



Review

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Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders

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The family of the mammalian small heat-shock proteins consists of 10 members (sHSPs/HSPBs: HSPB1–HSPB10) that all share a highly conserved C-terminal alpha-crystallin domain, important for the modulation of both their structural and functional properties. HSPB proteins are biochemically classified as molecular chaperones and participate in protein quality control, preventing the aggregation of unfolded or misfolded proteins and/or assisting in their degradation. Thus, several members of the HSPB family have been suggested to be protective in a number of neurodegenerative and neuromuscular diseases that are characterized by protein misfolding. However, the pro-refolding, anti-aggregation or pro-degradative properties of the various members of the HSPB family differ largely, thereby influencing their efficacy and protective functions. Such diversity depends on several factors, including biochemical and physical properties of the unfolded/misfolded client, the expression levels and the subcellular localization of both the chaperone and the client proteins. Furthermore, although some HSPB members are inefficient at inhibiting protein aggregation, they can still exert neuroprotective effects by other, as yet unidentified, manners; e.g. by maintaining the proper cellular redox state or/and by preventing the activation of the apoptotic cascade. Here, we will focus our attention on how the differences in the activities of the HSPB proteins can influence neurodegenerative and neuromuscular disorders characterized by accumulation of aggregate-prone proteins. Understanding their mechanism of action may allow us to target a specific member in a specific cell type/disease for therapeutic purposes.

1. Introduction

The family of the mammalian small heat-shock proteins, also called the HSPB proteins, consists of 10 members (HSPB1–HSPB10, see alternative names of each member in table 1), all of a relatively low molecular weight (MW, ranging from 15 to 45 kDa) and sharing some structural similarities, like a highly conserved C-terminal alpha-crystallin domain (ACD, [52]). This ACD plays an important role in the modulation of both structural and functional properties of the HSPBs. Indeed, monomers of the HSPB proteins associate (partially via their ACDs) into dimers that are thought to act as basic units/building blocks, capable of generating oligomers ranging from *ca* 200 to 600 kDa [49,53]. The various HSPB monomers can form both homo- and hetero-dimers as well as

Table 1. The mammalian small heat-shock proteins. n.a., not analysed as far as we know; AD, Alzheimer's disease; ALD, Alexander disease; DMPK, dystrophin myotonia-protein kinase; DRG, dorsal root ganglia; MNDs, motor neuron diseases; NPD, Neuman Pick's disease; PD, Parkinson's disease; RT qPCR, real-time quantitative polymerase chain reaction.

HSPB name (alt. name)	stress-induced changes after brain injury/during neurodegeneration					biochemical function (as detected in cells after upregulation)						
	expression in brain or sciatic nerves	cardiac muscles	skeletal muscles	ischaemia or nerve injury	misfolding diseases (neurons)	misfolding diseases (astrocytes/glia)	promoting refolding			anti-aggregation		
							polyQ	ALS	other	polyQ	ALS	other
HSPB1 (hHsp27, mHsp25)	low/moderate in: cortex, cerebellum, striatum, hippocampus, spinal cord [1–3]	high [4]	high [4]	UP in neurons [5,6]	generally UNCHANGED UP in Purkinje cell (NPD) [7,8]	generally UP [9–16] extracellular in AD brain [17]	strong [3,18]	no [1,19] [20] TDP-43: no [21]	SOD-1: +/- n.a.	cytoskeletal stabilization [22] anti-oxidant [23]	n.a.	
HSPB2 (MKBP)	low in: cortex, hippocampus, sciatic nerves [1,2]	high [4]	high [4]	n.a.	n.a.	extracellular in AD brain [17]	no [1]	no [1]	n.a.	activation of DMPK [24]; maintaining myofibrillar integrity [25]	n.a.	
HSPB3 (HSP127)	low in: cortex, cerebellum, spinal cord, sciatic nerve (RT qPCR) [1–3]	high [4]	high [4]	n.a.	n.a.	n.a.	no [1]	no [1]	n.a.	maintaining myofibrillar integrity [25]	n.a.	
HSPB4 (α A- crystallin)	brain: not reported low in: sciatic nerve [2]	none	none	n.a.	n.a.	n.a.	++ [1]	no [1]	n.a.	maintaining the proper refractive index in the lens [26]	n.a.	
HSPB5 (α B- crystallin)	high in: white matter, cortex, cerebellum, striatum, hippocampus, spinal cord, sciatic nerve [1–3]	high [4]	high and increasing with aging [4]	n.a.	UP in: neurons (in MNDs, NPD, AD, PD, CID) [27–32]	UP in: reactive glial cells, microglia, oligodendrocytes [11,13,16,17, 33, 34]	++ [1]	no [1]	no [21]	PD: ++ [35] ALD: ++ [36]	cytoskeleton stabilization; maintaining the proper refractive index in the lens [37,38]	

(Continued.)

Table 1. (Continued.)

HSPB name (alt. name)	stress-induced changes after brain injury/during neurodegeneration				biochemical function (as detected in cells after upregulation)				
	expression in brain or sciatic nerves	cardiac muscles	skeletal muscles	ischaemia or nerve injury	misfolding diseases (neurons)	misfolding diseases (astrocytes/glia)	promoting refolding	anti-aggregation	other
							polyQ	ALS	other
HSPB6 (Hsp20, p20)	high in: cortex, cerebellum cortex, choroid plexus of the ventricles, telencephalon, spinal cord, sciatic nerve [1–3]	high [4]	high [4]	UP after hypoxic stress (in neonatal hippocampus) [39,40]	n.a.	n.a. extracellularly in AD brain [17]	+ [1]	no [21]	n.a. cytoskeleton stabilization [41]
HSPB7 (cvHsp)	low in brain [1,3]	high [4]	moderate/high increasing with aging [4]	n.a.	n.a.	no [1]	+ + + [1]	no [21]	n.a. maintaining myofibrillar integrity [42]
HSPB8 (Hsp22, H11, E2I/G1)	low in: cortex, cerebellum, striatum, hippocampus, spinal cord, sciatic nerve. low/moderate in motoneurons [1–3,43]	high [4]	high [4]	n.a.	UP in ALS in spinal cord and motoneurons [10,43]	UP in astrocytes in AD, PD, polyQ [44] and mildly UP in glial cells (not in microglia) in ALS [43,45]	no [1]	+ [3, 46–48] [43] TDP-43: + + + [21]	n.a. protein synthesis inhibitor; autophagy simulator [43,46–48]
HSPB9 (CT51)	n.a. (testis only?)	n.a. (testis only?)	n.a. (testis only?)	n.a.	n.a.	no [49]	+ + + [49]	+/- [21]	n.a. cancer/testis antigen [50]
HSPB10 (ODFP, ODF1)	n.a. (testis only)	n.a. (testis only)	n.a. (testis only)	n.a.	n.a.	no [49]	no [49]	n.a.	n.a. cytoskeleton stabilization [51]

homo- and hetero-oligomeric complexes [54,55]. The dynamic association/dissociation of the oligomers has been suggested to be key to the function of the HSPB proteins and is often regulated by their phosphorylation state [49,56]. HSPB proteins are biochemically classified as molecular chaperones and participate in protein quality control; in fact, several HSPB family members have been shown to be able to bind to (partially) unfolded or to misfolded, aggregation-prone proteins [57] preventing their aggregation. In conjunction with ATP-dependent chaperones (e.g. HSP70s/HSPAs), the HSPB-bound clients can either be refolded or degraded; the mechanisms for either refolding or degradation is not fully understood, but may depend both on the state of the client and on the specific HSPB member that is bound to it (see later).

The chaperone activity of small HSPs has been discovered and explored mainly in cell-free experiments with purified proteins [58–60] and it accounts for, for example, the role that HSPB4 plays in maintaining eye transparency [26]. Whether this chaperone function is also underlying other cellular functions of HSPB members is less clear. For example, some HSPBs members (e.g. HSPB1 and HSPB5) have the capability to modulate the assembly and stabilization of cytoskeleton components, such as actin and intermediate filaments [22,37,38,41,61–64], but how far these actions rely on their chaperone activity is unknown. It is also not clear whether other HSPB family members serve in cytoskeletal protection or whether different cytoskeletal and contractile elements may require different HSPB members. Other functional endpoints that have been shown to be affected by HSPB members include the maintenance of proper cellular redox state, protecting cells from oxidative stress conditions (HSPB1, [65]), a general anti-apoptotic function (HSPB1, [66,67]) and a role in skeletal muscle cell differentiation (HSPB2 and HSPB3, [25]). The biochemical mechanisms underlying these different cellular effects of the various HSPB members are often still elusive and not always directly linked to the *in vitro*-defined chaperone-like activities.

Several HSPB family members have been suggested to be protective in a number of neurodegenerative and neuromuscular diseases that are characterized by protein aggregation and axonal transport defects. This directly relates back to the two most postulated actions of these HSPBs: their chaperone action and cytoskeletal stabilizing function, respectively. On the other hand, mutations in some members of the HSPB family (namely, HSPB1, HSPB3, HSPB4, HSPB5 and HSPB8) have been associated with neurological and muscular alterations, suggesting that loss of their function as general chaperones or/and cytoskeletal protectors is crucial for maintaining neuronal and muscular cell function and viability. Here, we will discuss the implication of HSPB proteins in neurodegenerative and neuromuscular diseases, focusing our attention on how the differences in the HSPB activities influence their protective functions.

2. The role of HSPBs in neurodegenerative and neuromuscular diseases

(a) Some HSPBs are upregulated in neurodegenerative diseases

The formation and accumulation of insoluble aggregates containing misfolded/mutated proteins is a pathological

hallmark of many neurodegenerative and neuromuscular disorders with late onset, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), polyglutamine (polyQ) diseases (e.g. Huntington disease (HD), spinal and bulbar muscular atrophy (SBMA), etc.), and Creutzfeldt–Jacob disease (CJD). These diseases include both sporadic and genetically inherited forms. Some inherited forms are linked to mutations in a specific protein that misfolds and/or is prone to aggregation. Notably, often the proteinaceous aggregates are found in the corresponding degenerated tissues. Protein aggregation is a multi-step nucleation-dependent process starting from the oligomerization of the self-associating misfolded protein, which generates pre-fibrillar aggregates (detergent-soluble). Pre-fibrillar aggregates can subsequently generate fibrillar structures (detergent-insoluble). During this aggregation process, the mutated protein interacts with and entraps different intracellular components. These include several HSPs (including several HSPB members; [68]), components of the proteasome system, elements involved in vesicular transport and transcription factors. Sequestration of HSPs and proteasomal components may reflect their failure to degrade the misfolded proteins as well as their failure to prevent their aggregation or their unsuccessful attempts to disaggregate the inclusions. As a consequence, the HSPs might be diverted away from their normal functions. Similarly, entrapping of vesicular transport components and transcription factors may lead to their reduced activities in essential neuronal processes. Finally, aggregates might physically impair cellular processes (e.g. axonal transport) [69]. Probably, a combination of these events contributes to disease progression and, therefore, aggregation has long been considered the key pathogenic mechanism. Nevertheless, the precise role of aggregates in neuronal cell death and disease progression is still largely debated. Several studies have suggested that not all forms of aggregates may be toxic [70–72]. In fact, large (amyloidic) aggregates that entrap these various key cellular elements have been found to be protective in certain systems and under certain conditions [70–75]. It has thus been proposed that smaller (amorphous) oligomeric and/or heteromeric species are more toxic as they are capable of freely moving around in the cellular milieu and perturb various neuronal functions. Besides the aforementioned effects of large aggregates (e.g. impairment of cellular proteostasis, alterations of the degradative systems, disruption of axonal transport, dysregulation of the transcription of specific genes), this could also include, for instance direct effects on membrane integrity and/or synaptic functioning [70,73,76,77]. However, irrespective of the presumed differential toxicity attributed to these different aggregates, it is clear that any factor/approach able to decrease mutated protein accumulation, to prevent the initiation of their aggregation or to facilitate the clearance of (early) aggregates will greatly contribute to restore (or maintain) the normal neuronal proteostasis and function, thus slowing down disease progression. This potentially can be achieved by boosting specific molecular chaperones/HSPs (e.g. HSP70s/HSPAs and HSP40s/DNAJs and some sHSPs/HSPBs), which will avoid or attenuate protein aggregation, or by stimulating the degradative pathways (i.e. autophagy), which will help to clear the aggregates. The efficacy of both approaches has been experimentally demonstrated using cellular and/or animal models of aggregate diseases (e.g. polyQ diseases, AD and PD) [19,43,46,78–91].

Here, we will focus on boosting the activity of the HSPB proteins as a potential approach to counteract protein toxicity in neurodegenerative diseases, keeping in mind that not all HSPB family members share the same functional properties [1,92]. Therefore, the putative protective efficacy of the 10 members may largely differ depending on the specific protein causing the aggregation disease.

In addition, the various members present very different tissue/cell-specific distribution. In basal conditions, only some HSPB family members are expressed in the central nervous system (CNS) and very few in neurons (some are mainly confined in glial components of the CNS; table 1). However, misfolded protein expression and the consequent cell stress during neurodegeneration might trigger both the overexpression of some HSPBs already present in target cells or of HSPBs normally silent in neurons (table 1). Thus even the HSPBs normally absent in the brain might become players in the intracellular response to mutant misfolded protein neurotoxicity [92]. Regarding the specific pattern of expression of the HSPB family members, HSPB2 and HSPB3 are mainly expressed in the skeletal muscle cells [25], while HSPB9 and HSPB10 are only found in testis [52]. HSPB7 was originally termed cvHSP and indeed is highly expressed in cardiac tissue, although it is also expressed at lower levels in several other tissues, including the brain [1,92]. HSPB6 is constitutively and highly expressed in smooth, cardiac and skeletal muscles and plays a role in muscle function [93]. The other members (HSPB1, HSPB5 and HSPB8) are highly expressed in muscle tissues, but are also expressed in many other tissues, including in the CNS, with peculiar cell-type-dependent differences in expression (table 1). For example, while HSPB1 is highly expressed in peripheral sensory neurons [94], HSPB8 is highly expressed in motor neurons [17,33,43,44,95]. As it will be discussed later, some members of the HSPB family can prevent aggregation of (some) disease-associated mutant proteins and that is thought to be protective in neuronal cells. Thus, HSPB upregulation (both at the level of neuronal and glial cells) might represent a protective cellular response to neuronal damage. However, it is also possible that this over-induction occurs as a consequence of neuronal stress, without an active participation to the protective processes. While the presence of HSPB1 and HSPB5 in ballooned neurons (e.g. in AD, CJD, etc.) could suggest that they interact with aggregates in neurons (either actively engaged in protein quality control here or reflecting failed function or being trapped here), the upregulation of the same proteins in astrocytes is more difficult to understand. But several lines of evidence suggest that misfolded proteins may act in a non-cell autonomous way. For example, the primary toxicity can be exerted on the glial (and microglia) cells surrounding affected neurons, which may thus also be indirectly affected via release of neurotoxic factors from glia or reduced removal of neurotoxic agents by the glia, i.e. through an altered dialogue between these cells [96,97]. Indeed, recent evidence suggests that astrocytes, together with other glial cells (especially microglia), participate in the maintenance of the extracellular milieu containing debris and aggregates from dying neurons. A comparable non-cell autonomous mechanism may play a role in motor neuron diseases, involving interactions between motor neurons and the target muscle cells [98] in which muscle denervation from motor neurons rather than being causative to muscle degeneration also may result as consequence of

initial muscle damage/death [99,100]. It can be hypothesized that HSPB8, for example, somehow is involved in this process: in anterior horn spinal cord of ALS mice, HSPB8 is highly induced not only in motor neuron (at very high levels in this primary target of mutant protein toxicity), but also in the glial cells (at lower levels) of the affected regions [43], and in muscle tissues (at the highest levels; P. Rusmini, V. Crippa, E. Giorgetti, A. Boncoraglio, R. Cristofani, A. Poletti 2013, unpublished data). Studies should therefore not only focus on elucidating the role of HSPBs in individual populations of cells, but also look at cell non-autonomous and cell–cell interactions.

Finally, low or even a complete lack of constitutive and induced expression in target cells of individual HSPB members that did reveal protective power in cell models of disease should not be a reason to imply that these members cannot exert protective functions in disease. Obviously, such members are not part of the canonic intrinsic (neuronal/glial/muscular) response to the diseased protein, but could be still considered potential therapeutic targets if one were able to induce them by pharmacological means, with the specific aim to assist mutant neurotoxic protein clearance.

3. Refolding and anti-aggregation are distinct properties of the HSPBs

As previously mentioned, a number of neurodegenerative diseases are characterized by the progressive accumulation of aggregation-prone proteins. Upregulation of specific members of the HSPB family could slow down or completely inhibit the aggregation process in cell models [43,46–48,92,95,101], with significant differences among the 10 HSPB family members. In particular, using mutated polyQ-containing proteins (e.g. huntingtin and ataxin-3) we showed that HSPB6, HSPB7, HSPB8 and HSPB9 overexpression inhibited protein aggregation and protected against its mediated toxicity, while overexpression of all the other members had no effect [1]. Curiously, HSPB6, HSPB7, HSPB8 and HSPB9 could not efficiently facilitate the refolding of denatured substrates (e.g. heat-denatured firefly luciferase), but rather were linked to protein degradation by either the proteasomal or the autophagic systems (table 1; see also later, [1,46]). Inversely, the strongly heat-stress-regulated HSPB1, HSPB4 and HSPB5 members, which were found to be very efficient in facilitating the refolding of heat-denatured substrates, both in cells and *in vitro*, showed poor anti-aggregation effects against polyQ proteins (table 1) [1]. These data suggest that the different HSPB members may either have different client specificity and/or have a different impact on client processing.

Despite the lack of anti-polyQ aggregation activity, HSPB1 and HSPB5 showed some protective effects in some cell models of polyQ disease. As HSPB1 and HSPB5 can increase the resistance of the cytoskeletal network [22,37,38,41,62–64] and are upregulated both in ballooned neurons and astrocytes [9,27,28], one may speculate that they protect axonal transport and vesicular trafficking against disruption by the protein aggregates and thus delay the consequences of aggregation, without affecting aggregate formation as such. Yet, when directly comparing the cytoprotective effects of, for example, HSPB5 to that of HSPB7 in cell models, we found HSPB5 protection was marginal ([1], figure 1c).

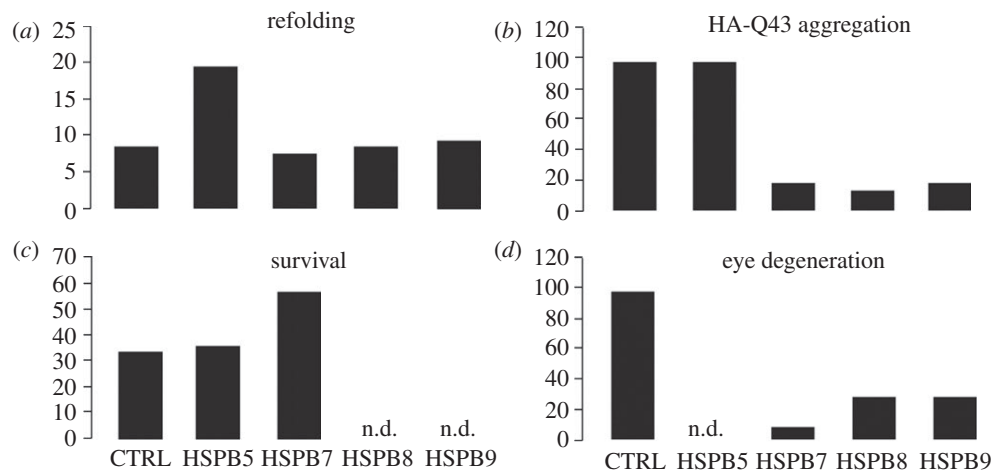


Figure 1. Comparison of effects of overexpression of HSPB5, HSPB7, HSPB8 and HSPB9 on the cellular capacity to refold heat-denatured luciferase (a) or to suppress protein aggregation (b) and toxicity (c) of polyQ proteins in cells or to reduced polyQ-mediated eye degeneration in flies (d). Data compiled and replotted from [1,101] and (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data).

Consistent with the cellular data, the three most potent suppressors of polyQ aggregation (HSPB7, HSPB8 and HSPB9) also reduced polyQ-mediated eye degeneration in a *Drosophila melanogaster* *in vivo* model [1,101] (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data; figure 1). These latter three HSPB members have been implied in polyQ degradation via the proteasome (HSPB9: M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data), by supporting autophagic clearance of polyQ aggregates [1] or by enhancing autophagic flux [43,46,95,101,102], see below).

4. Anti-aggregation activity of HSPBs: stimulating degradative pathways

As mentioned earlier, HSPB7, HSPB8 and HSPB9 seem to have the potential to trigger (or facilitate) proteasomal and/or autophagic degradation of certain misfolded proteins. Among these HSPB members, HSPB8 is probably the most studied in this context. Overexpression of HSPB8 efficiently prevents aggregation of different polyQ-containing proteins (huntingtin, ataxin-3 and androgen receptor (AR), responsible for HD, spinocerebellar ataxia type 3 (SCA3) and SBMA, respectively) [1,47,101], as well as SOD1 (protein responsible for ALS) and various truncated forms of TDP-43s (associated with both ALS and frontotemporal dementia (FTD; table 1)) [43,95].

Figure 2 illustrates this for the mutant ARpolyQ that causes SBMA. Overexpression of HSPB8 in SBMA motor neurons leads to a substantial decrease in mutant ARpolyQ aggregates (IF, figure 2a) and large insoluble species (filter retardation assay, FRA; figure 2b,c), which in this disease are triggered by the AR ligand testosterone. Several data indicate that this anti-aggregation activity of HSPB8 is caused by a facilitation of autophagy-mediated degradation of the mutated proteins or their (initial) aggregates. In this process, HSPB8 collaborates with BAG3 [46] and HSPA8 (Hsc70) and CHIP [43,104]. Indeed, as shown in figure 2, the anti-aggregation effects of HSPB8 were greatly reduced in the presence of the autophagy inhibitors 3-methyladenine (3-MA) or bafilomycin (figure 2c).

Differently from many other members of the HSPB family (e.g. HSPB1 and HSPB7), HSPB8 forms a stable complex with the HSPA8 (Hsc70) co-chaperone BAG3, which may explain

why HSPB8 is so efficient in autophagy-mediated degradation of misfolded polyQ substrates. We previously showed that not only HSPB8 stability, but also its anti-aggregation and pro-degradative functions, depend on its association with BAG3, whose knock-down prevented HSPB8 from exerting its protective role [46]. Also, within the complex, BAG3, but not HSPB8, is responsible for the stimulation of autophagy [46]. This suggests that HSPB8 might play a role in the recognition and delivery of the cargo, via BAG3, to the autophagosomes for degradation. In addition, we found that HSPB8 (in complex with BAG3), besides participating in autophagy-mediated degradation of misfolded proteins, is also involved in the translational shut-down mediated by the induced phosphorylation of eIF2 alpha that occurs during proteotoxic stress. Interestingly, induction of phospho-eIF2 alpha, which we observed upon overexpression of HSPB8, BAG3 or of the complex, leads to both protein synthesis inhibition (which decreases the total load of proteins to be refolded or degraded) and autophagy stimulation (which clears the aggregated proteins accumulating during proteotoxic stress) [48]. While *in vitro* translation experiments indeed have revealed that HSPB8 can cause translational shut-down, precise insight into how HSPB8 can modulate translation upon stress is still missing. Similarly, future studies will be needed to reveal the precise role of HSPB8 in the complex and if and how client recognition and targeting to autophagy is coupled to its action on translation.

5. The anti-aggregation power of HSPBs depends on several factors

The aggregation propensity of misfolded proteins depends on several factors, including the exposition of hydrophobic residues, the alteration of specific conformed domains, the capability to generate beta-plated sheets, and so on. These factors may affect the kinetics of aggregation, the biophysical nature of aggregates that are ultimately formed (e.g. insolubility and reversibility) and also the location inside the cells where these proteins may accumulate [105]. All of these may obviously also affect the possibilities of diverse HSPBs to interact and deal with these various proteins before or after they have aggregated. Even for apparently similar clients like the proteolytic polyQ-containing fragments, the length of the polyQ stretch and the kinetics of

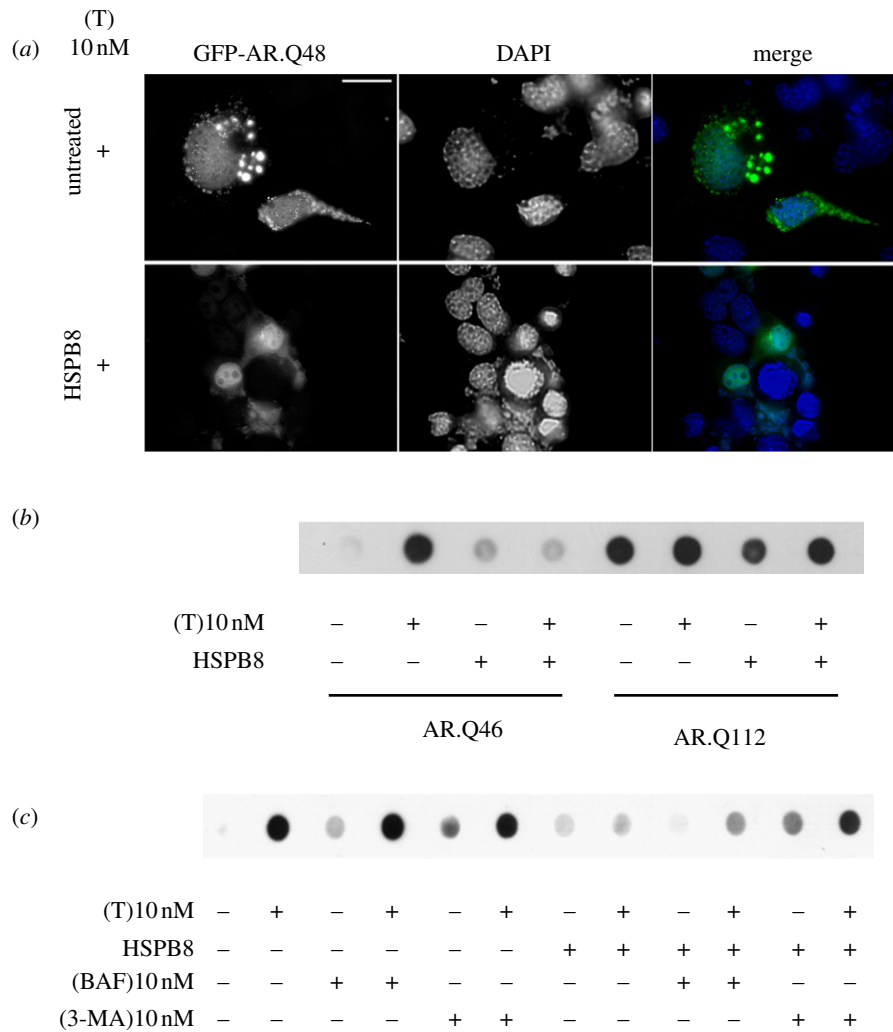


Figure 2. (a) High-resolution fluorescence microscopy analysis of NSC34 cells transfected with GFP-AR.Q48 and an empty vector (pCDNA3/untreated) or pCNEO-cMyc-HSPB8 (expressing rat HSPB8). Cells were treated with 10 nM of testosterone (+T) for 48 h and fixed using a 1:1 solution of 4% saccharose and 4% paraformaldehyde in 0.2 N PB (25 min at 37°C under weak agitation), and then in ice-cold methanol (10 min, RT). Nuclei were stained with DAPI (dark grey, blue). Images were obtained at 63 \times magnification. Fluorescence analysis showed that T-treated GFP-AR.Q48 formed intracellular aggregates that are completely removed by HSPB8; a massive decrease of mutant ARpolyQ protein levels was also detectable, suggesting that HSPB8 induces the removal of mutant ARpolyQ from immortalized motor neurons. Scale bar, 10 μ M. (b) The effect of HSPB8 on the aggregation propensity of two AR mutants characterized by a polyQ of different length was tested using filter retardation assay (FRA). NSC34 cells co-transfected with plasmids coding for AR.Q46 or AR.112 and either an empty vector (pCDNA3) or pCNEO-cMyc-HSPB8 (expressing rat HSPB8) were treated with ethanol (–T) or with 10 nM of testosterone (+T) for 48 h. Cell lysates were prepared for FRA as previously described [86]. (c) The involvement of the autophagic pathway in the pro-degradative effects of HSPB8 on mutant AR.Q46 was tested by FRA after selective blockage of the autophagic flux. NSC34 cells were co-transfected with plasmids coding for AR.Q46 and an empty vector (pCDNA3) or pCNEO-cMyc-HSPB8 (expressing rat HSPB8) and were treated with ethanol (–T) or with 10 nM of testosterone (+T) for 48 h. Autophagic flux was modulated using treatments for 24 h with either 10 mM of 3-methyladenine (3-MA, acting on autophagosome assembly) or 10 nM of bafilomycin (BAF, acting on the process of fusion between autophagosome and lysosome as well as on the ATP-dependent H⁺-pump [103]). Cell lysates used for FRA were prepared as previously described [86]. (Online version in colour.)

aggregation (the longer the polyQ repeat, the faster the aggregation [1]) were found to impede the possibility of the various HSPB members to suppress aggregation. While HSPB8 and HSPB9 were equally (or even a bit more) effective compared with HSPB7 on huntingtin fragments with relatively short expansions (43Q, figure 1 [1]) only HSPB7 was efficient in suppressing aggregation of the longest expansions tested (119Q; see [1]). For HSPB8, similar observations were made in our previous findings showing that HSPB8 efficiently decreases both soluble and insoluble levels of huntingtin fragments with relatively short expansions (43Q; [46,47]), but has no effect on long expansions (Q119; [1]). We also tested the selective effect of HSPB8 on ARpolyQ containing a stretch of different size (Q46 versus Q112), both being in the pathological range (even if no SBMA patients with Q112 have been described so far). The two types of ARpolyQs

have a marked difference in their aggregation power, in response to the AR ligand [72,73]. While HSPB8 almost completely counteracts AR.Q46 aggregation, induced by testosterone, its effects on AR.Q112 are much lower, since there is only a relatively small decrease in the total amount of insoluble ARpolyQ (figure 2b).

These differences in the efficiency of HSPB7, on the one hand, and HSPB8 and HSPB9, on the other hand, to prevent aggregation of polyQ proteins with different expansion sizes clearly suggests that their modes of action to deal with these disease-related proteins must be largely different. HSPB8 and also HSPB9 (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data) seem to act by lowering the level of soluble species (non-aggregated or/and early aggregate intermediates) apparently maintaining these substrates in a state competent

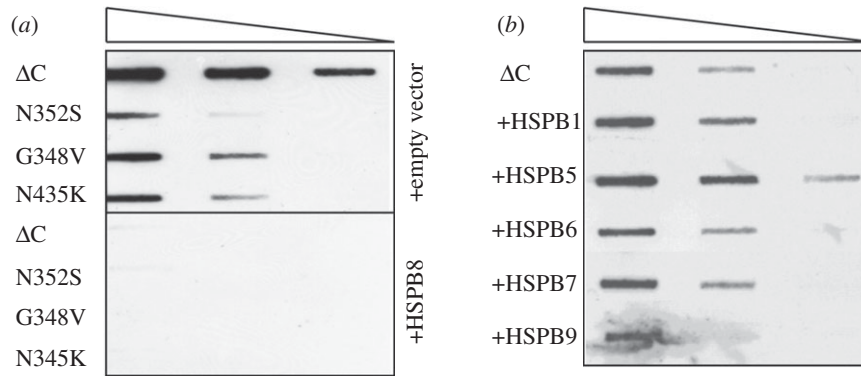


Figure 3. HSPB8 blocks the aggregation of several disease-associated mutant forms of TDP43. (a) HEK293 cells were transfected with vectors encoding for different disease-associated mutant forms of TDP43 (Δ C, N352S, G348V or N345K, kindly provided by Dr E. Buratti) and either an empty vector or a vector encoding for human HSPB8. Cellular lysates were prepared 72 h post-transfection and the aggregation of TDP43 mutants was analysed by FTA (as previously described; [43]). (b) HEK293 cells were transfected with a vector encoding for Δ C TDP43 and either an empty vector or vectors encoding for various members of the HSPB family (V5-HSPB1, V5-HSPB5, V5-HSPB6, V5-HSPB7 and V5-HSPB9). Aggregation of Δ C TDP43 was analysed as described in (a).

for degradation, thus disposing of them before they form large, insoluble species. In the case of HSPB8, the lowered levels of soluble mutant polyQ proteins might be due, at least in part, to its effects on eIF2 alpha phosphorylation and translational attenuation [48]. Also, HSPB8, together with the co-chaperone BAG3, the chaperone HSP70 and the ubiquitin-ligase CHIP, works through stimulating autophagic degradation [43,46,48,106], thus also taking care of early nucleating species. Concerning HSPB9, it was found to stimulate proteasomal degradation (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data), which may lower the number of nucleating species of the polyQ proteins; however, when nucleation is initiated, which occurs more readily with longer polyQs, HSPB9 might become ineffective. Since HSPB9 is only expressed in testis, HSPB9 upregulation should not play a role in neurodegenerative diseases. However, as stated before, it is important to underline that pharmacologically induced upregulation of specific members of the HSPB family (normally not expressed or upregulated) in a specific neuronal cell type might still result in protection, therefore representing a good therapeutic approach. For those HSPB members that are already expressed in target cells, drugs may be designed that stimulate their (chaperone-like) activity, e.g. by acting on the phosphorylation or oligomeric status of the HSPB members. Alternatively, or in case HSPB members are not already expressed in the target cells, analysis on expression regulation of the various HSPB members combined with drug screens using reporter constructs may identify routes towards boosting or inducing expression of individual disease-ameliorating members.

HSPB6, which only effectively suppresses shorter polyQs [1,107], also plays a role in modulating autophagy [108]. By contrast, HSPB7 does not change the rate of proteasomal degradation and does not increase the autophagic flux; rather, it appears to prevent early aggregates from nucleating into inclusions with sizes that are too large to be handled by the autophagic machinery, probably by marking these early seeds, which enables their shuttling into the autophagosomes [1,57,109,110]. This HSPB7 action thus does not rely on the speed of seed formation but rather on the rate at which these seeds grow (and thus is less dependent on the size of the expansion).

Another aspect that might influence the efficacy of the diverse HSPBs to prevent mutated protein aggregation is represented by specific physical properties of the mutant protein

and aggregate itself. Indeed, the effects of HSPB members on different aggregation-causing mutants seem to differ widely (table 1). It has been shown that, while polyQ proteins form aggregates with a core that is inaccessible to nascent proteins, mutated SOD1 (G85R/G93A), associated with ALS, forms a porous aggregate, through which nascent proteins can diffuse [110]. As stated above, we have already shown that overexpression of HSPB8 efficiently prevented the aggregation and facilitated the autophagy-mediated degradation of mutated SOD1 and of various mutated forms of TDP43, which is associated with both ALS and FTD [43] (figure 3a). When we compared the HSPB8 activity to that of other HSPB family members with anti-aggregation activity towards mutated polyQ proteins, namely HSPB6, HSPB7 and HSPB9, none of these blocked the aggregation of the truncated mutant form of TDP43 (TDP43 Δ C; figure 3b: HSPB1-5-6-7-9; table 1). HSPB1 and HSPB5, which mainly showed pro-refolding activity, were also unable to prevent aggregation of TDP43 Δ C (figure 3b). HSPB9 seems to have a mild effect on TDP43 Δ C. Why HSPB6 and HSPB7 showed no anti-aggregation activity towards TDP43 Δ C, while being very active towards mutated polyQ proteins [1,107] (table 1), is still unclear and will be investigated in the future.

Finally, another factor that can explain the differential efficacy of the various members of the HSPB proteins in inhibiting protein aggregation is the stage at which they act. As previously mentioned, many neurodegenerative diseases, including AD, polyQ diseases and PD, are characterized by the accumulation of fibrillar proteinaceous aggregates in specific neuronal types [111–117]. The formation of these fibrillar aggregates consists of a multi-step process involving a nucleation step and the subsequent elongation of the fibrils [118]. Interestingly, it has been shown that some HSPBs can only prevent protein aggregation at a specific stage. For example, HSPB5, which showed very high refolding capacity in cells and *in vitro* but no anti-aggregation activity towards mutated polyQ proteins [1], inhibits fibrillar aggregate formation of mutated alpha-synuclein, associated with PD, only at early stages. HSPB5 binds to partially folded monomers of mutated alpha-synuclein, thereby preventing mature fibril formation and shifting the equilibrium to monomer fibrils, which can be easily disposed/degraded [35,119,120]. A similar mechanism has been shown for ataxin-3. HSPB5 can significantly inhibit the first stage of

ataxin-3 aggregation [121], by directly interacting with the Josephin domain of ataxin-3, which has an intrinsic tendency to aggregate and form fibrils [122]; however, HSPB5 is ineffective on already formed SDS-insoluble fibrils of mutated ataxin-3. All together, these data strongly suggest that HSPB5 acts as a chaperone specifically towards growing fibrils at an early stage and may have only limited protective powers because it cannot block protein aggregation at later stages and does not seem to target its bound substrate to the proteolytic pathways. They also suggest that, in order to be efficient in inhibiting protein aggregation, upregulation of HSPB5 should either take place at a very early stage of disease or occur concomitantly with the upregulation of other chaperones able to bind to and target intermediate species and/or fibrils to degradation.

6. Subcellular localization of the aggregate-prone species and HSPBs

Finally, subcellular localization can influence the efficiency of the various members of the HSPB family in inhibiting the aggregation of a specific misfolded substrate involved in neurodegenerative diseases. In fact, while the ubiquitin proteasome system is present and active both in the nucleus and in the cytoplasm, autophagy (which can be facilitated by some HSPB members; see earlier) is confined to the cytoplasm [123,124]. HSPB8 and HSPB6, which mainly rely on autophagy, are expected to mainly act on cytoplasmic aggregating proteins. Indeed, HSPB8 could only efficiently block the aggregation and facilitate the autophagy-mediated clearance of ARQ46, which is located in the cytosol, but had very limited effect on ARQ112, which is aggregating inside the nucleus. Considering that the cytoplasmic retention of a mutated form of AR containing a Q112 tract ameliorates disease via autophagy [125], strategies that allow keeping the mutated proteins in the cytosol, where several HSPBs (and other molecular chaperones) can participate directly or indirectly in their targeting to the autophagosomes for degradation, will slow-down disease progression. Whether some HSPBs can also participate in modulating the shuttling of the mutated proteins from the nucleus to the cytoplasm and whether this may contribute to their protective effects is still unknown.

7. HSPB-mediated protection can also be independent of anti-aggregation/pro-refolding activities and/or autophagy facilitation

HSPB1, which shows good refolding capacity, had no anti-aggregation activity in cells against mutated huntingtin, both with short and long polyQ stretches [1] (table 1). However, overexpression of HSPB1 could inhibit the aggregation of mutated SOD1 [20] (table 1) and could also prevent the toxicity mediated by several polyQ-containing proteins [19]. This may depend on the different physical properties of the aggregating substrates (e.g. immobile versus mobile aggregates), as stated before, although so far no experimental proof supporting such a hypothesis exists. However, in the case of HSPB1, the protective effects may also be due to other specific functions, which are not related to refolding

or inhibition of aggregation, but rather to its ability to prevent the activation of APAF-1 by the cytochrome C released from the mitochondria, that will trigger the apoptotic caspase cascade, as well as on its anti-oxidants effects [67]. In fact, overexpression of HSPB1 exerts a protective effect and significantly decreases cell death in cellular models of HD, characterized by high oxidative stress, by maintaining the redox state of the cell without showing effects on aggregation [19]. Moreover, in neuronal-like cells, HSPB1 could also protect against the toxicity mediated by mutated ataxin-3, associated with SCA3, where increased oxidative stress has been suggested to play a role in disease pathogenesis, again without effects on aggregates. Interestingly, overexpression of mutated ataxin-3 correlated with a reduction in the expression levels of HSPB1, suggesting that a decrease in the function of HSPB1 (not directly related to effects on the diseased protein as such) may play a role in disease progression. Such a decrease in HSPB1 expression has also been documented in other forms of SCA diseases, like SCA7 [126,127] and in transgenic mice models of SCA-17 [128]. This would imply that re-introduction of HSPB1 can compensate for such a loss of function of HSPB1 as a factor contributing to disease progression. However, data in HD mice did not reveal such an effect [129], suggesting that this may not apply to all polyQ diseases.

8. Conclusions and perspectives

Although similar in terms of primary sequence, the various members of the mammalian HSPB family are differentially expressed in tissues and cells, have different abilities to form homo- and hetero-oligomers, show other non-HSPB partner interactions and display different functions. The tissue-selective expression pattern of the members probably reflects a highly specific need of a given HSPB to assure proper function and viability of that cell type/tissue, and a particular HSPB may exert a protective function in a specific cell type. Some functional redundancy does exist between the various members, as evidenced by findings that several HSPB members can handle the same (un- or misfolded) protein equally (e.g. assist their (re) folding). However, HSPB members possess different affinity and specificity for clients and may handle the same client differently (e.g. routing them to proteasomal or autophagosomal degradation). Thus, depending on their client specificity and mechanism of action, only upregulation of specific HSPB members would exert protective functions against neurodegeneration. Moreover, impaired HSPB function may harm certain tissues more than others and explain why certain HSPB mutants have been linked to tissue-specific (e.g. motor neurons and muscle cells) degeneration. In addition, the same HSPB may have different biochemical activities: depending on their oligomeric status, they may independently function as chaperones for soluble proteins, as stabilizers/chaperones for cytoskeletal elements, or as modifiers of the cellular redox state. As a consequence of these properties, the same HSPB member may protect more or less efficiently and certain HSPB members may be better targets than others.

In general, a better understanding of the client specificity and functional diversity of the various HSPBs will be required in order to target a specific member or functionality thereof for therapeutic purposes.

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