Small heat shock proteins
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A comparative screen of the human HSPB family of molecular chaperones reveals HSPB7 as a superior suppressor of aggregation of polyglutamine containing proteins

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To be submitted
Abstract

A small number of heat shock proteins have previously been shown to act protectively on aggregation of several proteins containing an extended polyglutamine tract, which are linked to several neurodegenerative diseases. A specific subfamily of heat shock proteins is formed by the HSPB family of molecular chaperones, of which a few members are known to act as anti-aggregation proteins in vitro. Furthermore, mutations in a subset of HSPB members are linked to neuropathies, underlining the importance of this subfamily in protein folding, aggregation and neurological diseases.

Using overexpression, we screened the HSPB family for novel inhibitors of polyglutamine aggregation and provide a comparative overview on the human HSPB family in view of preventing polyglutamine aggregation. This screen revealed several HSPB members as novel suppressors of polyglutamine aggregation, with HSPB7 as the most active one.

Interestingly, this HSPB protein was found to function independently of the HSP70 machine and heat-shock response. Furthermore, it did not target polyglutamine proteins for proteasomal degradation, but was found to depend in part on autophagy for its activity. Moreover, it prevented polyglutamine induced toxicity in cells and in an in vivo model for SCA3, showing the potential of HSPB7 as a potential therapeutic target.
**Introduction**

Several inherited neurodegenerative diseases exist which are based on a CAG triplet expansion in the affected genes. This results in elongation of the glutamine (polyQ) tract present in the proteins where, in general, an extension beyond 35-40 glutamines causes disease whereby the age at onset is related to the length of the expansion (1). These diseases include amongst others polyQ extensions in the androgen receptor, the TATA-box binding protein, huntingtin and several ataxia-related genes (SCA genes). The corresponding diseases are characterized by protein misfolding and aggregate/inclusion formation of the affected proteins with a concurrent increase in proteotoxic stress.

From (cellular) studies it has become clear that certain heat shock proteins, as molecular chaperones, form a potent natural defense against proteotoxic stress induced by these protein misfolding diseases. Heat shock proteins are divided into several main families which are both structurally and functionally highly divergent (2). Within the HSPH (Hsp110), HSPA (Hsp70) and DNAJ (Hsp40) families, several members have been found that can reduce aggregation and toxicity associated with polyglutamine (polyQ) containing proteins and other misfolded proteins (3-10). Also within the mammalian small heat shock protein (HSPB) family, some members, in particularly HSPB1 and HSPB8, have been reported to suppress toxicity or aggregation of neuropathy-causing mutated proteins (11;12). In cells, HSPB1 suppresses mutant huntingtin-induced reactive oxygen species formation and cell death which is dependent on the phosphorylation status of HSPB1 (13). In a mouse model of Huntington disease (HD), however, HSPB1 overexpression did not improve the HD phenotype and furthermore it failed to form large HSPB1-substrate complexes which are implicated in chaperone activity (14). HSPB8 acts via another mechanism and modulates toxicity of HD via the induction of autophagy and a translational shut-down (15;16). Whether or not HSPB8 can also rescue HD pathology in vivo is yet unknown. Furthermore, the potency of the other HSPB members in reducing proteotoxic stress remains unknown.

The human HSPB family consists of eleven members and the amino acid sequence variation between members of this family is larger than in any of the other HSP families. Here, we present a systematic comparison of ten members of the human HSPB family for their ability to modulate either HD or SCA3 aggregation or assist in the refolding of a heat-denatured substrate. The effectiveness to prevent aggregation largely differed between members and, in part, was dependent on the type of the misfolded substrate. However, within the HSPB family, HSPB7 was overall the most potent suppressors of HD and SCA3 aggregation. In addition, HSPB7 was able to reduce polyQ related toxicity in cells and protected against polyQ-related eye-degeneration in a Drosophila SCA3 model. In contrast to e.g. HSPB1, HSPB7 is not a classical chaperone since it is unable to assist in refolding of heat denatured proteins. In addition, it does not depend on the HSPA chaperone machine for its anti-aggregation activity. Although its precise mode of action remains to be elucidated, HSPB7 is in part dependent on autophagy to decrease polyQ aggregation.

**Experimental procedures**

**Reagents and antibodies**

Chemicals were obtained from Sigma, beetle luciferin from Promega. Antibodies against the V5 tag (Invitrogen), EGFP (JL-8) (BD bioscience), GAPDH (6C5) (RDI research diagnostics), HA tag (Covance), HSPB7 (Abcam) and HSPA1A (SPA-800), HSPA6 (SPA-754) from Stressgen were mouse monoclonal. The antibodies against DNAJB1 (SPA-400) (Stressgen) and HSPB5 (Abcam) were rabbit-polyclonal. Reagents for Western blotting were obtained from Amersham.
Secondary antibodies for immunocytochemistry used were CY5 anti-mouse and FITC anti-mouse (Jackson) and CY3 anti-mouse (Amersham).

**Molecular techniques**

Standard recombinant DNA techniques were carried out essentially as described by Sambrook et al. Oligonucleotide primers (Biolegio). Restriction enzymes and DNA polymerases were used according to the manufacturer’s instructions (Invitrogen, New England Biolabs). DNA sequencing reactions were carried out by ServiceXS.

**Plasmid generation**

The truncated SCA3 fragments were PCR amplified from skin fibroblasts from heterozygous SCA3 patient with 64 and 82 CAG-repeat present in the Ataxin3 gene and cloned into pCMV-EGFP and pCDNA5.1-FRT/TO-EGFP respectively, in frame with EGFP. Plasmid containing either HDQ43 or HDQ74 were kindly provided by Dr. David Rubinsztein (Cambridge, UK). Generation of the human HSPB plasmid library was described before as well as the HSPB7 deletion mutants (Chapter 3). For retroviral transduction, the pQCXIN vector (Clontech) was used for cloning. The Ub-R-EGFP construct was kindly provided by Dr. Dantuma (Karolinska Institutet, Sweden) The construct encoding His-BAG3 and HA-BAG1 have been described before (21;44). The construct encoding the dominant negative HSF-1 was kindly provided by Prof. dr. N.H. Lubsen (Nijmegen, the Netherlands).

**Cell lines, cell culture, transfections and transductions**

NG108, mouse embryonic fibroblasts (MEF) and human embryonic kidney 293 cells expressing the tetracycline repressor (Flp-In T-Rex HEK293, Invitrogen) were grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal calf serum (Griener Bio-one) at 37 °C under a humidified atmosphere containing 5% CO₂. The generation of HEK293-HDQ119, with integration of a tetracycline regulatable expression vector coding for EGFP-HDQ119, has been described before (Hageman et al. submitted elsewhere). Gene expression in Flp-In T-Rex HEK293 cells was induced with a final concentration of 1 μg/mL tetracycline. HEK293 cells and MEF cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions using 1μg of plasmid DNA per 35-mm dish. NG108 cells were differentiated into neuronal-like cells by addition of 2 mL differentiation medium (DMEM containing 1 mM sodium pyruvate, 50 μM 3-Isobutyl-1-methylxanthine, 10 μM 5’-(N-Ethylcarboxamido)adenosine, 0,5% FCS) to 35000 cells in a 35mm dish. Seven days later, cells were transfected using Effectene (Qiagen) according to the manufacturer’s instructions using 0,6 μg of plasmid DNA per 35-mm dish. For transduction, retroviral particles were generated by co-transfection of HEK293-T cells with pCL-ampho and pQCXIN endoding either HSPB5 or HSPB7 using Fugene (Roche) according to the manufacturers instructions. The supernatant was collected after 48 hours and filtered using a Millex-HV filter (Millipore). Cells were transduced by adding 1,5 mL of filtrated medium containing 4 μg/mL polybrene. Medium was replaced after 24 hours and transduced cells were selected using puromycin selection.

**Drosophila**

Fly stocks were maintained at 22°C according to standard protocols. GAL4 driver stocks were obtained from the Bloomington Stock Centre (Indiana University, USA). The GMR-UAS-SCA3Q78 fly used for the eye-degeneration screen was generously provided by N. Bonini (University of Pennsylvania, USA) and maintained at 25 °C. HSPB7 transgenic lines were generated.
by Genetic Services Inc. (Sudbury, USA) by injection of the pUAS transformation vector into the W1118 genetic background.

**Westernblot analysis**

Samples were prepared in Laemmli sample buffer. Equal amount of protein were separated on 10% or 12.5% SDS-PAGE gels followed by transfer onto nitrocellulose membranes. Primary antibodies were used at the following dilutions: V5 (1:5000), EGFP (1:5000), GAPDH (1:10000), HSPA1A (1:5000), HSPA6 (1:5000), DNAJB1 (1:5000). After a one hour incubation with the primary antibody in PBS-TWEEN20, membranes were incubated with HRP-conjugated secondary antibodies at a 1:5000 dilution. Detection was performed using enhanced chemiluminescence.

**Filtertrap assay**

The filtertrap assay was performed basically as described by Carra et al. (12). Briefly, cells grown in a 35mm dish were lysed in 200 μL FTA buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 50 mM dithiothreitol) containing 2% SDS. 100 μL, 20 μL and 4 μL sample was applied onto a prewashed (FTA, 0.1% SDS) 0.2 μM cellulose acetate filter contained in a Bio-Dot microfiltration apparatus (BioRad). Gentle suction was applied to filtrate the samples followed by one washing step using FTA, 0.1% SDS. Trapped material was probed with anti-EGFP (GL-8) or anti-HA at a 1:5000 and 1:2000 dilution respectively followed by incubation with HRP-conjugated secondary antibodies at 1:5000 dilution. Detection was performed using enhanced chemiluminescence.

**Microscopy**

For microscopy, cells were plated 24 hours before transfection. For fixation, the coverslips were washed with cold PBS and fixed for 15 minutes with methanol (-20°C). Cells were permeabilized in blocking solution (100 mM glycine, 3% BSA, 0.1% triton) for 1 hour followed by 1 hour incubation with the primary antibody (V5 anti-mouse, 1:200, SC-35 anti-mouse, 1:10000, MYC anti-rabbit 1:200). After three washing steps, coverslips were incubated for 1h with CY3-conjugated anti-rabbit secondary antibody (Amersham Biosciences) at 1:200 dilution or with FITC-conjugated anti-mouse secondary antibody (Jackson) at 1:200 dilution. After three washing steps, the coverslips were mounted using Citifluor mounting medium (Citifluor Ltd). Images were obtained using an inverted confocal laser scanning microscope (TCS SP2, DM RXE, Leica) with a 63×/1.32 NA oil objective.

**Luciferase refolding assay**

Chaperone activity of HSPB members was assessed by using the luciferase refolding assay (41). Briefly, HEK293 cells were co-transfected with luciferase (NucSuperluc-EGFP) (59) together with HSPB encoding plasmids (1:9 ratio). Two hours after transfection, expression was induced by addition of tetracycline. After 24 hours, cells were resuspended and divided into 1 mL portions in tissue-grade 10 mL tubes. The following day, cells were given a heat-shock (30 minutes at 43°C) in the presence of cycloheximide (20 μg/mL) and 4-morpholinoethanesulfonic acid (MOPS; 20 mM, pH7.0) in order to inhibit protein synthesis and increase the buffer capacity of the medium respectively. After heat treatment, cells were allowed to recover before luciferase activity was determined (3 hours at 37°C). Luciferase activity measurements were performed using a Berthold Lumat 9510 luminometer (Berthold Technologies).

**Sucrose gradient centrifugation**

Cells from a 35mm dish were scaped in 200 μL cold lysis buffer (150 mM NaCl, 50 mM NaH2PO4, 10 mM imidazole, 0.5% NP-40, 1.5 mM MgCl2 and 3% glycerol, pH8). Cells were lysed by five passages through a 25 gauge needle, followed by centrifugation at 300G for 15 minutes.
The cell lysate was loaded on top of a 10-80% sucrose gradient (10 mM Tris-HCl, pH 8, 5 mM EDTA, 50 mM NaCl) and centrifuged for 18 hours at 100000g using a Sorvall Discovery 90SE ultra centrifuge and the SW55 rotor. Fractions of 400 μL were precipitated by adding an equal volume of 25% (w/v) trichloroacetic acid. After a 30 minute incubation on ice, proteins were pelleted at 14000 rpm for 15 minutes. The pellet was washed twice with 80% acetone (-20°C) and allowed to dry. The pellet was dissolved in 20 μL 0,1M NaOH containing 1% SDS. An equal amount of Laemmli buffer was added and samples were boiled for five minutes followed by Western blot analysis.

**Clonogenic assay**
A clonogenic assay was used to determine cellular survival when overexpressing EGFP-HDQ119 without or with HSPB members. Exponentially growing cells were plated in triplicate in 10 cm dishes at a density of 200 cells per dish. EGFP-HDQ119 expression was activated by addition of tetracycline. Cells were allowed to grow and form colonies for two weeks, after which they were fixed and stained (0,1% Coomassie Brilliant Blue, 50% methanol, 10% glacial acetic acid followed by washing (10% methanol, 7.5% glacial acetic acid). The effect of EGFP-HDQ119 expression and co-expression of HSPB5 and HSPB7 was determined by comparing colony reduction in the presence of EGFP-HDQ119 expression.

**Proteasome inhibition, proteasome activity measurement and autophagy analysis**
Proteasomal activity was blocked by addition of 10 μM MG132 to the culture medium for 12 hours. Proteasomal activity was monitored by co-transfection of UB-R-EGFP followed by Westernblot- and FACS analysis. The role of autophagy in reducing polyQ aggregation was analysed using mouse embryonic fibroblasts lacking the ATG5 gene which was kindly provided by Dr. Mzushima, Okazaki, Japan.

**Results**

**HSPB7 is the most potent HSPB member in preventing polyglutamine aggregation**
Certain members of the HSPB family have been reported to suppress protein aggregation in vitro using several model proteins (17-21). Specific substrates include amongst others, amyloid beta (19), desmin filaments (20) and mutated huntingtin (21). Apart from these specific substrates, HSPB proteins can bind to many other non-native proteins in general (22-24). In cell free systems, several HSPB members were shown to be rather generic in preventing aggregation of a number of unrelated proteins and support their refolding via interacting with the HSPA1A (HSP70) machine (25). In cells, HSPB proteins are found to interact with cytoskeletal elements but may also here act rather promiscuously in preventing protein aggregation and support protein refolding (26-28).

To compare the effectiveness of the HSPB family in dealing with disease-related misfolded proteins, we co-expressed them together with either mutant Huntingtin exon 1 (Htt) or a fragment of Ataxin-3 (SCA3), both containing an expanded glutamine tract. Using the filtertrap assay (29) to detect protein aggregates, we found that aggregation of a moderately expanded HD protein (HA-HttQ43) was strongly reduced by co-expression of four HSPB members (HSPB6, HSPB7, HSPB8, HSPB9), whereas HSPB1 and HSPB4 were only marginally effective (Figure 1a). HSPB2, HSPB3, HSPB5 and HSPB10 showed no activity at all. Using a HD fragment containing a longer polyQ tracts (EGFP-HttQ74), co-expression of HSPB7 and HSPB9 resulted in a considerable reduction of aggregated material, while co-expression of either HSPB1, HSPB4
**HSPB7** is a novel suppressor of polyglutamine aggregation

HEK293 cells were co-transfected with V5-tagged HSPB members and polyglutamine encoding plasmids at a 9:1 plasmid ratio. Cells were lysed after 24 or 48 hours and analysed by the filtertrap-binding assay using five-fold dilutions. Several HSPB members were able to suppress polyglutamine aggregation of a protein with (A) 43 glutamines; HA-HttQ43 (B) 74 glutamines; EGFP-HttQ74 and (C) 119 glutamines; EGFP-HttQ119. (D) To compare the effect of the V5-tag, also non-tagged HSPB members were co-expressed with the EGFP-HttQ74 encoding plasmid, (E-G) Expression levels of the polyglutamine proteins and HSPB members were analyzed by Western blot analysis.

**Figure 2. The human HSPB family contains potent suppressors of polyQ expanded SCA3 aggregation.** (A) HEK293 cells were co-transfected with V5-tagged HSPB members and truncated EGFP tagge dSCA3 containing a stretch of 82 glutamines at a 9:1 plasmid ratio. After 24 hours cells were lysed and analyzed by the filtertrap-binding assay using five-fold dilutions. (B) Expression levels of EGFP-SCA3-Q82 and HSPB members were analyzed by Western blot analysis.
or HSPB6 was less effective (Figure 1b). At a glutamine length of 119, only HSPB7 was still effective in reducing the amount of aggregated material, while HSPB9 showed only a marginal effect (Figure 1c). Repeating the screen with the non-tagged HSPB library and EGFP-HttQ74 led to similar results, i.e. that HSPB9 and especially HSPB7 are the most potent suppressors of polyQ aggregation (Figure 1d), indicating that the V5 tag had not affected the function of most HSPB members. Under all conditions of overexpressed HSPB members, the expression levels of the soluble fraction of the various Huntingtin proteins was not largely affected (Figure 1 e-g) meaning that different substrate concentrations could not explain differences in effectiveness of the various HSPB members. The difference in aggregation suppressive function of the various HSPB members was also not due to differences in expression levels or higher stability of the HSPB proteins themselves; in fact, HSPB7 and HSPB9 expression levels were amongst the lowest of the whole HSPB family (Figure 1e,f). This means that their effectiveness may even be somewhat underestimated here.

We next repeated the screen using another polyglutamine expanded protein, Ataxin-3 (EGFP-SCA3Q82) (Figure 2a). Also for this substrate, HSPB7 and HSPB9 were the most effective
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suppressors; HSPB1, HSPB4 and HSPB8 also showed some, but minor activity (Figure 2a). In accordance with data from the HD screen (Figure 1e,f,g), HSPB effectiveness against aggregation was not accompanied by a detectable reduction in soluble SCA3Q82 levels (Figure 2b).

**HSPB7 activity is concentration dependent and active in the nucleus**

Transient overexpression can often lead to non-physiologically high levels of expression per cell. Therefore, we titrated the levels of HSPB7 by varying the ratio between HSPB7 and polyQ expressing plasmids and analyzed the effects on suppression of polyQ aggregation. Exclusively cytosolic inclusions were found when EGFP-HttQ74 was overexpressed (Figure 3a). This was markedly reduced when HSPB7 was co-expressed (Figure 3a). An increase in the HSPB7:EGFP-HttQ74 ratio (Figure 3b), was paralleled by a concurrent reduction in the extend of polyQ aggregation (Figure 3c). HSPB7 overexpression did not result in a reduction of soluble polyQ levels (Figure 3b), suggesting that rapid substrate degradation or translational inhibition are not the major pathways by which HSPB7 prevents aggregate formation. Next, we tested if HSPB7 could also prevent nuclear polyQ aggregation, a main feature of polyQ diseases related to cytotoxicity (4). Expression of a nuclear localization signal (NLS) containing EGFP-HttQ74 construct shows the formation of solely nuclear aggregates (Figure 3d). Clearly, co-expression of HSPB7 also prevented aggregate formation in the nucleus (Figure 3d) in a concentration dependent manner (Figure 3e,f). Again, no large effects on soluble polyQ levels were observed (Figure 3e).

**HSPB7 does not target polyQ proteins for proteasomal degradation**

Classical chaperone activity towards misfolded and non-foldable proteins is generally found to intertwine with cellular degradation pathways. Heat shock proteins such as HSPA1A, DNAJB1 and DNAJB2 were shown to promote degradation of misfolded proteins through the ubiquitin-proteasome system (30;31) while e.g. the Drosophila HSP40 member Mrj (ortholog of mammalian DNAJB6/DNAJB8) and human HSPB8 were shown to induce autophagy (15;32). Although HSPB7 did not visibly reduce soluble HD or SCA3 levels (Figure 1f, 2b, 3b,e), part of the misfolded or aggregated substrate could specifically be targeted for degradation by either of these pathways. To test whether HSPB7 could promote proteasomal degradation we co-transfected cells with HSPB7 together with HA tagged HttQ43 and treated cells with the proteasome inhibitor MG-132. Under such conditions, HSPB7 was still active in preventing aggregate formation (Figure 4a), whereas the protective action of DNAJB1, known to prevent polyQ aggregation by stimulating proteasomal degradation (30), was clearly reduced (Figure 4a). In addition, we used the proteasome activity reporter Ub-R-EGFP (33); when co-expressed together with DNAJB1 the Ub-R-EGFP levels were strongly reduced, confirming the role of DNAJB1 in proteasomal degradation (Figure 4b). Co-expression of HSPB7, however, did not significantly affect the expression level of this proteasome activity reporter (Figure 4b). Co-expression of polyglutamine proteins (HA-HttQ43), that is sometimes suggested to negatively affect proteasomal activity (34), did not have an effect on proteasomal activity in our assays irrespective of chaperone co-expression (Figure 4b). Analysis of Ub-R-EGFP levels by fluorescent-activated cell sorting gave comparable results (Figure 4c). DNAJB1 enhanced proteasomal degradation while HSPB7 did not. These data suggest that HSPB7 does not reduce polyQ aggregation by enhancing proteasomal degradation.

**HSPB7 is partly dependent on autophagic clearance of aggregated polyQ proteins**

Although HSPB7 was also effective in the nucleus of cells, which would not support that it acts via autophagy, we decided to directly test the possibility that HSPB7 could prevent polyQ ag-
ggregation by stimulating autophagosomal clearance of misfolded proteins at least in the cytosol. Hereto, we co-expressed EGFP-HttQ74 with either HSPB7 or BAG3 in mouse embryonic fibroblasts with or without a disruption of ATG5, a key regulator of autophagy (35). BAG3 was used as a positive control as it was shown that, in complex with HSPB8, it induces elf2α phosphorylation and hereby reduced polyQ aggregation via protein synthesis inhibition and clearance of aggregates via autophagy (15;21). In cells positive for ATG5, polyQ aggregates are reduced when HSPB7 is co-expressed (Figure 5a) with no visible changes is soluble EGFP-HttQ74 levels (Figure 5b) unlike overexpression of BAG3 that also reduced polyQ aggregation in these ATG5 positive cells (Figure 5a), but with a concomittant large reduction in soluble EGFP-HttQ74 levels (Figure 5b). Cells lacking the ATG5 gene show more polyQ aggregation in control conditions (Figure 5c), consistent with the role of constitutive autophagy in clearance of polyQ aggregates (36;37). As demonstrated before, BAG3 overexpression in ATG5 -/- cells was less effective in reducing polyQ aggregation than in ATG5 +/- cells, although some activity remained, which can be attributed to its effect on protein synthesis leading to a large reduction of soluble EGFP-HttQ74 levels also in ATG5 -/- cells (Figure 5d) (16). In ATG5 -/- cells, HSPB7 activity was largely reduced independent of an effect on soluble EGFP-HttQ74 levels (Figure 5c, d). This suggests that, at least in the cytosolic compartment, HSPB7 co-operates with autophagic mechanisms to reduce aggregated polyQ material but in a manner that is distinct from the BAG3/HSPB8 complex.

**HSPB7 is not a classical chaperone**

The canonical mechanism by which small heat shock proteins are believed to function is that they form oligomeric complexes that, via dy-

**Figure 4. HSPB7 is not involved in proteasomal degradation.** (A) HEK293 cells were co-transfected with V5-tagged DNAJB1 or HSPB7 together with HA-HttQ43 at a 9:1 plasmid ratio. After 24 hour and 48 hours, samples were generated and analysed by the filtertrap binding assay. In addition, cells were treated for 12 hours with the proteasome inhibitor MG132. (B) Co-transfection of V5-tagged DNAJB1 or HSPB7 with the proteasomal activity reporter Ubr-EGFP, shows a reduction in Ubr-EGFP levels for DNAJB1 co-expression, while HSPB7 does not influence Ubr-EGFP clearance. Co-expression of HA-HttQ43 did not influence Ubr-EGFP clearance. (C) Fluorescent activated cell sorting measurements on Ubr-EGFP fluorescence levels show that DNAJB1, but not HSPB7, reduces the Ubr-EGFP fluorescence.
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Dynamic de- and re-oligomerization steps, bind unfolded substrates that are subsequently transferred to HSPA protein machines for further processing (38;39). First, we tested whether HSPB7 forms oligomers in cells, like the classical small HSPs, such as HSPB1 and HSPB5 do. Here, we expressed V5-HSPB5 or V5-HSPB7 in HEK293 cells and separated the cell lysates on sucrose gradients. While HSPB5 was found throughout the gradients, indicating formation of oligomeric species of various sizes, HSPB7 was mainly localized at low density indicative of its existence as a mono- or dimeric species (Figure 6a). Native gel analysis confirmed these findings: V5-HSPB1 clearly migrated consistent with oligomeric species like V5-HSPB5, while V5-HSPB7 migrated mostly as a single molecular species (Figure 6b). So, in living cells HSPB7 does not seem to form the canonical large HSPB oligomers.

To test whether, despite its non-classical characteristic, HSPB7 still could act as a classical chaperone, we used heat-denatured firefly luciferase as a model substrate (40-42). Cells expressing luciferase with or without co-expressed HSPs were heated for 30 minutes at 45 °C to denature luciferase and (chaperone-assisted) refolding was measured by allowing a 1 hour recovery period at 37 °C. Overexpression of the classical V5 tagged chaperones HSPB1, HSPB4 and HSPB5 increased luciferase refolding compared to cells co-expressing mRFPruby (a non-chaperone control) (Figure 6c), consistent with previous data (39;43). Overexpression of the other HSPB members, including HSPB7, showed no comparable activities on luciferase refolding (Figure 6c). Overexpression of non-tagged HSPB1 and HSPB7 gave similar results compared to the V5-tagged versions (Figure 6d), demonstrating that V5-tagging did not negatively influence refolding activity. This suggests that HSPB7 does not act as a classical chaperone at least not when firefly luciferase is used as a substrate.

Figure 5. HSPB7 efficacy is reduced in autophagy-deficient ATG5 -/- cells. (A) Wildtype MEF cells (ATG5 +/+) were co-transfected with V5-tagged HSPB1, HSPB7 or His-tagged BAG3 together with EGFP-HttQ74 (9:1 ratio). Cell lysates were analysed by filtertrap binding 48 hours after transfection. (B) Western blot analysis shows that BAG3, but not HSPB7, reduces the soluble pool of EGFP-HttQ74. (C) V5-tagged HSPB1, HSPB7 or His-tagged BAG3 were co-transfected with EGFP-HttQ74 (9:1 ratio) in MEF cells lacking the ATG5 gene (ATG5 -/-). Both BAG3 activity and HSPB7 activity was reduced. (D) Western blot analysis shows that BAG3, but not HSPB7, also reduced the soluble pool of EGFP-HttQ74 in ATG5 -/- cells.

V5-HSPB1  V5-HSPB7  His-BAG3
Control  V5-HSPB1  V5-HSPB7  His-BAG3
Signal intensity 0 20 40 60 80 100
Signal intensity 0 20 40 60 80 100
\(\alpha\)-GAPDH  \(\alpha\)-V5  \(\alpha\)-EGFP
V5-HSPB1  V5-HSPB7  His-BAG3
Signal intensity 0 20 40 60 80 100
Signal intensity 0 20 40 60 80 100
\(\alpha\)-GAPDH  \(\alpha\)-V5  \(\alpha\)-EGFP
HSPB7 is not dependent on a functional HSPA machine or activity of HSF-1

To explore whether the action of HSPB7 on poly-Q aggregation was dependent on collaboration with HSPA members, we initially aimed to down regulate HSPA8 and HSPA1, the endogenously expressed HSPA members in HEK293 cells (Hageman and Kampinga, unpublished observations). However, this turned out to be rather toxic to these cells. We therefore used an indirect approach to address this question and co-transfected the HEK293 cells with HSPB7 and EGFP-HDQ74 with or without BAG1. BAG1 is a nucleotide exchange factor that accelerates the ATPase cycle of HSPA and we previously showed that BAG1 overexpression leads to a negative effect on HSPA1A-mediated refolding (44) and hereby also interferes with the effect of HSPB1 on protein refolding (39). Co-expression of HSPA1A and BAG1 with either cytosolic or nuclear luciferase resulted in a decrease in refolding activity (Figure 7a), showing that HSPA mediated refolding is affected in both cellular compartments. However, BAG1 overexpression did not significantly affect HSPB7 activity on polyQ aggregation (Figure 7b), showing that HSPB7s major route of anti-aggregation does not require HSPA activity. To exclude that HSPB7 overexpression might induce a general stress response that upregulates other chaperones that in turn ensure clearance of poly-Q aggregates, we first measured levels of the stress inducible HSPA1A and DNAJB1. HSPB7 overexpression did not lead to a detectable induction of these classical heat-shock proteins (Figure 7c). In addition, we co-expressed EGFP-HttQ74 with either HSPB5 or HSPB7 in cells carrying a tetracycline inducible dominant negative HSF-1 (dnHSF-1) transgenic
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Figure 7. HSPB7 does not require a functional HSPA machine, induces no general stress response and is not dependent on HSF-1 responsive genes to reduce polyglutamine aggregation. (A) HSPA1A was cotransfected with BAG1, cytoplasmic luciferase or nuclear luciferase (4:5:4:5:1 ratio). Cells were heat shocked at 43°C for 30 minutes and allowed to recover after which luciferase activity was determined. In both compartments, co-expression of BAG1 inhibits the HSPA machine. (B) Co-expression of BAG1 with HSPB7 and EGFP-HttQ74 shows that BAG1 mediated inhibition of the HSP70 machine only marginally reduced the effect of HSPB7 on preventing polyQ aggregation. (C) HEK293 cells were transfected with either mRFPruby, V5-tagged HSPB5 or HSPB7 with (+) or without (-) HA-HttQ43 encoding plasmids. Levels of the stress-inducible HSPA1A and DNAJB1 were not altered under these conditions, indicating that none of them induced a general stress response. (D) The dominant negative HSF-1 cell line lacks HSF-1 inducible gene expression as can be seen by a failure of these cells to induce the expression of strictly heat-inducible HSPA6 within 12 hours after a 30 minutes heat shock at 43 °C. (E) Using either HEK293 cells or the dominant negative HSF-1 celline, V5-tagged HSPB5 or HSPB7 were co-transfected with EGFP-HttQ74 (9:1 ratio). In both cell lines, HSPB7 was found equally effective in suppressing HttQ74 aggregation.

construct which blocks HSF-1 dependent HSP expression. Expression of the dnHSF-1 construct effectively blocked the heat shock induced-expression of the HSF-1 inducible HSPA6 protein (Figure 7d). In these cells, that can no longer induce a stress response, HSPB7 retained its full activity to suppress poly-Q aggregation (Figure 7e). Thus, HSPB7 does not induce the general heat shock response and does not require induction of other heat shock proteins through HSF-1 in order to be functional.

HSPB7 sequence features in relation to anti-aggregation activity

HSPB7 was originally discovered as a cardiovascular HSPB member (cvHSP) but although it is indeed highly expressed in cardiac tissue (45), database analyses revealed that HSPB7 is more ubiquitously expressed than originally thought. In fact, it was found to be one of the
highest expressed HSPB members in human tissues at the messenger level which suggests a general function in cells (Chapter 3). Furthermore, HSPB7 homologs can be found throughout vertebrate species (46) (Figure 8a). To test whether HSPB7 from different species are functional homologs of the human HSPB7, we co-expressed EGFP-HttQ74 with either human, mouse, fish or frog HSPB7 and compared their efficacy on preventing polyQ aggregation in human HEK293 cells. All HSPB7 species variants were able to suppress polyQ aggregation (Figure 8b), strongly suggesting that they are true orthologs of human HSPB7 and that they have a general function that is maintained when expressed in cells derived from a different species.

The main sequence-related feature that makes HSPB7 unique within the HSPB family is the presence of a conserved N-terminal serine-rich region of approximately eighteen residues. In addition, HSPB7 contains a conserved C-terminal region (C-box) of nine residues. Both these sequence features are also conserved in mouse, fish and frog orthologs (Figure 8a). In addition, we recently found that the N-terminus contains targeting motifs required for HSPB7 to be located to nuclear SC35 splicing speckles (Chapter 3). To investigate the role of these features on preventing polyQ aggregation, we co-expressed EGFP-HttQ74 with several human HSPB7 deletion mutants (Figure 8c). Deletion of the serine-rich region, previously shown to be redundant for SC35 speckle association, did not affect the activity of HSPB7 against polyQ aggregation (Figure 8d). Deletion of the N-terminal domain, which leads to displacement of HSPB7 from SC35 speckles, strongly reduced the activity of HSPB7 against polyQ aggregation (Figure 8d). Deletion of the nine C-terminal residues of HSPB7 did not affect the activity of HSPB7 against polyQ aggregation (Figure 8d). Thus, both the HSPB7-specific serine-rich region and the C-box are redundant features not required for preventing polyQ aggregation. A combination of these two deletions still resulted in an active HSPB7 mutant protein (data not shown). On the other hand, the role of the N-terminus seems indispensable in preventing polyQ aggregation. This
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could merely reflect the deletion of a substrate interaction domain, as implicated for other small HSP proteins (40;47;48), or that SC35 speckle association of the HSPB7 N-terminus has a role in preventing polyQ aggregation.

**HSPB7 prevents polyQ aggregation in neuronal-like cells**

Although the precise mechanism remains to be elucidated, the above data show that HSPB7 is a novel and very potent, non canonical modulator of polyQ aggregation. To test whether it

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**Figure 9. HSPB7 co-localizes with polyQ aggregates and reduces aggregate formation in neuronal cells.** (A) NG108 cells were differentiated (see materials and methods) into neuronal cells during a seven day period. Cells were transfected after differentiation with EGFP tagged SCA3-Q64. After 48 hours cells were fixed with methanol and analysed by confocal microscopy. (B) After differentiation, cells were co-transfected with EGFP-SCA3-Q64 and V5-HSPB7 encoding plasmids (1:9 ratio). In the low fraction of cells expressing HSPB7 that did show aggregates, V5-HSPB7 colocalized with these intranuclear SCA3 aggregates. The number of cells positive for either EGFP-HQ74 (C) or SCA3-Q64 (D) inclusions in transfected differentiated NG108 cells overexpressing either HSPB7, V5-HSPB7, HSPB5 or V5-HSPB5 were counted using immunofluorescence. Clearly, HSPB7 strongly reduced inclusion formation. See Appendix 4 for colour print.
is also effective in reducing polyQ aggregation and toxicity in neurons, we first co-transfected differentiated NG-108 neuroblastoma x glioma cells with HSPB7 and EGFP-HttQ74 or EGFP-SCA3Q63. Interestingly, overexpression of polyQ proteins (without NLS) in these cells led to an exclusive nuclear accumulation of polyQ aggregates, leaving the cytoplasm almost void of polyQ inclusions (Figure 9a), which is entirely different from what was found in HEK293 cells, unless we used NLS-mediated targeting. HSPB7 showed co-localization with the nuclear polyQ aggregates (Figure 9b). Since transfection efficiencies were very low, we could not use biochemical endpoints to test whether HSPB7 reduced the process of aggregation also in these NG108 cells. Therefore, we calculated the fraction of green, EGFP-HttQ74 or EGFP-SCA3Q63 expressing cells with visible inclusions (Figure 9c,d). Clearly, also in differentiated NG-108 cells

Figure 10. HSPB7 reduces polyQ induced toxicity in cells and in vivo. PolyQ induced cellular toxicity was analysed using a HEK293 cell line containing a tetracycline inducible EGFP-HttQ119 construct (53) that stably expressed either non-tagged HSPB5 or HSPB7. (A) Addition of tetracycline to induce the expression of EGFP-HttQ119 (panel A, upper lane) (B) Long term toxicity of polyQ expression was evaluated using the colony formation assay showing reduced colony formation (within two weeks) upon poly-Q expression. (C) Quantitative analysis of colony formation reveals that expression of EGFP-HttQ119 alone or together with HSPB5 results in a major reduction of colonies. Co-expression of HSPB7 results in a significant protection against polyQ toxicity. (D) The Drosophila SCA3 model (32) was used to evaluate the effect of HSPB7 expression on eye-degeneration. HSPB7 transgenic flies were generated and expression of HSPB7 alone caused no phenotypic changes and these flies had normal eye morphology (D1). Expression of HSPB7 was tested by crossing the Actin-GAL4 driver fly with the HSPB7 transgenic fly (D1, bottom right insert). Expression of SCA3trQ78 resulted in a visible degeneration (D2). A mild reduction upon co-expression of HSPB7 on SCA3trQ78 induced eye degeneration was observed, but this effect was not statistically significant (data not shown & D3). (E) By analysing positive phototaxis, a significant reduction in blindness could be observed when HSPB7 was co-expressed with SCA3trQ78. The level of statistical significance was determined using Chi-square analysis.
HSPB7 (both V5- and non-tagged) effectively reduced polyQ aggregation, while overexpression of HSPB5 had only a minor effect.

**HSPB7 reduces polyQ related toxicity**

We next analyzed if HSPB7, in addition to reducing polyQ aggregation, could also reduce polyQ-induced cytotoxicity. Hereto, we used a HEK293 cell line containing a tetracycline inducible EGFP-HttQ119 construct. These cells were next used to generate stable lines constitutively expressing either non-tagged HSPB5 or non-tagged HSPB7 (Figure 10a). Expression of EGFP-HttQ119 was subsequently induced by addition of 1 µg/ml tetracycline (Figure 10a) and long term toxicity was evaluated using colony formation (i.e. the ability of individual cells expressing polyQ proteins to form colonies containing at least 50 cells within 2 weeks). Expression of EGFP-HttQ119 severely affected colony-forming ability, indicating polyQ-induced cytotoxicity (Figure 10b,c). While co-expression of HSPB5 did not significantly protect against cytotoxicity, co-expression of HSPB7 led to a significant reduction in polyQ mediated cytotoxicity (Figure 10b,c). HSPB7 expression alone did not affect cell growth or colony formation (data not shown). Thus, HSPB7 is not only able to both reduce polyQ aggregate formation in cells but also reduces polyQ induced cytotoxicity.

To test if HSPB7 can also prevent polyQ-induced neuronal toxicity in vivo, we targeted HSPB7 overexpression in the compound eyes of the Drosophila SCA3 model (32). This fly model is characterized by continuous expression of a truncated polyQ-expanded SCA3-gene (SCA3-trQ78) in the compound eyes, resulting in neurodegeneration and blindness. Overexpression of polyQ expanded SCA3 resulted in visible eye degeneration (Figure 10d) and loss of positive phototaxis, a measure for blindness (Figure 10e). HSPB7 transgenic flies showed normal eye-morphology and phototaxis (Figure 10d,e). Although expression of HSPB7 in the SCA3 transgenic flies did not result in statistically significant amelioration of the SCA-3 induced eye-degeneration (data not shown), a significant improvement in positive phototaxis was seen (Figure 10d,e). So, HSPB7 is not only functional in preventing polyQ aggregation in cells in vitro, but is also able to reduce polyQ-induced toxicity in vivo.

**Discussion**

In this work, we have compared the various members for their ability to prevent polyQ aggregation and identified, HSPB7 as the most active member. Its mode of action was demonstrated to be of a non-canonical nature: although HSPB7 was in part dependent on autophagy, it functions independently of proteasomal degradation. Furthermore, HSPB7 did not form high-molecular weight complexes like HSPB1 and HSPB5, which are implicated as being important for their chaperone activity. In agreement with this, HSPB7 was unable to chaperone heat-denatured luciferase, a well known substrate for both HSPB1 and HSPB5. Although the precise mechanisms remains to be elucidated, HSPB7-like activities are evolutionary conserved and also prevent aggregate formation in neuronal-like cells and even reduce cytotoxicity and neuronal toxicity in cells and in vivo respectively, making it an excellent candidate for future therapeutic strategies.

**HSPB members show diversity in cellular activity**

By screening the HSPB family for activity against amyloid fibril formation of polyQ proteins, a clear difference in their respective capacities to handle this substrate was found. Whereas the classical, well-studied HSPB1 and HSPB5 assist in refolding of heat denatured luciferase, and show substantial activity against aggregation of diverse other substrates (27;49;50), they were merely ineffective in preventing polyQ aggregation (12) (this study). Strikingly, HSPB9 and especially HSPB7 were most effective in reducing polyQ aggregation, but did not sup-
port refolding of heat unfolded luciferase. This set of data suggests at least two points. Firstly, diverse HSPB members can act on different substrates consistent with a rather promiscuous substrate recognition ability. Secondly, the effectiveness of the various members to handle (the same) substrates differs. This could mean that binding of a certain substrate to one HSPB is more efficient for its further processing (refolding, proteasomal degradation, autophagosomal routing etc.) than binding to another HSPB member. The latter may occur via additional interaction partners of the diverse HSPB members and or via differential substrate binding affinity e.g. impaired hand-over to HSPA members. Whereas relatively lower substrate affinity (e.g. HSPB1) could favor transfer to HSPA members and subsequent substrate folding (luciferase), tentative stronger substrate affinities (or other non-HSPA dependent mechanisms: e.g. HSPB7) may avoid the danger of transfer to HSPA members for non-refoldable substrates like polyQ that are poorly handled by the proteasome. Whereas such a model requires further biochemical evidence, it is supported by several other observations. Those HSPB members that function in stimulating luciferase refolding depend on a functional HSPA machine (39;this study) and behave like classical dynamic oligomers as has been described in cell free experiments (23;51). Such members are usually poor suppressors of polyQ aggregation (12;this study). Inversely, the effect of HSPB7 on polyQ aggregation does not require a functional HSPA machine (this study) and a mere independence of the HSPA machine in preventing polyQ aggregation was recently also found for strong suppressors in the family of DNAJ proteins (9;52) that also do not support protein refolding reactions (53).

Clearly HSPB7 is not the only non-canonical member of the HSPB family. Also HSPB8 does not form the classical oligomeric complexes in cells but rather forms a stoichiometric complex with BAG3 (2:1). This complex induces autophagy (15) and inhibits protein synthesis in a HSP70-independent but elf2α-dependent manner (16). Hereby it also can lead to reduced polyQ aggregates, although this mechanism seems less effective in handling long polyQ expansions (this study). Although HSPB7 in part also depends on active autophagy, its mechanism of action seems distinct from the BAG3/HSPB8 complex as its activity does not seems to affect soluble protein levels, meaning that unlike the BAG3/HSPB8 it does not act on protein synthesis.

**Oligomerization of HSPB members and anti-aggregation**

One striking distinction between the non-canonical polyQ chaperoning HSPB proteins (HSPB6, HSPB7, HSPB8) and the classical HSPB proteins (HSPB1, HSPB5), seems to be the finding that they do not exist as large oligomers within living cells. While active chaperone activity of HSPB1 towards unfolded proteins is dependent on oligomeric dynamics (51;54), HSPB7 was found to exist as either mono- or dimeric sizes. Also HSPB6 and HSPB8, active in reducing HA-HttQ43 aggregation, are reported to mainly exist as dimers or small oligomers respectively (55;56).

Why would HSPB7, that can reduce amyloid fibril formation of polyQ containing proteins, not also support refolding of heat-denatured luciferase, which forms amorphous aggregates. Successful chaperoning of this specific substrate rather seems to require shuttling between dimers and large oligomers (39). As we were unable to detect clear degradation-related effect on reducing polyQ aggregation by HSPB7 and in addition a lack of requirement of HSPA and other stress-induced chaperones, we propose that HSPB7 might function by transiently shielding the polyQ stretch and effectively preventing polyQ-oligomer formation and halting the process of inclusion formation. These chaperone-polyQ oligomers next may be (slowly) cleared by autophagy, which could explain why HSPB7 is less efficient in ATG5 -/- cell defective in macroautophagy In this way, seed formation for further aggregation is reduced and other cellular degradation pathways are allowed a larger time-window to clear the aggregation-prone substrate without the requirement of boosting either proteasomal or autophagic clearance. A similar mode of action has been suggested for HSPB5 in preventing nucleation of amyloid fibril forming substrate in vitro (57;58),
a process leading to aggregate formation. Yet, HSPB5 seems unable to act in reducing polyQ aggregation, again suggesting specificity in substrate chaperoning. An alternative hypothesis could be that HSPB7 somehow has indirect effects on poly-Q aggregation via an effect on RNA metabolism. In chapter 3 we showed that HSPB7 is associated with SC35 speckles that play a role in RNA splicing and processing. By acting as a possible RNA chaperone, HSPB7 may effectively decrease aggregation of triplet expanded RNA species hereby maintaining cellular homeostasis and the ability of reducing the burden of unfolded peptides via constitutively active protein quality control pathways.

HSPB7 is able to reduce polyQ aggregation and toxicity in several models used here, which are all associates with an overwhelming induction of polyQ expression in a very short time. As negative effects of expanded polyQ protein expression in patients accumulates over many years, HSPB7 activity in vitro might translate in such situations in a highly effective strategy to handle polyQ fibril formation. Taken together, HSPB proteins in general show a diverse and often specific chaperone activity towards proteins tending to form either amorphous or amyloidal aggregates, with sometimes diverse outcomes on cellular survival. The comparative study presented here forms a beginning in our understanding on substrate-specificity of HSPB proteins and differences in their mode of action. Further comparative studies using different substrate proteins are embraced to clarify the role of substrate in relation to HSPB chaperone activity.

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References

Chapter 4


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