ring) extends its apical domain, so

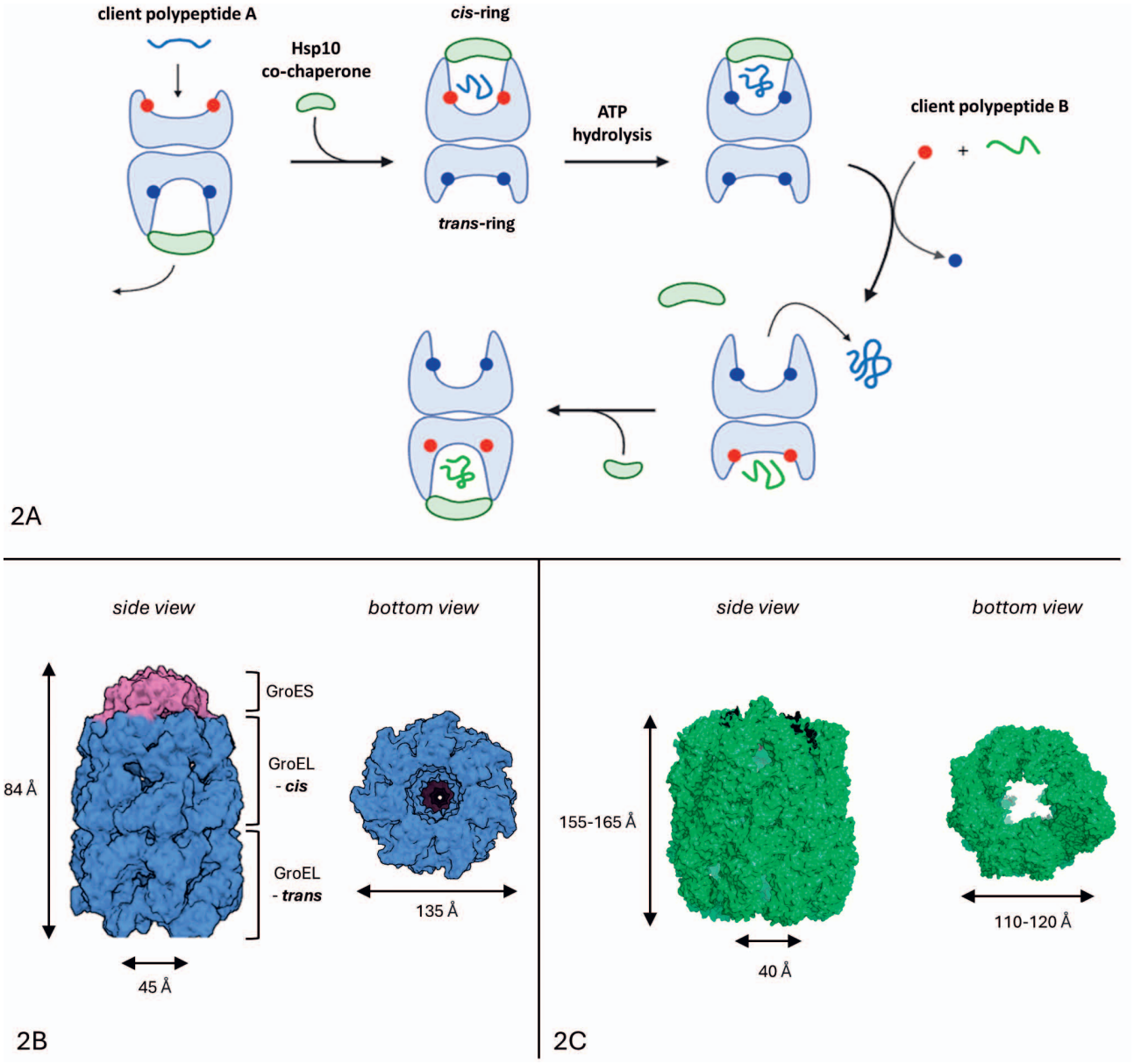

Fig. 2. HSP60. (**A**) The working cycle of chaperonin (as an example, type 1 HSP60, GroEL-GroES complex is depicted). After the *cis*-ring is bound to seven ATP molecules, the cavity is ready to bind unfolded client protein A and changes the conformation. The Hsp10 molecule (GroES) then docks onto the *cis*-ring and closes the aperture as "a lid", allowing the ATP hydrolysis and folding catalysis. Then, the simultaneous exchange of ADP for ATP in the *trans*-ring stimulates the dissociation of Hsp10 and the folded client protein A. The *trans*-ring now becomes the new *cis* and can bind the client protein B. (**B**) The structure of the bacterial GroEL-GroES complex (PDB code 1AON). (**C**) The structure of the human TRiC/CCT complex, type 2 HSP60 (here in ATP-state) (PDB code 6NR8). Adapted from Horwich et al. (2007).

the overall conformational change results in the "lid" opening (the mechanism similar to a camera aperture opening/closing) (Ditzel et al., 1998, Pappenberger, 2002). In contrast to this, type 1 HSP60s do not possess this apical domain; instead, they require an external "lid", which is HSP10. Initially, TRiC/CCT was discovered to be crucial for the correct folding of actin and tubulin; however, it was later demonstrated that approximately 10 % of all newly translated proteins undergo TRiC-mediated folding (Gao et al., 1992; Yaffe et al., 1992; Thulasiraman et al., 1999). TRiC has an important role in protecting the cell from protein aggregation: mutations in TRiC/CCT lead to affecting axonal transport in distal neurons, leading to sensory neuropathy (Bouhouche et al., 2005; Min et al., 2014). This chaperonin is probably also involved in Alzheimer's disease, as decreased levels of TRiC/CCT mRNA were detected in brain tissue samples of AD patients (Brehme et al., 2014).

HSP70

One of the most abundant and well-studied chaperone systems, HSP70, was identified in the cytosol of bacteria, certain archaea (*Thermotoga maritima, Aquifex pyrophilus*) and in various compartments of eukaryotic cells (Lindquist and Craig, 1988; Michels et al., 1997; Pockley et al., 1998; Gribaldo et al., 1999; Gehrmann et al., 2005). The main difference between HSP70 and the HSP60 system discussed above is that HSP70 works as a monomer. However, recent data suggest the existence of an HSP70 dimer; nonetheless, its exact role in the HSP70 function is to be investigated (Mayer, 2021). All HSP70s share a high homology level and sequence identity, not lower than 40 % (Lindquist and Craig, 1988). In both the bacterial and eukaryotic genomes there are several Hsp70s encoded, for instance, by three genes in the *E. coli* K-12 strain and at least 13 in *H. sapiens* (Genevaux et al., 2007; Radons, 2016). Human HSP70 representatives differ in their subcellular localization and expression levels. Most of them (HSPA1L, HSPA2, HSPA5, HSPA8, HSPA9, HSPA12A, HSPA12B and HSPA13) are constitutively expressed and fulfil "housekeeping" functions (i.e., functions unrelated to cellular stress) (Radons, 2016).

These functions include:

a) *De novo* posttranslational or co-translational protein folding in cooperation with other ribosome-associated chaperones (for instance, BiP/HSPA5 assists translation on the ER membrane) (Haas and Wabl, 1983).
b) Assembly and disassembly of oligomeric complexes (for instance, HSPA8 (alias HSC70) disassembles clathrin coats from the intracellular vesicles) (Chappell et al., 1986).
c) Protein protection from early proteolytic degradation by shielding ubiquitination sites on the proteins (Rosenzweig et al., 2019).
d) Facilitating the membrane translocation of proteins into organelles. For example, HSPA8/HSC70 assists the import of polypeptides into mitochondrial matrix (Craig, 2018).

On the other hand, expression of five other human HSP70 homologues (HSPA1A, HSPA1B, HSPA6, HSPA7 and HSPA14) is induced by cellular stress, namely heat shock, lack of nutrients or oxidative stress (Lindquist and Craig, 1988; Radons, 2016). Their stress-related activities comprise refolding of misfolded proteins; protein targeting to degradation; impeding protein aggregation or disaggregation, and other processes (Rosenzweig et al., 2019).

The general domain organization of HSP70 can be explained on the example of well-studied bacterial homologue DnaK. The 66.6 kDa protein consists of two distinctive domains. The larger 45 kDa N-terminal domain is named nucleotide-binding domain (NBD) and binds and hydrolyses ATP. The smaller 15 kDa C-terminal substrate-binding domain (SBD) is responsible for client polypeptide binding. A disordered C-terminal tail of variable length protrudes from SBD (Rosenzweig et al., 2019). Binding of a single ATP molecule to the NBD entails a drastic conformational change in SBD: the α-helical lid of SBD gets opened, whereby a client protein can fit into the SBD cleft (Fig. 3).

The ATPase cycle of NBD is highly connected to the SBD, the second major domain capable of binding exposed hydrophobic regions of unfolded polypeptides (Morshauser et al., 1995). These two functionally unrelated domains are joined by a short linker, essential for the HSP70 cycle. The linker allosterically couples the ATP binding and hydrolysis performed by the NBD to the substrate binding and release executed by SBD. In the ADP-state of HSP70, the linker remains disordered, so that the NBD and SBD are mobile relative to each other. However, upon ATP binding, the linker docks onto NBD, forming a much less flexible conformation (Fig. 3; Vogel et al., 2006). The disordered C-terminal tail contains a conserved motif (sequence EEVD) interacting with the short sequence present in many HSP70 protein cofactors (Mayer and Bukau, 2005).

The allostery cycle is highly dependent on several proteins, which mediate substrate binding and stimulate the ADP/ATP exchange or ATP hydrolysis. Ones of the most extensively studied classes of such proteins are J-domain proteins (JDPs/HSP40) and nucleotide-exchange factors (NEFs). JDPs are homodimeric proteins, which interact with both NBD and SBD through their N-terminal J-domain, promoting NBD activity upon the substrate binding to SBD (Kityk et al., 2018). The promotion of ATP hydrolysis is tightly coupled to this role of JDP, since together they represent an integrated stimulus leading to the conformational switch of HSP70 from low-affinity to high-affinity state (Fig. 3). Moreover, JDPs serve as a "substrate scanner", preselecting client polypeptides for HSP70 (Jiang et al., 2019). There are three types of JDPs encoded in humans (types A, B and C), which are distinguished by their affinity to different HSP70 and different substrates. Another class of proteins important for the HSP70 cycle are NEFs: they accelerate the exchange of ADP for ATP, and therefore the SBD lid opening and substrate release. In general, there are four non-homologous types of HSP70 NEFs: GrpE type, present in bacterial cytosol, mitochondria and plastids, and three other classes functioning in eukaryotic cytosol (Bag, Hsp110 and Armadillo families); they differ in the specificity for HSP70 homologues. It is noteworthy that HSP60 chaperonins (see previous chapter) extensively interact with HSP70 and work in conjunction. The transfer of newly translated polypeptide clients from ribosomes to DnaK (bacterial HSP70) and consequently to GroEL-GroES was observed in bacteria (Teter et al., 1999). In a similar fashion, the interaction between eukaryotic HSP70 and TRiC/CCT and the sequential substrate transfer from HSP70 to HSP60 was described (Frydman et al., 1994; Frydman and Hartl, 1996). *In vivo*, TRiC/CCT is commonly associated with prefoldin, an ATP-independent hexameric co-chaperone, which targets client proteins to the TRiC

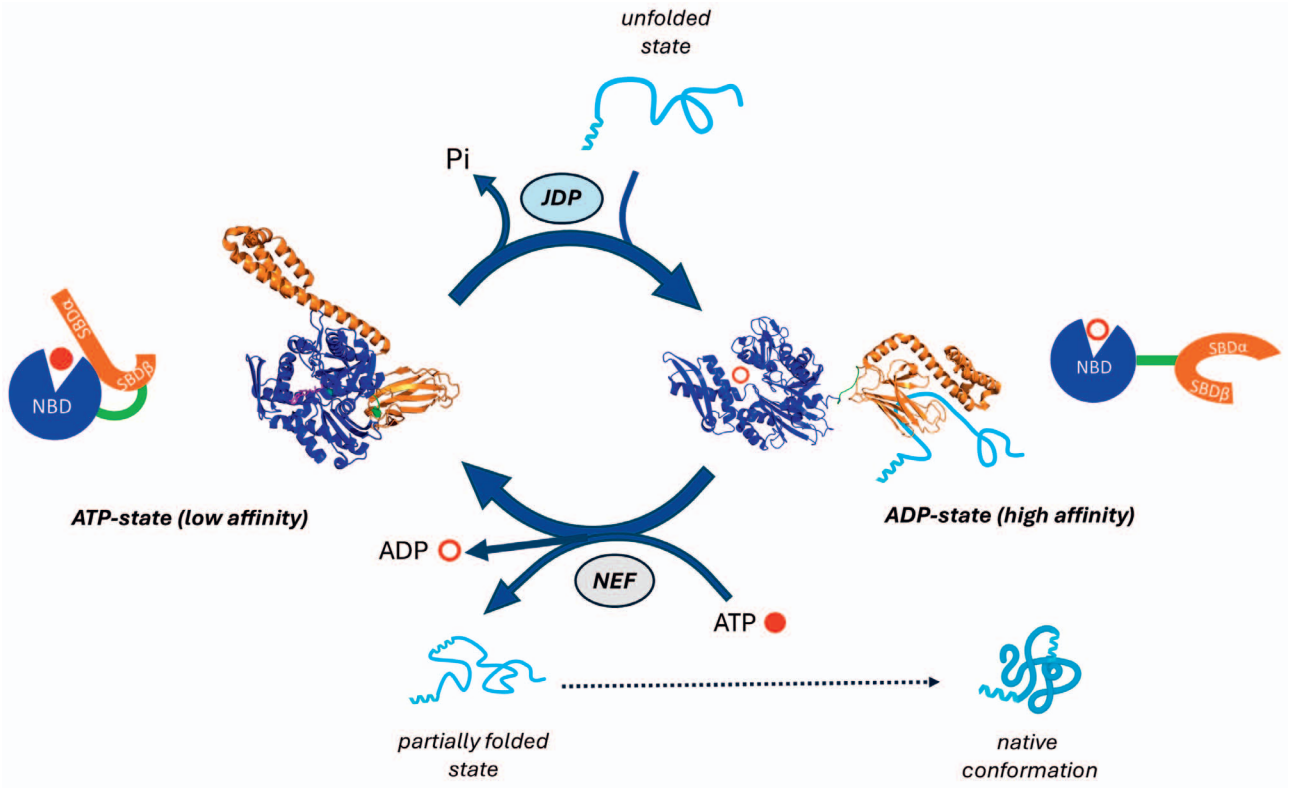

Fig. 3. The structure and allosteric cycle of HSP70. In the low-affinity, ATP-bound state (PDB code 4B9Q), HSP70 is ready to bind a misfolded/unfolded protein substrate. The binding of the substrate is coupled to the ATP-hydrolysis event; the whole step is facilitated by a JDP. Once the lid is closed and the client is bound to SBD (high-affinity, ADP-bound state, PDB code 2KHO), the ADP/ATP exchange enhanced by NEFs is required for the substrate release and the Hsp70 conformational switch. The released partially folded client is therefore able to proceed in the native folding pathway and acquire its functional form. Based on Rosenzweig et al. (2019).

aperture (Vainberg et al., 1998; Gestaut et al., 2019). The possible reason for this directionality of substrate transfer may be the much quicker binding of HSP70 molecules to the unfolded protein; the potential time required for a polypeptide to enter the HSP60 folding chamber is much longer. In the context of ribosome-associated HSP70, which will be discussed later (see chapter *"Ribosome-Associated Proteins"*), the specific HSP70-HSP40 complex (eukaryotic RAC) is physically associated to ribosomal subunits and the exit tunnel. This ensures that the exiting translated protein is firstly bound by RAC and only then possibly transferred to a chaperonin complex (Otto et al., 2005).

As mentioned earlier, different human HSP70 representatives have specific roles in various cellular processes. For example, HSPA8/HSC70 is a constitutively expressed cytosolic foldase, which also acts as an uncoating enzyme for clathrin-coated vesicles, in concert with protein auxillin (JDP). Disrupted interaction between HSC70 and auxillin leads to impaired recycling of synaptic vesicles in axons (Chappell et al., 1986; Augustine et al., 2006). Additionally, HSPA8 participates in membrane translocation, i.e., the protein transport into cellular organelles, by binding and keeping the proteins to be translocated in unfolded state, a condition required for the translocation through the membrane transporters (Sheffield et al., 1990). HSPA8 also plays a role in targeting proteins to the ubiquitin-proteasome degradation pathway, exposing the recognition site on client proteins to E3-ligase (Bercovich et al., 1997). Finally, HSPA8 enables a switch to "heat-shock response" under heat stress: HSPA8 enters the nucleus, and binds to and modulates the activation of transcription factor HSF1 (Ahn et al., 2005). HSF1 is responsible for the induction of heat shock-related genes, for instance, *HSPA1A* and *HSPA1B*. These chaperones share the general HSP70 mode of action, i.e., carrying out the refolding of heat-denatured cell proteins in the cytosol (Radons, 2016). Another example of specific functions carried out by HSP70s is protein HSPA12B, constitutively expressed in human endothelial cells. HSPA12B was shown to be indispensable for angiogenesis in humans (Hu et al., 2006). Therefore, the expression of certain HSP70s is tissue- and context-specific in humans, illustrating the functional diversity of this protein family.

There are numerous observations of tumour cells exhibiting elevated levels of heat-inducible HSP70s. For instance, they were detected in patients with acute mye-

loid leukaemia, colorectal, breast, endometrial, uterine, cervical and bladder cancers (Hwang et al., 2003; Thomas et al., 2005; Ciocca and Calderwood, 2005). The cancer cells might use the HSP70 system as a protective mechanism against stress conditions; however, the exact roles are still under scientific investigation. Besides oncogenesis, altered HSP70 expression was reported in neurodegenerative pathologies. For instance, BiP/HSPA5, ER-resident chaperone and the main effector in the unfolded protein response (UPR) pathway, is up-regulated in Alzheimer's and Parkinson's diseases. The activated UPR induces the apoptotic pathways in the affected cells (Holtz and O'Malley, 2003; Lee et al., 2010). In bacteria, it was shown that HSP70 protein DnaK is responsible for resistance to several antibiotics, since a deletion of the gene produces less resistant strains (Wu et al., 2015). Multiple inhibitors of bacterial HSP70 were developed and tested in recent decades; for instance, pyrrhocoricin, a proline-rich antibacterial peptide, which specifically binds to SBD of *E.coli* DnaK and arrests its ATP-driven cycle (Kragol et al., 2002). Promising isoprenoid compound, geranylgeranylacetone (GGA), was shown to be a selective inhibitor of *Helicobacter pylori* DnaK while simultaneously making the pathogen cells more susceptible to antibiotic treatment. In addition, GGA stimulates the expression of HSPA1A in human cells, increasing their protection from stress-induced damage (Otaka et al., 2007; Grave et al., 2015).

HSP90

Another important chaperone, HSP90, was identified in bacteria and eukaryotic cytoplasm, ER, mitochondria and plastids. HSP90 is an approximately 90 kDa protein, which functions as a homodimer. In the human genome, 17 genes encoding various HSP90 variants were identified, including the stress-inducible (HSP90AA1) and constitutively expressed proteins (HSP90AB1), as well as organelle-specific homologues such as HSP90B1/GRP94, which is an ER-resident chaperone (Spence et al., 1990; Chen et al., 2005). HSP90s suppress protein aggregation and increase the fraction of correctly folded proteins *in vitro* (Wiech, 1992). It is noteworthy that the majority of known HSP90 clients are represented by enzymes and receptors involved in signal transduction, for instance glucocorticoid receptors, p53, cyclin-dependent kinases, etc. (Picard, 2002). For some of the client proteins, HSP90 is crucial during the initial protein folding or participates in the formation of many important protein complexes, which are later imported into the nucleus, e.g., snoRNPs (complexes modifying ribosomal RNA and required for ribosome synthesis), RNA polymerase II, RNA-induced silencing complex (RISC, one of the main components of RNA interference pathway), telomere complexes and kinetochores. Additionally, HSP90s facilitate the assembly of cytosolic 26S proteasome (Makhnevych and Houry, 2012).

Similarly to HSP70 and HSP60, HSP90 belongs among ATP-dependent chaperones; its working cycle and structure is depicted in Fig. 4. ATP binding and hydrolysis are the driving force of the mutual conformational shift of the HSP90 subunits. However, this functional cycle demands multiple HSP90 co-chaperones. For example, bacterial Aha1 (human AHSA1) is an HSP90 ATPase activator, while Sba1 (p23) is an HSP90 ATPase inhibitor; Hop1/Sti1 (STIP1) functions as an adaptor protein linking HSP70 to the HSP90 system (Wandinger et al., 2008). The human STIP1 is responsible for physical transfer of a substrate protein from HSP70 to HSP90. In this cooperation, HSP90 plays a rather protective role for the client protein and induces the correct client folding after release from HSP70 (Wegele et al., 2006; Morán Luengo et al., 2018).

Several studies indicated the role of HSP90 in cancer development due to its ability to stabilize and enhance activity of the signal transduction proteins. HSP90 binds and stabilizes HER2, the membrane tyrosine kinase, which is up-regulated in breast cancer and stimulates cell survival and proliferation. Similarly, proto-oncogenic kinase RAF1 from the MAPK pathway is stabilized in a complex with HSP90, which was shown to be indispensable for RAF1 activity (Schulte et al., 1996; Münster et al., 2002). HSP90 and its co-chaperone p23 were identified in the active complex with telomerase catalytic subunit (hTERT) *in vivo*; telomerase is very often activated in tumour cells, which ensures their unlimited proliferation potential (Holt et al., 1999).

In addition, HSP90 can biochemically "buffer" mutated signalling pro-oncogenic proteins, which would normally be targeted for degradation. Mutated "oncogenic" versions of these proteins can be stabilized by HSP90, which allows them to function and promote cancer cell survival and proliferation (Whitesell and Lindquist, 2005). Not surprisingly, HSP90 became a potential target for anti-cancer therapy. For instance, geldanamycin, a benzoquinone compound isolated from *Streptomyces hygroscopicus,* was discovered to be a competitive inhibitor of the HSP90 ATPase domain. Similarly, the synthetic purine-like PU3 compound was developed to specifically bind and inhibit the ATPase domain (Jhaveri et al., 2012).

It was also established that HSP90 is utilized by some viruses (i.e., influenza A, HSV-1 and HCV) for folding and assembly of both capsid and non-capsid proteins. This indeed also stimulated the research on potential HSP90 inhibitors: many of them exhibited a suppressing effect on viral replication and thus represent a promising treatment against certain viral infections (Geller et al., 2012).

Finally, HSP90 was found to associate with amyloid-β plaques, neurofibrillary tangles (NFTs) and α-synuclein-formed Lewy bodies in Alzheimer's and Parkinson's disease, respectively (Ramirez-Alvarado et al., 2010). Promising results were observed in mouse models of Alzheimer's disease. Knock-out of the HSP90 system (together with HSP70) in the mouse cells led to enhanced formation of NFTs, while increased expression of HSP70 and HSP90 helped to keep most of the tau-protein (i.e., the main component of NFTs) in the solu-

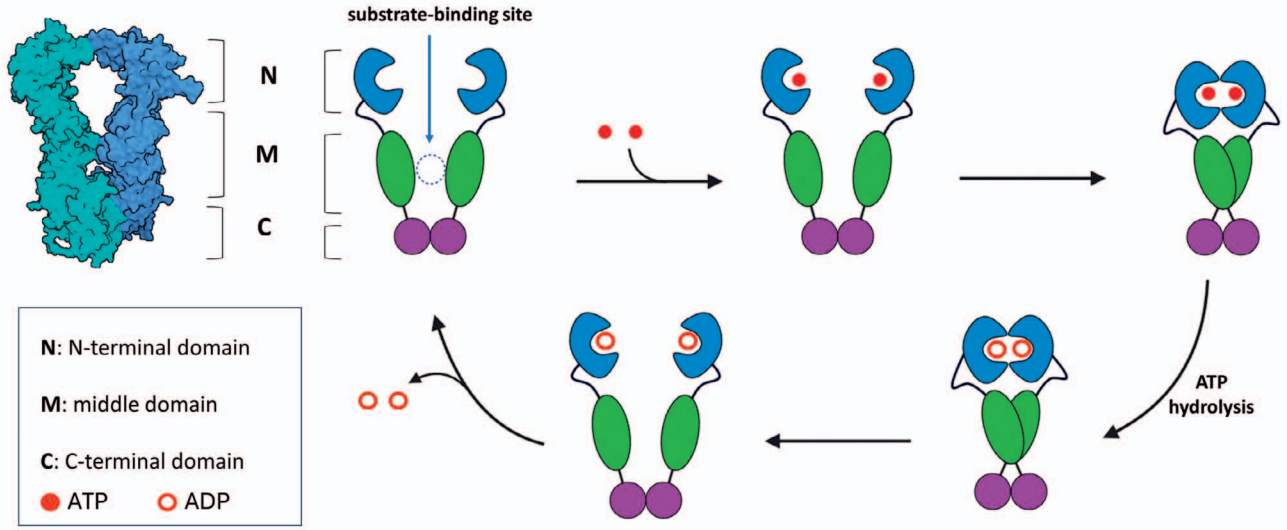

Fig. 4. The structure and ATP-driven cycle of HSP90. Its functional form is an almost parallel homodimer (PDB code 2IOP), where each subunit is capable of ATP binding and hydrolysis by its N-terminal (**N**) domain. A client polypeptide is bound between two adjacent middle (**M**) domains, while the C-terminal (**C**) domains are responsible for constitutive dimerization. After binding two ATP molecules, HSP90 undergoes substantial conformational changes, which result in N-domains docking onto M-domains and the twist of the whole assembly. When ATP is hydrolysed, the conformation returns to its initial state and the client protein and ADP molecules are released. Based on the study by Wandinger et al. (2008).

ble form and promote its normal binding to microtubules (Dou et al., 2003).

However, in later studies, HSP90 inhibitors significantly decreased the concentration of soluble tau and enhanced the heat shock response *in vivo:* this effect was attributed to the ability of HSP90 to inhibit HSF1, the main heat shock response transcription factor mentioned earlier in the chapter on HSP70 (Dickey et al., 2005). These observations clearly documented that the role of HSP90 in Alzheimer's disease is quite complex and several possible mechanisms explaining the effects of HSP90 inhibitors can be proposed.

Ribosome-Associated Chaperones

As suggested by the name, members of this chaperone group are situated on ribosomes or in their vicinity and facilitate the folding of a newly translated polypeptide as it emerges from the ribosomal tunnel. In all life domains, there is a specific class of such chaperones: bacterial trigger factor (TF), eukaryotic ribosome-associated complex (RAC), and eukaryotic and archaeal nascent polypeptide-associated complex (NAC) with its numerous cofactors.

NAC is the initial component of human ribosome-associated chaperone machinery; it is bound to the ribosome in an 1 : 1 ratio (Fig. 5A**)**. NAC is a heterodimer consisting of an α-subunit (human BTF3) and a β-subunit (NACA), which share the homologous NAC domains responsible for the dimerization. The α-subunit has an additional C-terminal ubiquitin-associated (UBA) domain with an unknown function. The N-terminus of the β-subunit is responsible for interaction with the ribosome; however, no data on the structure of this part of the protein is available, similarly to the N-terminus of the α -subunit. Interestingly, both subunits are responsible for the interaction with client polypeptides emerging from the ribosomal tunnel exit as translation occurs (Preissler and Deuerling, 2012). The analysis of mRNA sequences isolated from the ribosome-NAC complex suggests that NAC is a general chaperone for the whole cell proteome and is always associated with actively translating ribosomes in the yeast model (Alamo et al., 2011). NAC is essential and its impaired activity is lethal in eukaryotes (Deng and Behringer, 1995; Markesich et al., 2000). The NAC activity is tightly coupled with RAC in eukaryotes.

RAC in humans is represented by a unique ribosome-specific HSP70-HSP40 system, consisting of proteins HSP70L1 (HSP70) and MPP11 (HSP40) (Otto et al., 2005). However, more is known about the yeast counterparts, Ssz1 (Hsp70) and Zuo1 (Hsp40). It was shown that the Ssz1-Zuo1 heterodimeric complex is stable and does not dissociate, unlike the canonical HSP70-HSP40 intermediate complex, where HSP40/JDP interacts with HSP70 only transiently during the working cycle (see chapter "*HSP70*"). Moreover, Zuo1 possesses additional domains allowing the whole complex to interact with both ribosomal subunits. Interestingly, the yeast RAC serves as an ATPase-stimulating factor for cytosolic Hsp70, Ssb1, where the J-domain of Zuo1 is the key interactor with Ssb1; the main Hsp70 chaperoning activity in the ribosomal vicinity is carried out by Ssb1. In

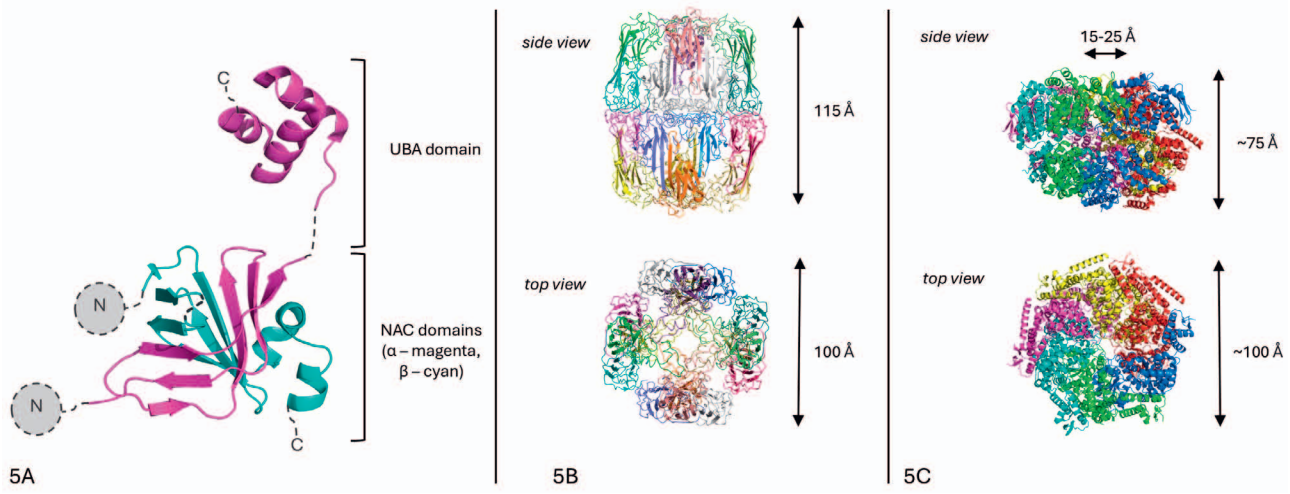

Fig. 5. Representatives of the various chaperone classes. (**A**) The model of human NAC, PDB code 3LKX; for the UBA domain, the model of archaeal orthologue was used (PDB code 1TR8). The structure of the N-terminal domains has not been resolved. (**B**) The homo-16-meric assembly of human αA-crystallin/HSPB4 (PDB code 6T1R). The present central pore has not been reported to fulfil any specific function. (**C**) The ClpB complex (Hsp100, *E. coli*), hexameric, in ATP-bound state (PDB code 6QS8). The central pore is dynamic and changes its diameter throughout the threading of an unfolded polypeptide through it. Based on Preissler and Deuerling, 2012; Deville et al., 2017; Kaiser et al., 2019.

mammalian cells, the role of Ssb1 is apparently fulfilled by classic cytosolic HSPA8, as an extensive interaction of MPP11 and HSPA8 has been detected. The role of the HSP70L1 subunit is not fully understood, since no experimental evidence of HSP70L1 interaction with the nascent polypeptide chain was found (Hundley et al., 2005; Zhang et al., 2017).

Similarly to NAC, RAC is essential for the correct ribosome functioning and maturation, which was shown by the experiments on yeast deletion mutants, where elevated protein aggregate levels and decreased protein synthesis were detected (Albanèse et al., 2010; Koplin et al., 2010) . Several models of NAC/RAC cooperation were suggested recently (reviewed by Preissler and Deuerling, 2012). Experimental confirmation of the structure and function of the human homologues of NAC and RAC complex are still required; however, from the studies of NAC/RAC in yeasts it can be concluded that the ribosome-associated chaperone machinery is indispensable for the protein synthesis.

Small HSPs

Small heat shock proteins (sHSPs) are an ATP-independent, diverse group of chaperones with molecular weight in the range from 12 to 43 kDa. They were identified in all domains of life; the number of homologues per genome increases from prokaryotes to eukaryotes, with the highest number (19) found in plants. The human genome has 10 known sHSP homologues (HSPB1–10). The physiological function of sHSPs is to prevent the aggregation process by binding the exposed regions of client proteins and forming stable complexes, especially under (heat) stress conditions. Therefore, sHSPs are characterized as holdases (Jakob et al., 1993; Haslbeck, et al., 2005). Noteworthy, sHSPs tend to form large barrel-shaped oligomeric assemblies, resembling chaperonin double-ring complexes. For instance, αA-crystallin (HSPB4) oligomers from *H. sapiens* contain 12, 16 or 20 subunits; the model of 16-mer is depicted in Figure 5B. Small HSP assemblies can bind several unfolded substrates at once through the exposed hydrophobic regions; the formed "substrate-oligomer" complexes are then very stable at normal physiological conditions. As was mentioned in the previous chapters, for the release of bound client proteins, interactions with other ATP-dependent chaperone systems, e.g., HSP70-HSP40 or HSP100 (see the next chapter), are necessary; without them, the "chaperone-client" complex cannot dissociate. Therefore, even though sHSPs cannot use ATP directly, ATP still fuels the substrate release from sHSP assemblies (Lee et al., 1997; Mogk et al., 2003).

Some sHSPs are tissue-specific in mammals, including HSPB4 (αA-crystallin), which utilizes its anti-aggregation features to maintain the inner environment of the eye lens. Various mutations in αA- and αB-crystallin were shown to cause cataract and other eye pathologies, which are characterized by protein aggregate formation in the lens (Kaiser et al., 2019). Among human sHSPs, several homologues (HSPB5/αB-crystallin, HSPB2, HSPB1, HSPB6 and HSPB7/cvHSP) are highly expressed in skeletal and heart muscle tissue, and their aberrant expression and altered localization in cardiomyocytes contribute to ischemic state of the heart. However, it is difficult to speculate whether they are required to normal myofibril functions or their major role here is connected to the prevention of ischemia-caused cel-

lular stress and its consequences (Golenhofen et al., 2000).

Several neurodegenerative diseases were shown to be associated with altered sHSP expression and function. It was demonstrated that human HSPB1 inhibited the initial stages of amyloid fibril growth *in vitro*, and it was speculated that age-related decrease in sHSP expression in human neurons might be one of the mechanisms contributing to the age-related amyloid formation and Alzheimer's disease development (Kudva et al., 1997). In the *Caenorhabditis elegans* model of human Alzheimer's disease, transgenic Aβ-peptide was co-precipitated with three different endogenous sHSPs (HSP16-1, HSP16-2 and HSP16-48), together with HSP70, suggesting their participation in the pathological process (Fonte et al., 2002). Aggregation of mutated α-synuclein into protein inclusions was inhibited by the action of HSPB1 and αB-crystallin in mammalian cell lines (Cox and Ecroyd, 2017). Nevertheless, the cell toxicity assays conducted on rat neurons indicated that HSP27 enhanced the Aβ harmful effects: while preventing the amyloid fibril formation, it stimulated Aβ to associate into Aβ/αB-crystallin-rich aggregates (Stege et al., 1999).

HSP100

The HSP100 family belongs to the ATP-dependent disaggregases, i.e., proteins capable of disassembling large protein complexes or aggregates potentially toxic to cells. It is another representative of ring-shaped oligomeric chaperones (among chaperonins and sHSPs). The whole assembly is typically hexameric; each subunit has one or more ATP-hydrolase domains (Fig. 5C; Schirmer et al., 1996). The HSP100 complexes may or may not have a central pore. The pore is, in case of yeast Hsp104, only 25 Å wide and cannot allow even a partially folded substrate to pass through (versus 45 Å in the GroEL-GroES complex). Instead, the whole hexameric assembly dissolves protein aggregates and apparently pulls an unfolded polypeptide chain through itself. The process is controlled by the ATP-driven working cycle (Parsell et al., 1994; Deville et al., 2017).

The activities of HSP100 are often coupled to proteolysis. For instance, bacterial HSP100 ClpA and ClpX chaperones associate with ClpP protease in bacteria, forming a complex that resembles the 26S proteasome assembly in eukaryotes. Two possible mechanisms can be engaged in this proteolysis coupling: a) by binding and keeping the target protein in an unfolded state, HSP100 enables the protease to digest it; b) HSP100 can pass the target polypeptide in an unfolded state through the HSP100 central pore, directing it to the protease (Schirmer et al., 1996). In yeasts, Hsp104 is highly expressed during the respiratory metabolism and growth and confers tolerance to heat, ethanol and arsenic compounds. Similarly, Hsp101 in plant cells and Clp proteins in bacteria modulate the cell viability under heat shock (Squires et al., 1991; Sanchez et al., 1992; Queitsch et al., 2000). Human HSP100 homologue SKD3/CLPB was identified in the mitochondrial intermembrane space. Unlike its counterparts from the bacteria and yeasts, SKD3 can assemble into multiple unique oligomers during its working cycle – a homo-14-mer without a bound substrate protein and a homo-12-mer upon substrate binding. In addition, the SKD3 monomer contains an additional ankyrin (ANK) domain, which is the distinguishing feature of human homologue and whose disruption abolishes the SKD3 disaggregase activity (Wu et al., 2023). It was discovered that SKD3 is responsible for maintaining the mitochondrial cristae shape (Chen et al., 2019). The same study demonstrated that SKD3 is up-regulated in human acute myeloid leukaemia (AML); it is further induced upon acquisition of resistance to venetocalx, and SKD3 ablation resensitizes AML to this BCL2 inhibitor. Mutations in SKD3 were reported in a number of human diseases, such as 3-methylglutaconic aciduria, progressive brain atrophy, intellectual congenital neutropenia, cataract and several others (Wortmann et al., 2015).

Conclusion

In this review, the main HSP classes were discussed, including their structural and functional characteristics. Nevertheless, the degree of variation between different chaperone families, their cooperation and the plethora of specific functions make the topic too complex to cover in a single manuscript.

The importance of continuing studies of chaperones is especially emphasized in the context of human diseases. As was mentioned previously in this work, some chaperones are involved in maintaining deleterious protein conformations (e.g., HSP90), a process with potential clinical relevance in cancer, or drug resistance. Indeed, many potential HSP inhibitors were developed and tested in recent years (Whitesell and Lindquist, 2005; Lianos et al., 2015).

On the other hand, a failure or insufficiency of the chaperone network may result in malfunctioning of protein degradation and disaggregation processes, which eventually can lead to accumulation of toxic protein aggregates in the cell. This can be exemplified by the malfunctioning HSP90/HSP70 system and its role in the development of some neurodegenerative diseases (Lackie et al., 2017). Indeed, attempts to increase the activity or expression of HSPs could potentially lead to development of new HSP-stimulating drugs (Kampinga and Bergink, 2016). Such drugs are being developed to target the HSF1 transcription factor, which is the key activator of the corresponding HSPs (e.g., stress-induced HSP70). Geldanamycin, GGA and celastrol belong among such compounds (Kampinga and Bergink, 2016) HSF1-independent strategies for the drug development are considered as well. Compound 115-7c was designed and tested to directly bind and enhance the biochemical functions of a given HSP system, showing promising results for further testing on human patients (Calamini and Morimoto, 2012).

Finally, chaperones participate in bacterial resistance to antibiotic treatments, as it was shown for bacterial DnaK (Wu et al., 2015). The compounds such as GGA and pyrrhocoricin specifically target and inhibit the bacterial DnaK while decreasing the bacterial resistance to other antibiotics (Kragol et al., 2002; Otaka et al., 2007).

Summarizing the examples above, the delicate protein homeostasis is hugely supported by chaperones. However, their role in pathophysiological cellular processes is sometimes contradictory and, in some cases, seems deleterious rather than beneficial (e.g., HSP70 in tumour cells). The ambiguous effects of certain HSPs on human pathology development are highly dependent on the physiological context of the cell (cancer, neurodegeneration, infection, etc.). Moreover, all HSPs are involved in a multitude of interactions, and a crosstalk between various chaperone systems is often observed. This underlies the complexity of the HSP network, which is yet to be fully elucidated.

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