HSPA1A-Independent Suppression of PARK2 C289G Protein Aggregation by Human Small Heat Shock Proteins

Melania Minoia,* Corien Grit,* Harm H. Kampinga

University Medical Center Groningen, University of Groningen, Department of Cell Biology, Groningen, The Netherlands

The C289G mutation of the parkin E3-ubiquitin protein ligase (PARK2) is associated with autosomal recessive juvenile onset Parkinson’s disease and was found to be associated with protein aggregation. Members of the human small heat shock proteins (HSPBs) have been implicated in protein degradation and prevention of protein aggregation. In this study, we show that of the 10 HSPB members, individual overexpression of HSPB1, HSPB2, HSPB4, and HSPB7 suppresses PARK2 C289G-associated protein aggregation. Intriguingly, the protective actions of these HSPBs are not impaired upon inactivation of the ATP-dependent HSP70 chaperone machines. Depending on the HSPB member the protective actions involve either autophagic or proteasomal degradation pathways.

Parkinson’s disease (PD) is the second most common neurodegenerative disorder and is characterized by degeneration of dopaminergic neurons and the presence of cytoplasmic inclusions, called Lewy bodies (LB), in the substantia nigra. PD is mostly sporadic; however, several familial forms are known, including mutations in the PARK2 gene. The PARK2 gene codes for the parkin RBR E3-ubiquitin protein ligase, which has been associated with autosomal recessive juvenile onset Parkinson’s disease (ARJPD) (1).

PARK2/parkin is an E3-ubiquitin protein ligase, predominantly expressed in the muscles and the brain (1, 2). PARK2 consists of a ubiquitin-like (UBL) domain at the N terminus and two RING domains at the C terminus, of which the latter are essential for PARK2’s ubiquitin ligase function (2, 3). In addition to its ubiquitin ligase function, PARK2 is a key player in the mitochondrial quality control system (4). One of the recessive mutations in PARK2 is the PARK2 C289G mutation, located at the RING1 domain, and it suggests loss of function as the prevalent cause in juvenile onset PD. However, the expression of PARK2 C289G is associated with its sequestration into protein aggregates, since the affected cysteine residue is important for the structural stability of the PARK2 protein (5–7), which suggest that this PARK2 mutant may (also) exert dominant-negative effects or have a gained toxic (aggregation-related) function. In line, Khan et al. reported in 2003 on 24 ARJPD cases, of which 10 patients had only one allele that was mutated (8). In addition, non-PD patients with a single PARK2 mutant allele were found to manifest behavioral disorders and nigrostriatal dysfunction (9, 10).

A potential strategy to counteract the protein aggregation associated with PARK2 C289G expression may be achieved by the action of members of the family of small heat shock proteins (HSPBs). All HSPBs contain an alpha-crystallin domain, flanked by variable C- and N-terminal regions (11, 12). For several of the HSPB members, it was shown that they can bind to nonnative proteins and either facilitate their correct folding or assist in their degradation (11, 13). The HSPBs lack ATPase activity and therefore are generally thought to require the ATP-dependent HSP70 chaperone machinery for their activities (13), although HSP70-independent chaperone actions have also been suggested (12, 14).

The importance of HSPBs in neuromuscular functioning appears from the many muscular and neurodegenerative diseases caused by mutations in the HSPBs members (11, 12, 15) and their up-regulation in numerous pathological conditions (16, 17). Moreover, upregulation of several HSPBs was found to be protective in a number of protein aggregation disease models for polyglutamine (polyQ) diseases and amyotrophic lateral sclerosis (ALS) (11, 14, 15, 18, 19). These data prompted us to test if members of the HSPB family might also be able to suppress aggregation related to expression of the PARK2 C289G mutant. Our dedicated screen revealed that HSPB1, HSPB2, HSPB4, and HSPB7 were potent suppressors of PARK2 C289G aggregation in mammalian cells. This result differs from what we found for polyQ diseases, where HSPB7, HSPB8, and HSPB9 were the most protective members (14, 20). The antiaggregation activity of the HSPBs was not found to depend on the Hsp70/HSPA chaperone system but either involved support of clearance of PARK2 C289G aggregates via autophagy (HSPB7) or via proteasomal degradation of PARK2 C289G (HSPB1).

MATERIALS AND METHODS

Plasmids and reagents. The human HSPB plasmid library used here was described before (14). Flag-PARK2 WT and Flag-PARK2 C289G constructs were kindly provided by Michael E. Cheetham. Bortezomib (100 nM) was obtained from Selleck Chemicals. 3-Methyladenine (3-MA; 10 mM), wortmannin (200 nM), and VER-155008 (40 μM) were obtained from Sigma.

Cell culture and transfection. Mouse embryonic fibroblasts (MEFs) and human embryonic kidney 293 (HEK293) cells expressing the tetracycline repressor (Flp-In T-Rex HEK293; Invitrogen, Carlsbad, CA) were

Received 20 May 2014 Published for modification 18 June 2014 Accepted 7 July 2014

Address correspondence to Harm H. Kampinga, h.h.kampinga@umcg.nl.

* Present address: Melania Minoia, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden; Corien Grit, University Medical Center Groningen, University of Groningen, Department of Neuroscience, Groningen, The Netherlands.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.00698-14.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/MCB.00698-14
grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal calf serum (Gibco), penicillin at 100 U/ml, and streptomycin at 100 mg/ml (Gibco). Cells were grown at 37°C in 5% CO2. HEK293 and MEF cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions using 1 μg of plasmid DNA per 35-mm dish. Gene expression was induced with 1 μg of tetracycline/ml. Cells were transfected for 24 h with Flag-PARK2 C289G alone or together with the HSPBs (ratio, 1:3). pcDNA3 or pcDNA3-FRTTO-mRFP were used as control conditions. Transfection of small interfering RNA (siRNA) for HSPA1A was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence. HEK293 cells were plated on poly-ε-lysine (Sigma)-coated coverslips. At 24 h after transfection, the cells were washed twice with phosphate-buffered saline (PBS; Gibco) and fixed with 2% formaldehyde (Sigma-Aldrich) for 10 min at room temperature. The cells were permeated using 0.1% Triton X-100 (TX-100) in PBS for 7 min at room temperature and later incubated in PBS+ (0.5% bovine serum albumin and 0.15% glycerine in PBS). The cells were incubated with primary antibodies overnight at 4°C. Cells were washed four times with PBS+ and incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen) or Alexa Fluor 594-conjugated donkey anti-mouse IgG (Invitrogen) for 1.5 h at room temperature, washed two times, and mounted in glycerol (Agar Scientific). Confocal images were obtained using a confocal laser scanning microscope (Leica TCS SP8) with a ×63/1.32 oil objective lens. The percentage of aggregates in Flag-PARK2-positive cells was calculated.

Protein extraction and Western blotting. PARK2 C289G aggregation was measured according to the method of Rose et al. (21). In short, transfected cells were washed with PBS, lysed in 1% Triton X-100 buffer containing protease inhibitors (Roche), incubated in the lysis buffer for 15 min on ice, and then separated by centrifugation at 14,000 rpm for 15 min at 4°C to obtain Triton X-100-soluble (supernatant) and -insoluble (pellet) fractions. Pellet fractions were resuspended by sonication in Laemmli sample buffer (62.5 mM Tris–HCl buffer [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 10% β-mercaptoethanol, and 0.001% bromophenol blue). The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and then processed for Western blotting. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) at a 1:7,000 dilution. Visualization was performed with enhanced chemiluminescence and Hyperfilm (GE Healthcare).

Mouse monoclonal anti-FLAG (M2) antibody was obtained from Sigma, while mouse monoclonal anti-HSPA1A/Hsp70, antiubiquitin (FK2), and anti-HSPB1 antibodies were obtained from Enzo Life Science. Mouse monoclonal anti-PARK2 antibody was obtained from Cell Signaling Technology. Mouse monoclonal anti-GAPDH, anti-HSPB2, anti-HSPB4, and anti-HSPB7 antibodies were obtained from RDI Research Diagnostics, Transduction Laboratories, Abcam, and Abnova, respectively. Rabbit polyclonal anti-FLAG and anti-H2A were from Abcam.

Statistical analysis. Results are expressed as means ± the standard errors of the mean (SEM). The statistical significance was analyzed by using an independent t test. A P value of <0.05 was considered statistically significant. All experiments were carried out at least three times, but only the data of one representative experiment are shown.

RESULTS

HSPB1, HSPB2, HSPB4, and HSPB7 are able to suppress PARK2 C289G aggregation. The C289G missense mutation in the PARK2 RING domain alters the intracellular distribution pattern of PARK2 and decreases its solubility (5, 21). We confirmed that in HEK293 cells ectopically expressed wild-type (WT) PARK2 is diffusely distributed, whereas PARK2 C289G forms inclusions in 56.3% of the cells within 24 h (Fig. 1A). In line with this, the majority of the PARK2 WT (50 kDa) was found in the Triton X-100-soluble fraction, whereas a major fraction of the PARK2 mutant had become TX-100 insoluble (Fig. 1B). The appearance of a smear above the 50-kDa band of the PARK2 mutant has previously been shown to be attributed to the loss of inhibition of its auto-ubiquitination activity (7). The lower 42-kDa band is an N-terminal truncated PARK2 species resulting from an internal initiation site within the PARK2 gene (22). In addition, a significant amount of PARK2 mutant was found in the stacking gel (high molecular weight [HMW]: Fig. 1B), implying formation of SDS-insoluble aggregates.

HSPBs have been shown to prevent the aggregation of several aggregation-inducing proteins, their effectiveness seemingly depending on the substrate and physical properties of the aggregate it forms (11). When coexpressing different HSPB members with PARK2 C289G (see Fig. S1A in the supplemental material), a major reduction in the aggregation of PARK2 C289G was seen in cells coexpressing HSPB1, HSPB2, HSPB4, or HSPB7 but not in cells coexpressing the other HSPB members (Fig. 1C). Immunocytochemistry confirmed these effects, showing significantly lower percentages of PARK2 puncta in cells coexpressing HSPB1, HSPB2, HSPB4, or HSPB7 compared to control conditions (Fig. 1D and E). Expression of HSPB6, which did not affect PARK2 insolubilization (Fig. 1C), also did not affect the puncta formation of PARK2 C289G (Fig. 1E). Categorizing PARK2 C289G-aggregates in abundance and size (see Fig. S1B in the supplemental material) reveals that all effective HSPB members especially reduced the number of cells with large(r) aggregates (Fig. 1E). Confocal analysis further revealed that HSPB2, HSPB4, and HSPB7 colocalize with PARK2 aggregates, whereas HSPB1 does not (Fig. 2A, arrow). Interestingly, HSPB2 and HSPB4 were found inside the aggregates, whereas HSPB7 appears to be more at the rim of the aggregates, similar to what was observed previously for polyQ aggregates (14). Therefore, although the four HSPBs all suppressed PARK2 insolubilization, their mode of action may actually differ, especially that of HSPB1.

The Hsp70/HSPA chaperone machinery is not required for the HSPBs antiaggregation activity. In vitro studies have shown that many HSPBs can prevent irreversible aggregation of clients directly and without the need for additional activities. However, the HSPBs generally are thought to require cooperation with ATP-dependent chaperones, like the Hsp70/HSPA machines, for client release and subsequent processing (23–26). To investigate whether the effects of HSPB1, HSPB2, HSPB4, and HSPB7 on parkin C289G aggregation depend on a collaboration with Hsp70/HSPA family (22), we confirmed the siRNA data. Furthermore, as with the knock-
down of HSPA1A, the VER-155008 treatment did not abolish the protective effects of HSPB1, HSPB2, and HSPB4 on PARK2 C289G aggregation, even though VER-155008 has led to an increased burden of aggregation-prone PARK2 C2989G (Fig. 3B). Together, these results suggest that these HSPBs are able to suppress PARK2 C298G aggregation in an Hsp70/HSPA-independent manner.

HSPBs use the proteasome system or the autophagy pathway to degrade mutated PARK2. HSPB proteins have been repeatedly associated with enhanced protein degradation through either the ubiquitin-proteasome system (28–30) or through autophagy (14, 20). To test whether HSPB1, HSPB2, HSPB4, and HSPB7 depend on a functional autophagosomal machine to exert their protective effects on PARK2 C289G aggregation, we inhibited autophagy with 3-methyladenine (3-MA) and wortmannin. The inhibition of autophagy enhanced PARK2 C289G aggregation (see Fig. S2 in the supplemental material), suggesting that constitutive autophagy is involved in the normal clearance of misfolded PARK2.

FIG 1 Suppression of PARK2 C289G aggregation by HSPBs. (A) HEK293 cells were transfected for 24 h with Flag-tagged PARK2 WT or PARK2 C289G. Subcellular distribution of PARK2 (green) was investigated by immunofluorescence. The diagram shows the percentage of PARK2 aggregates. (B) Cells, transfected as in panel A, were fractionated in TX-100-soluble and -insoluble proteins and analyzed by Western blotting. (C) HEK293 cells were transfected for 24 h with Flag-tagged PARK2 C289G alone or together with each of the different HSPB proteins. Cells were fractionated in TX-100-soluble and -insoluble proteins and analyzed by Western blotting. The HSPB expression levels are shown in Fig. S1A in the supplemental material. (D) Immunofluorescent staining of Flag-tagged PARK2 (green), HSPBs (red), and DAPI (blue) in HEK293 cells, transfected as in panel C. (E) Diagram showing the percentages of flag-tagged PARK2-expressing cells with aggregates (*, \( P < 0.05 \); **, \( P < 0.001 \); \( n > 3 \) independent samples, means ± the SEM). Cells were divided in different categories, as shown in Fig. S1B in the supplemental material. HSPB6 was added as a negative control. HMW, high molecular weight.

FIG 2 Colocalization of the HSPBs with PARK2 C289G aggregates. (A) Immunofluorescent staining of Flag-tagged PARK2 (green), HSPBs (red), and DAPI (blue) in HEK293 cells, transfected as for Fig. 1D.
as has been suggested before (31). Under such conditions HSPB1, HSPB2, and HSPB4 were still able to prevent PARK2 aggregate formation. However, the protective action of HSPB7 was strongly reduced (Fig. 4A). To further test whether HSPB7 indeed inhibits PARK2 C289G aggregation using autophagy, we tested the capacity of HSPB7 to suppress PARK2 C289G aggregation in autophagy-deficient (ATG5−/−) MEFs. In these cells, HSPB1, HSPB2, and HSPB4 still led to a reduction in PARK2 C289G aggregates (Fig. 4B), which is consistent with their action being unrelated to autophagy. However, HSPB7 activity on PARK2 C289G aggregates was abrogated in ATG5−/− cells (Fig. 4B).

These results are in line with our previous findings on polyQ proteins, in which HSPB7 was found to prevent polyQ aggregation in an autophagy-dependent manner (14).

To test whether HSPB1, HSPB2, and HSPB4 might suppress PARK2 C289G aggregation via the ubiquitin-proteasome system, we analyzed their antiaggregation effects upon proteasomal inhibition. Treatment with bortezomib successfully inhibited the proteasome as evidenced by the accumulation of ubiquitinated proteins (Fig. 4A). Surprisingly, treatment with bortezomib alone (see Fig. S2 in the supplemental material) or in combination with the four HSPBs (Fig. 4A) reduced the level of soluble PARK2. It has been recently shown that bortezomib treatment leads to activation of autophagy (32, 33); hence, we conclude from these data that mutant PARK2 C289G may be primarily degraded through autophagy under such conditions. However, bortezomib treatment significantly (albeit not completely) reduced the protective effects of HSPB1 and HSPB4 on PARK2 C289G aggregation, while having only a modest effect on HSPB7-mediated protection and no effect on HSPB2-mediated protection (Fig. 4A). These data suggest that HSPB1 and HSPB4 antiaggregation activities, at least partially, require an active proteasome. In conclusion, the different protective HSPBs prevent PARK2 aggregation via mechanistically distinct pathways.

**DISCUSSION**

In this paper we show that HSPB1, HSPB2, HSPB4, and HSPB7 can protect cells from C289G PARK2-aggregation in an Hsp70/
HSPA-independent manner and via different degradation pathways.

The ability of the several HSPB family members to protect against protein aggregation seems to differ substantially. Until now, HSPB1, HSPB5, and HSPB8 have been the most widely studied members. They have been found to reduce protein aggregates caused by proteins containing an expanded polyQ tract (HSPB8 [20]), tau (HSPB1 [34–36]), amyloid-beta (HSPB1, HSPB5, and HSPB8 [37, 38]), TDP43 (HSPB8 [15]) and SOD1 (HSPB1, HSPB5, and HSPB8 [18, 39]). However, only little is known about

![Graphs and images showing the role of ubiquitin proteasome system or the autophagy pathway in HSPB effects on PARK2 C289G aggregates.](image-url)

**FIG 4** Role of ubiquitin proteasome system or the autophagy pathway in HSPB effects on PARK2 C289G aggregates. (A) HEK293 cells, transfected as in Fig. 3A, were treated with bortezomib (100 nM) or with 3-MA (20 mM) and wortmannin (200 nM) overnight. Cells were fractionated in TX-100 and analyzed for PARK2 C298G aggregates (*, P < 0.05; **, P < 0.001; n > three independent samples, means ± the SEM). (B) ATG5−/− MEF cells were transfected for 24 h with Flag-tagged PARK2 C289G and mRFP-, HSPB1-, HSPB2-, HSPB4-, or HSPB7-encoding vector and analyzed for PARK2 C298G aggregates (*, P < 0.05; **, P < 0.001; n > three independent samples, means ± the SEM). HMW, high molecular weight.
the protective effects of the other HSPBs. It should also be emphasized that the endogenous expression levels of the various HSPB members substantially differ between cell types (11–13) and that HEK293 cells normally only express substantial levels of HSPB1, with the other members being expressed at very low levels only (40). Given that some of the HSPB members also can form hetero-oligomers (11, 12), this complicates interpretation of functional comparisons from studies in different cell lines. Over the last years, only a few comparative studies have been done in which the 10 HSPB members were directly compared for their activity toward various substrates within the same cell models systems. Comparing the 10 HSPB members for canonical refolding activity, using the firefly luciferase as a model substrate, revealed that only HSPB1, HSPB4, and HSPB5 upregulation lead to improved refolding after heat shock (14, 41). In the same HEK293 cells, upregulation of especially HSPB7 but also HSPB6, HSPB8, and HSPB9 was found to be effective in preventing polyQ-protein aggregation (mutant huntingtin and ataxin-3) (20, 41), whereas HSPB8 was the only HSPB able to prevent aggregation of mutant TDP43 (associated with ALS) (15). In cardiomyocytes, upregulation of especially HSPB1 but also HSPB6, HSPB7, and HSPB8 was effective in protecting cytoskeletal elements from tachypacing (42). Here, we show, using again HEK293 cells, that HSPB1, HSPB2, and HSPB4, as well as HSPB7, suppress aggregation of the PARK2 C298G mutant (Fig. 1). These data clearly indicate that the different human HSPB members display substrate specificity, albeit with some overlap (11). The physical properties of the substrates and aggregates are likely important factors that determine which HSPB member may be able to handle them. While, for example, polyQ proteins and amyloid-β aggregates are amyloidogenic, mutants of SOD1 and TDP-43 form more amorphous aggregates. Although Parkinson’s disease is characterized by the presence of amyloid containing LB, only in a minority of cases of PARK2-associated ARJPD have LB or LB-like α-synuclein-positive inclusions been found (43–45). Moreover, PARK2-associated aggregates are largely detergent insoluble and SDS soluble, whereas, for example, polyQ proteins are both detergent and SDS insoluble (5, 21), which is consistent with our findings that different HSPBs are needed to handle these two different substrates.

In addition to substrate specificity, the mechanisms of action of the different HSPB members seem to differ drastically. Cell-free studies have shown that some HSPB members prevent substrate aggregation, hereby keeping them competent for HSP70-dependent refolding (24–26). In cells, the handling of heat-denatured luciferase by some HSP members also was found to be dependent on a functional Hsp70 machinery (14, 41). Besides supporting (re)folding, HSPB substrate holding in cells might also assist in proper client disposal. In the present study, such seems to be the case for HSPB1 and HSPB4 that require a functional proteasomal degradation pathway to prevent PARK C298G aggregation (Fig. 4). Interestingly, our siRNA and inhibitor studies demonstrated that endogenous Hsp70/HSPA proteins play an important role in combating the aggregation associated with mutant PARK2 expression (Fig. 3). In line, Rose et al. found that endogenous Hsp70 is required to support actions of DNAJ proteins on mutant parkin-associated aggregation (21). However, the effects of HSPB1 and HSPB4 on the enhanced degradation of mutant parkin were independent of the Hsp70/HSPA chaperone machinery (Fig. 3). It is tempting to speculate that the proteasome capping proteins might be able to release clients from HSPB1 directly, without the need of the Hsp70/HSPA machine. In support of such a hypothesis, HSPB1 was found to colocalize with both ubiquitinated proteins and proteasomal inclusion bodies (28) and HSPB1 was found to directly interact with the proteasome (30, 46). Such a mode of action would also explain why HSPB1 is not located with the aggregates (Fig. 2) since such an action would be prior to aggregation. However, direct evidence for such a scenario remains to be established.

On the other hand, the mode of action of HSPB7 seems to be related to autophagic degradation since we found that it associates with misfolded proteins in a later stage, i.e., when they already have started to form oligomers. At this stage, HSPB7 seems to form a ring around the aggregates (14) (Fig. 2) by which it may prevent aggregate propagation such that the aggregates can be handled by the autophagy machinery. Furthermore, HSPB2 may play an even more intricate role, since neither HSP70s, autophagy, nor proteasome inhibition impaired its antiaggregation activity (Fig. 3 and 4). Recent findings based on a cell-free in vitro model showed that HSPB2 has protein-dependent chaperone activity and a significant ability to inhibit amyloid fibril formation of α-synuclein (47). HSPB2 could represent an atypical chaperone by a unique, yet to be established mechanism.

In conclusion, the different HSPB members have both overlapping and divergent substrate specificities. The HSPBs support substrate refolding after acute stress but also have degradation supportive activities in a disease mutant-specific manner. Thus, boosting HSPB expression or activity should be further explored as potential target for delaying folding diseases.

ACKNOWLEDGMENTS

We thank Klaas Sjollema (UMCG Microscopy and Imaging Center) for assistance with the confocal microscope.

This study was supported by a grant from Senter Novem (IOP-IGE07004) awarded to H.H.K.

REFERENCES


