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Chapter 7

DNAJ family shows J-domain dependent anti-aggregation activity on RING1 mutant parkin

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ABSTRACT

Parkinson’s disease is one of the most common neurodegenerative disorders and several mutations in different genes have been identified to contribute to the disease. A loss of function parkin RING1 domain mutant (C289G) is associated with autosomal-recessive juvenile-onset Parkinsonism (AR-JP) and displays altered solubility and sequesters into aggregates. The heat shock protein 70 (Hsp70) chaperone machine has been shown to play a role in the prevention of aggregation of different mutant or stress unfolded proteins. For neurodegenerative CAG repeat diseases, we previously found that overexpression of members of the DNAJB co-chaperone subfamily, rather than Hsp70 itself, suppressed aggregation of the disease causing polyglutamine (polyQ) proteins. For the most potent members, DNAJB6b and DNAJB8, this activity was largely independent of the Hsp70 machine. Here, we show that upon upregulation, all cytosolic DNAJ family members are equally efficient in reducing parkin C289G aggregation. Upregulation of the DNAJs alone was sufficient to provide protection, but the action of all DNAJs tested was dependent on interaction with and activity of Hsp70s. Our data imply that different aggregation-prone, disease-causing proteins pose different challenges to the protein quality control system and require specific chaperones for their proper handling. The DNAJs can have both Hsp70 dependent and independent activities, highlighting the versatility of the DNAJs with respect to the disease causing proteins, thus making them possible interesting potential therapeutic targets.
INTRODUCTION

Maintenance of protein quality control (PQC) is critical for cellular and organismal health. When PQC is lost either due to stress conditions or mutations, intracellular proteins are prone to misfolding and aggregate formation. Proteins need to be folded correctly to prevent them from attaining conformations that can cause them to function improperly or aggregate into, potentially cytotoxic, complexes (1). The accumulation of misfolded and aggregated proteins is a hallmark of many ageing-related neurodegenerative diseases like Amyotrophic Lateral Sclerosis, Alzheimer’s, Parkinson’s and Huntington’s disease, also called proteinopathies. Maintenance of proteostasis is achieved by an integrated network of several hundred proteins, including the autophagy system, the ubiquitin-proteasome system (UPS), and molecular chaperones and their regulators (2, 3). Molecular chaperones play a crucial role in various ways in the prevention of misfolding and aggregation of different mutant proteins. The largest group of molecular chaperones are the heat shock proteins (HSPs). Most HSPs can recognize exposed hydrophobic regions of non-native proteins and hereby can prevent protein aggregation. In doing so, they not only assist in protein (re)folding but also in the degradation of misfolded client proteins by targeting them to the protein degradation machineries (4, 5). Due to the different characteristics of the HSPs as modifiers of proteostasis, they have been considered as a possible therapeutic target for multiple diseases involving aggregation of mutant proteins or disruption of the proteostasis network. However, there are large differences between proteinopathies with regard to which proteins form aggregates and the type of aggregates they form (6–8) and this likely will be important to determine which class of HSPs might be most suited in the prevention of aggregation of these proteins (9).

In Parkinson’s disease, progressive accumulation of stable protein aggregates in the cytoplasm, named Lewy bodies, is one of the pathological hallmarks and the cause of selective loss of dopaminergic neurons in the substantia nigra (10–13). Several dominantly inherited mutations are known, including mutations in the genes for α-synuclein (SNCA) and parkin (PARK2), which cause protein aggregations and the disease phenotype (11, 14–16). Parkin is an E3 ubiquitin ligase that plays a role in ubiquitination of several candidate substrate proteins and thereby targeting them for proteasomal degradation. Parkin is also involved in the elimination of damaged mitochondria through autophagy, called mitophagy (17, 18). Parkin contains a N-terminal ubiquitin-like (Ubl) domain, a central RING (Really Interesting New Gene) domain (RING0) and a C-terminal RING domain consisting of two RING finger motifs (RING1 and RING2) separated by an In-between-RING (IBR) domain (19). A wide variety of PARK2 mutations have been found, including exon deletions, duplications and triplications, missense, nonsense, and frameshift mutations (20). Here, we focus on one
of the first reported mutations in the RING1 domain of parkin, the Cys289 to Gly (C289G) mutation, that is associated with an autosomal-recessive form of juvenile parkinsonism (AR-JP) (21, 22). Besides a loss of function, the C289G mutation results in alterations in parkin solubility and sequestration in aggresome-like protein aggregates and hence might also indicate a dominant toxic-gain-of-function phenomenon (23–27). Indeed, expression of parkin C289G in cells leads to impaired mitochondrial function, which is restored when this aggregation formation is prevented (18) and carrying a single allele with the C289G mutant is associated with a higher risk of parkinsonism (28–30).

Given the capacity of molecular chaperones to prevent aggregation of misfolded proteins and analogous to our previous work on polyQ aggregation (31, 32) we compared different members the Hsp70 machine for their ability to prevent aggregation of parkin C289G (6, 9, 31, 33–38). Using the same cell model as used for the polyQ screen, we now show that handling of parkin C289G requires different chaperone activities. In fact, unlike for polyQ aggregation, all cytoplasmic DNAJs were equally effective in preventing parkin C289G aggregation in an Hsp70-dependent manner. Specifically, DNAJB6 and DNAJB8, which we have previously identified as superior suppressors of polyQ aggregation in an Hsp70 independent but C-terminal SSF-SST stretch dependent manner, could prevent parkin C289G aggregation in a manner independent of its C-terminal SSF-SST stretch and only when it was able to interact with the Hsp70 machinery.

RESULTS

DNAJB2a, DNAJB6, and DNAJB8 are equally efficient in reducing Parkin C289G mediated aggregation

Previously, we have shown aggregation of polyQ proteins can be prevented most efficiently by DNAJB6b and DNAJB8 and to a lesser extent by DNAJB2a (31). To test whether these chaperones are also able to reduce parkin C289G aggregation, HEK293 cells were transiently co-transfected with V5-tagged chaperones and flag-tagged parkin wildtype (WT) or C289G mutant. Upon Triton X-100 (TX-100) lysis, most of the wild type protein distributes to the TX-100 soluble fraction (Fig. 1A, lane 1 and 6), whereas parkin C289G ends up in the TX-100 insoluble fraction (Fig. 1A, lane 2 and 7). In addition, for parkin C289G a high molecular weight (HMW) smear in the TX-100 insoluble fraction of the cell lysate can be seen (Fig. 1A, lane 7), consistent with earlier work (17) and indicative of parkin C289G aggregation. Furthermore, cells expressing parkin C289G show multiple large inclusions scattered throughout the cytoplasm and nucleus whilst parkin WT is uniformly distributed in the cells (Fig. 1B, panel 1 and 2) (18). Unlike for the inhibition of polyQ aggregation, where DNAJB6 and DNAJB8 were superior over DNAJB2a, co-transfection of all three
chaperones decreased parkin C289G aggregation to a similar extent (Fig. 1A, lane 8, 9 and, 10). Inclusion formation was also equally effective reduced by DNAJB2a (Fig. 1B, panel 3), DNAJB6 (not shown), and DNAJB8 (Fig. 2C, panel 1). For DNAJB2a, these data are in line with the findings of Rose et al., using different cell lines (18).

**A functional J-domain is critical for activity of DNAJ proteins against parkin C289G aggregation**

Since we found no differences in efficacy between DNAJB6, DNAJB8, and DNAJB2a in their ability to reduce parkin C289G aggregation, we wondered whether other members of the DNAJ family could also prevent aggregation of parkin C289G. In fact, co-expressing parkin C289G with various DNAJA (Fig. 1C) and DNAJB family members (Fig. 1D) revealed that most DNAJs are capable of preventing parkin C289G insolubilization. Exceptions are DNAJA3 which is localized to mitochondria (1, 39), DNAJA4 which is a membrane-associated protein, and DNAJB9 which is normally localized to the ER (1, 40) and was hardly expressed here. The inability of these chaperones to prevent parkin C289G aggregation can be attributed to their different localization as compared to parkin, which is predominantly cytosolic and forms perinuclear inclusions.

In case of expanded polyQ proteins, the serine rich region at the C-terminal of DNAJB6 and DNAJB8, and not the J-domain, was found to be essential for anti-aggregation activity (31). To investigate the role of the different domains of DNAJ proteins in preventing parkin C289G aggregation, different mutants of the DNAJ chaperones were co-transfected with parkin C289G (Fig. 2A). DNAJ chaperones contain a highly conserved ~70 amino acid J-domain with a histidine-proline-aspartic acid (HPD) motif which is necessary for the interaction between DNAJs and Hsp70s (41). Here, we used DNAJ mutants in which the histidine residue in the HPD motif was substituted by a glutamine (H/Q) leading to a J-domain that can no longer bind to Hsp70s. We also used a mutant of DNAJB6b in which the entire J-domain was deleted (ΔJ) (Fig. 2B). Mutations in the J-domain or deletion of the J-domain render all the DNAJs incapable of preventing aggregation of parkin C289G (Fig. 2A, lane 17, 18, 21, and 24). For DNAJB8, we confirmed this with immunohistochemistry, showing that in cells co-transfected with the H/Q mutant of DNAJB8, parkin C289G still forms large inclusions. Interestingly, unlike wild type DNAJB8, the DNAJB8 H/Q mutant was found to co-localize with parkin C289G aggregates, suggesting that it may be trapped here due to its inability to interact with Hsp70 (Fig. 2C, panel 2). Together, these data imply that Hsp70 interaction is required for all DNAJs to prevent parkin C289G aggregation.

Besides the J-domain mutants, we tested the F93L mutant of DNAJB6b with a missense mutation in the G/F-rich region. This F93L mutant was recently implicated in limb-girdle
Figure 1. Chaperones containing a J-domain can prevent aggregation of parkin C289G. (A) Cells were transfected with flag-tagged parkin WT, flag-tagged parkin C289G or co-transfected with flag-tagged parkin C289G and V5-tagged DNAJB2a, DNAJB6b, or DNAJB8. Expression of chaperones was induced with tetracycline. Triton X-100 (TX-100) soluble and insoluble fraction were obtained 24 hours after transfection. Parkin WT and C289G were assessed with anti-parkin and anti-flag antibodies on Western blot. In the TX-100 insoluble fraction, high molecular weight (HMW) species of parkin C289G are observed. Co-transfection with DNAJB2a, DNAJB6b, or DNAJB8 prevents formation of parkin C289G HMW species. Anti-parkin antibodies detect full-length parkin and an N-terminally truncated isoform (also removing the flag-tag), generated due to an internal start site, showing an extra band around 42 kDa (65). The anti-flag antibodies recognize only the full-length parkin protein. (B) Representative immunofluorescence pictures of cells co-transfected with flag-tagged parkin WT or parkin C289G (red) and V5-tagged chaperones (green). DAPI staining is shown in blue. Bar represents 10 μm. Parkin WT shows a diffuse pattern throughout the cytoplasm. Parkin C289G forms aggregates mainly concentrated into large perinuclear inclusions. Co-transfection with DNAJB2a reveals clearance of parkin C289G aggregates and co-localization of DNAJB2a with parkin C289G, indicated by arrowheads. (C) Accumulation of parkin C289G is prevented when co-transfected with members of the DNAJA subfamily. DNAJA3, which is localized to mitochondria, and DNAJA4, a membrane protein, are exceptions. (D) Co-transfection with DNAJB chaperones could also prevent parkin C289G aggregation. DNAJB9 shows no prevention, due to localization of the chaperone to the ER. Expression of chaperones was detected with anti-V5 antibodies. GAPDH was used as a loading control for the soluble fraction.
muscular dystrophy and suggested to be slightly impaired delaying polyQ mediated aggregation (42). The F93L mutant, however, was equally effective as the wildtype DNAJB6 in preventing parkin C289G aggregation (Fig. 2A, lane 19), implying that this disease-causing mutation has no impact on the functional DNAJB6-Hsp70 interaction in dealing with parkin C298G aggregates. Next, we used a deletion mutant of DNAJB8 that lacks the serine rich SSF-SST domain (ΔSSF-SST). In contrast to the crucial role of this domain in the prevention of polyQ aggregation (31), the ΔSSF-SST mutant fully retained its anti-aggregation activity on parkin C289G as revealed by fractionation (Fig. 2A, lane 22) and immunofluorescence (Fig 2C, panel 3). These combined data further illustrate different chaperone activity requirements for dealing with either polyQ or parkin C289G aggregation.

To further consolidate that DNAJs prevent aggregation of parkin C289G via an interaction with Hsp70s, we examined whether short interfering RNA (siRNA)-mediated knockdown of HSPA1A had an effect on the DNAJ mediated clearance of parkin C289G. Interestingly, the partial RNAi-mediated knockdown of HSPA1A alone, already increases parkin C289G insolubilization considerably (Fig. 3A, lane 12), implying that constitutively expressed HSPA1A contributes to handling of parkin C289G to reduce its aggregation (see also Fig. 4A).

In the RNAi-treated cells, the overexpressed DNAJs largely have lost their ability to prevent parkin C289G aggregation. Yet, a small reduction in parkin C289G aggregation is still seen for DNAJB2a, DNAJB6b, or DNAJA1 (but not DNAJB1) (Fig. 3A, lane 14, 16, and 20 compared to lane 12). Interestingly, in the DNAJB2a, DNAJB6b, and DNAJA1 co-expressing cells, HMW smears were now detected in the TX-100 soluble fraction (Fig. 3A, lane 4, 6, 8, and 10), suggesting some Hsp70-independent actions of the DNAJs prior to parkin C289G insolubilization. When cells were treated with VER-155008, an adenosine-derived Hsp70 inhibitor that targets the ATPase binding domain of all Hsp70s (43), similar results were observed (Fig. 3B). This data demonstrates that DNAJs are capable of partially solubilising parkin C289G when Hsp70s are depleted but that Hsp70s are required for full prevention of parkin C298G aggregation.

Effects on parkin C289G aggregation upon overexpression of other components of the Hsp70 machinery

From the above results, parkin C289G appears to be a substrate for the Hsp70 machine, in which upregulation of different DNAJs can enhance the cellular capacity to prevent degradation as long as sufficient Hsp70 activity is available (Fig. 1, Fig. 2, and Fig. 3). We next asked whether upregulation of different members of the Hsp70 family might also result in reduced parkin C298G aggregation. HSPA1A and HSPA14 indeed reduced TX-100
Figure 2. The anti-aggregation activity of DNAJ chaperones is dependent on the J-domain, not on the SSF-SST domain. (A) Aggregation of parkin C289G is prevented when co-transfected with DNAJB2a, DNAJB6b, DNAJB8, or DNAJB1. Mutations in the J-domain of the chaperones (H/Q) and deletion of the J-domain as a whole (ΔJ), results in the loss of prevention of aggregation of parkin C289G. DNAJB6b with a mutation in the G/F-rich region (F93L) retains its anti-aggregation activity. Deletion of the SSF-SST domain (ΔSSF-SST) of DNAJB8 does not affect its ability to prevent aggregation. (B) Schematic overview of DNAJB1, DNAJ2a, DNAJB6, and DNAJB8 and the mutants. (C) Representative immunofluorescence pictures of cells co-transfected with flag-tagged parkin C289G (red) and V5-tagged chaperones (green). DAPI staining is shown in blue. Bar represents 10 µm. Co-transfection with DNAJB8 shows a reduction in parkin C289G aggregates and co-localization of DNAJB8 with parkin C289G, indicated by arrowheads. Co-transfection with DNAJB8 H/Q still shows formation of parkin C289G aggregates mainly concentrated into large perinuclear inclusions, indicated by arrowheads. Parkin C289G and DNAJB8 H/Q co-localize but aggregate formation is not prevented. Co-transfection with DNAJB8 ΔSSF-SST reveals clearance of parkin C289G aggregates and in occasional small aggregations co-localization of DNAJB8 ΔSSF-SST with parkin, indicated by arrowheads.
insolubilization to some extent (Fig. 4A, lane 10 ad 16) but without a significant increase in the amount of parkin C289G in the TX-100 soluble fraction (Fig. 4A, lanes 2 and 8). Overexpression of HSPA1L, HSPA2, HSPA6, HSPA8, and HSPA9 was ineffective (Fig. 4A, lanes 11 - 15). A minimal Hsp70 machine contains, besides DNAJs and HSPAs, also a nucleotide exchange factors (NEFs) (1). NEFs are a heterologous group of co-factors required for the dissociation of the substrate from HSPA and may also - in part - determine the fate of the substrate (1), like HSPH2, which is able to remodel the Hsp70 machine to efficiently disaggregate aggregated proteins, and BAG3 (44), which has been implied in dealing with aggregation of polyQ proteins. However, neither overexpression of the cytosolic NEFs (HSPH1, HSPH2, or HSPH3) (Fig. 4B) nor the overexpression of the BAG family members (BAG1 and BAG3) (Fig. 4C) has an effect on the aggregation of parkin C289G. So, within the background of endogenous expression levels of HSPs in HEK293 cells, overexpression of DNAJs seems the most effective way (i.e. rate limiting) to increase the efficiency of the Hsp70 machine to prevent aggregation of parkin C298G.

DISCUSSION

In the current study we show that, when upregulated in cells, all cytosolic members of the DNAJA and DNAJB subfamilies can suppress parkin C289G aggregation in an Hsp70 dependent manner. As it was already shown for DNAJB2a, prevention of parkin C289G aggregation was associated with restoring its function in mitophagy (18), implying that such anti-aggregation effects are cytoprotective. Hsp70 inhibition increased parkin C289G aggregation and only upregulation of HSPA1A and HSPA14, but none of the other Hsp70 members, resulted in reduced parkin C289G aggregation. Further, overexpression of the cytosolic nucleotide exchange factors (HSPH1, HSPH2, HSPH3, BAG1, and BAG3) did not lead to reduced parkin C289G aggregation. Comparing our current observations on parkin C298G to our previous studies (31, 32, 44) on suppression of aggregation of polyQ containing proteins, performed in the same cell model, reveals a number of intriguing features. First, different aggregation-prone, disease-causing proteins pose different challenges to the PQC systems and require specific chaperones for their handling. Second, the different HSPA members show a certain degree of functional differentiation despite their high sequence similarities and molecular activity. Third, the DNAJB6b and DNAJB8 chaperones that we previously identified as the most potent but largely Hsp70-independent suppressors of polyQ aggregation (31), can also inhibit parkin C289G aggregation but do now depend on a functional Hsp70 machine.

Why do we find such substantial differences between the chaperone requirements for these two different aggregation-prone, disease-causing proteins? First, the aggregates caused by the two mutant proteins have different biochemical properties as revealed by detergent
Figure 3. The anti-aggregation activity of DNAJ chaperones is dependent on Hsp70s. (A) Knockdown of HSPA increases levels of TX-100 soluble and insoluble parkin C289G. In cells co-transfected with DNAJs, the HMW smear is still formed, indicating that DNAJs lose their ability to prevent parkin C289G aggregation. However, a reduction in the smear can be observed when parkin C289G is co-transfected with DNAJB2a, DNAJB6b, or DNAJA1 (not DNAJB1) compared to parkin C289G alone. There is an increase in the TX-100 soluble fraction when co-transfected with DNAJs, indicating more soluble parkin C289G under conditions of knockdown of HSPAs and suggesting some Hsp70-independent actions of the DNAJs prior to parkin insolublization. (B) Hsp70s are inhibited with VER-155008, an adenosine-derived HSPA inhibitor that targets the ATPase binding domain. Similar results are observed as in panel D.
Chapter 7

DNAJs and Parkin

A

B

C
solubility. Whilst parkin C289G aggregates are TX-100 insoluble but largely SDS soluble (18, this report), polyQ aggregates are both TX-100 and SDS insoluble (7, 8, 31, 45–47). Further, the morphology of the aggregates is more dispersed for parkin C289G (18, this report) whilst polyQ proteins form more amyloid like inclusions (31, 45). Finally, whereas the Cys289 to Gly mutation in the RING1 domain of parkin leads to a disruption of the zinc coordination of the protein and thus to protein misfolding (48), polyQ expansions within a full length huntingtin or ataxin-3 are hardly misfolded and only initiate aggregation when fragmented by the action of proteases (49–52). So, although several neurodegenerative diseases share protein aggregation as a common feature, the characteristics of the different aggregation-prone proteins impose a substantially different challenge on the cellular PQC system (9). Also, the idea that the PQC system is challenged differently is supported by the fact that several DNAJ members, which did not suppress aggregation of polyQ proteins (31), did protect against parkin C289G aggregation (this report). Inversely, overexpression of BAG3, which resulted in prevention of polyQ aggregation (44), did not protect against parkin C289G aggregation (this report). Within the HSPB family of small HSPs, HSPB7 was the most effective in preventing polyQ aggregation and HSPB1 was ineffective (32), while HSPB1 was very effective in preventing parkin C289G aggregation (Minoia et al, submitted). Finally, the mode of action of DNAJB6 and DNAJB8, which effectively reduced both types of aggregates, was different for polyQ proteins (largely Hsp70 independent) and parkin C289G (largely Hsp70 dependent, see also below).

We have previously shown that Hsp70 members are rather ineffective on polyQ aggregates (31). In contrast, we now find that inhibition as well as upregulation of HSPA1A did substantially affect parkin C289G aggregation. Besides HSPA1A, upregulation of only HSPA14 also reduced parkin C289G aggregation, whereas overexpression of all other cytosolic Hsp70 members tested was ineffective. This not only further supports that the two investigated aggregation-prone proteins require different chaperone handling, but also suggests that besides DNAJs and NEFs (1), the diverse members of the Hsp70 family may also contribute to the functional specificity of Hsp70 machines (53) and may specifically engage in different processes. Although this may seem striking giving the large sequence identity, the similar peptide recognition motifs, and biochemical activity that these Hsp70s members are assumed to have (54, 55), work by the Frydman group already suggested that distinct Hsp70 members may act in different chaperone networks, one predominantly dealing with protein translation and one with (environmental) stress (56). Further support for functional differentiation between the Hsp70 family members was provided by the group of Jäättelä (57) and our own findings that e.g. HSPA6 was largely ineffective in refolding unfolded substrates (58).

Irrespective of the effects of Hsp70 up- and downregulation, it was striking to find that, when
upregulated, all cytosolic DNAJA and DNAJB members tested were suppressing parkin C289G aggregation and that they were almost all equally effective. The anti-aggregation activity of the DNAJs was largely dependent on interaction with Hsp70s, since the H/Q mutants lost most of their activity, and on the activity of endogenously expressed Hsp70 machine, because HSPA1A RNAi and an Hsp70 inhibitor largely abrogated the effects of DNAJs. For DNAJB2a, it was already shown that it generally requires interaction with Hsp70 for its action as stimulator of proteasomal degradation of clients (59) and suppressor of parkin aggregation (18). This result is rather striking as DNAJB6 and DNAJB8 suppressed polyQ aggregation in a largely Hsp70 independent manner (31, 60). In fact, purified DNAJB6 alone could suppress aggregation of purified polyQ peptides (61) suggesting that DNAJB6 acts downstream proteolytic cleavage of full length polyQ proteins to prevent polyQ peptide mediated aggregate seeding (46, 60, 61). For inhibition of polyQ aggregation, DNAJB6 required a C-terminal SSF-SST stretch rather than a functional J-domain for interaction with Hsp70 (31). This SSF-SST stretch was also required for DNAJB6 oligomerization (31) and consistently, purified DNAJB6 that was effective \textit{in vitro} also formed large oligomers (61). For inhibition of parkin C289G aggregation, the SSF-SST deletion mutant which forms apparent dimers (31) like several other canonical DNAJs (1), was still effective (Fig. 3). This implies that DNAJB6 and DNAJB8 can shift from more canonical, Hsp70 dependent dimers, effective in e.g. prevention of parkin C289G aggregation (this report), to non-canonical, Hsp70 independent oligomers that are effective as peptide chaperones that can prevent aggregation of polyQ (46, 60, 61) and, as recently found, also of amyloid-beta (Månsson et al, submitted). Interestingly, the F93L mutant of DNJAB6b that is implicated in limb-girdle muscular dystrophy (42) shows no loss of anti-aggregation activity on parkin C289G. This mutant was reported to be slightly defective in suppressing polyQ aggregation (42). However, we could not detect a large defect of this mutant in polyQ aggregation in our own experimental setting (data not shown), suggesting that this mutation may have impact on yet another aspect of the function of DNAJB6.

What is the mechanism through which all the DNAJs can suppress parkin C289G aggregation? We observed lower levels of parkin C289G than of parkin WT 24 hours after transfection and co-expression of the DNAJs even further lowered parkin C289G levels (but not parkin WT levels). This effect was lost when co-expressing the various DNAJ H/Q mutants (data not shown). As revealed by our western analysis, parkin C289G is present as a high molecular weight smear in the TX-100 insoluble fraction, consistent with other studies that the mutant is polyubiquitinated (23, 62), but has aggregated before being targeted to and degraded by the proteasome. Whereas cycloheximide chase experiments did not reveal a clear effect of the DNAJs on protein degradation rates (data not shown), we hypothesize that the DNAJs can interact with the misfolded and ubiquitinated parkin C289G at a very early stage so that
more of it is kept soluble and can be delivered (via Hsp70) to the proteasome for degradation. It is interesting to note that DNAJs were also still slightly effective under condition of HSPA1A knockdown and that this was paralleled by an increase of high molecular weight species of parkin C289G in the TX-100 soluble fraction (Fig. 3A and 3B). Based on this, we propose a model for the handling of parkin C289G by the Hsp70 machinery (Fig. 5) in which the DNAJs primarily and transiently bind misfolding and aggregating polypeptides of parkin C289G keeping them soluble for proteasomal degradation (holding activity). Via Hsp70 (HSPA1A and/or HSPA14) the substrates are next transferred to the proteasome for degradation. The holding activity of DNAJB1 may be the weakest of the ones tested, explaining why it lacks any effect when it when Hsp70 is depleted or inhibited.

Overall, our data further strengthens the fact that DNAJ proteins may be an attractive target in prevention of aggregation of disease causing proteins. Besides that their upregulation does not seem to affect the chaperone network (data not shown), which may have adverse effects (63), they may allow for strong and specific interventions in protein aggregation diseases that are just not all the same. On the other hand, DNAJB6, which we already identified as suppressor of polyQ aggregation in vitro (31) and that recently also delayed disease onset in vivo (Kakkar et al, submitted), may also be a target in Parkinson’s disease due to its dual mode of action as canonical Hsp70 co-chaperone and non-canonical inhibitor of peptide aggregation.
MATERIALS AND METHODS

Cell line, cell culture, and transient transfections
HEK293 stably expressing the tetracycline (tet) repressor (Flp-In T-REx HEK293, Invitrogen) were grown in DMEM (GIBCO) plus 10% FCS (Greiner Bio-one), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen), and for Flp-In TREx HEK293 cells, 5 mg/ml Blasticidin (Invitrogen). For transient transfections, cells were grown to 70%–80% confluence in 35 mm diameter dishes coated with 0.001% poly-L-lysine (Sigma) and/or on coated coverslips for confocal microscopy analyses. Cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instructions with a 1:1 ration of parkin and molecular chaperones. When transfecting with molecular chaperones tetracyclin was added to the medium. For transfection with siRNA, Lipofectamine2000 (Invitrogen) was used according to the manufacturer’s protocol before transfection with Lipofectamine. For inhibition of Hsp70 activity, VER-155008 (Sigma) at a 40μM concentration was added to cells immediately after transfection and then cells were further incubated for 8 hours.

Gene cloning and generation of mutants
Information about the (construction of) the chaperone plasmids used in this study is described in Hageman et al. (31). Information on the plasmid used for parkin is described in Ardley et al. (64).

Protein extraction to obtain soluble and insoluble fractions for Western blotting
24 hours after transfection cells were washed once in cold phosphate-buffered saline (PBS) and lysed in 200 μL 1% Triton X-100 in PBS, containing 1% complete protease inhibitor cocktail for mammalian cells (PIC) (Roche Applied Science) for 15 minutes on ice. Cell lysates were scraped and centrifuged at 14000 rpm (Eppendorf, rotor F45-30-11) for 15 minutes at 4°C. Supernatants were transferred to eppendorfs (Triton X-100 soluble fraction). Pellets were resuspended in 200 μL 1% SDS in PBS, containing 1% PIC and sonicated before centrifugation at 14000 rpm (Eppendorf, rotor F45-30-11) for 15 minutes at 4°C. Supernatants were collected (Triton X-100 insoluble fraction). Samples were diluted in 2x SDS-sample loading buffer with 20% β-mercaptoethanol (Sigma) to a final concentration of 1x and were left unboiled. Samples were used immediately or kept frozen at -20°C.

Western blot analysis
From the unboiled samples, 10 μl of soluble fraction, 10 μl of pellet fraction and 3 μl PageRuler Prestained Protein Ladder (Thermo Scientific) were loaded on 12.5% SDS-PAGE gels. SDS-PAGE was performed using the BioRad Mini-PROTEAN 3 system. After SDS-PAGE, proteins were transferred to nitrocellulose membranes by wet electrotransfer with the BioRad Mini-
PROTEAN 3 system. The transfer was carried out for 1.5 hour at 100 V. To prevent non-specific protein binding, membranes were incubated in 5% (w/v) non-fat dried milk in PBS-Tween (PBS-T) for one hour at room temperature. Membranes were washed three times 5 minutes in PBS-T and incubated for at least one hour with mouse anti-parkin antibody (Cell Signaling Technology) at a 1:1000 dilution, mouse anti-flag antibody (Sigma-Aldrich) at a 1:2000 dilution, mouse anti-V5 antibody (Invitrogen) at a 1:5000 dilution, mouse anti-Hsp70 (Enzo Life Sciences) at a 1:5000 dilution, and mouse anti-GAPDH (Fitzgerald) at 1:10000 dilution. Membranes were washed three times 10 minutes and incubated for one hour in anti-mouse HRP-conjugated secondary antibody (GE Healthcare) at an 1:5000 dilution in PBS-T. After incubation, membranes were washed three times 10 minutes in PBS-T and enhanced chemiluminescence detection was performed using the ECL Western Blotting substrate kit (Thermo Scientific) using 750 μL solution 1, mixed with 750 μL solution 2 per membrane.

Immunolabeling and microscopy

24 hours after transfection, indirect immunofluorescence of the flag-tag and the V5-tag was performed to detect the exogenously expressed parkin and chaperones. Cells were fixed with 3.7% formaldehyde for 15 minutes, washed three times 5 minutes with PBS, permeabilized with 0.2% Triton-X100 and blocked 10 minutes with 0.1% glycine and 30 minutes with 0.5% BSA in PBS. Incubation with rabbit anti-V5 antibody (Sigma-Aldrich) and mouse anti-flag antibody (Sigma-Aldrich) at a 1:200 dilution in PBS-T was performed overnight at 4°C. Cells were washed three times 5 minutes with PBS-T followed by a 1 hour incubation with Alexa Fluor 488 anti-rabbit (Invitrogen) and Alexa 594 anti-mouse (Invitrogen) secondary antibodies at a 1:1500 dilution in PBS-T. To visualize nuclei, cells were stained 10 minutes with 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). Cover slips were mounted in Citifluor (Agar Scientific). Images of flag, V5 and DAPI fluorescence were obtained using a Leica microscope (Leica DM6000 M) with a 63X oil lens. The captured images were processed using Leica Software and Adobe Photoshop CS5.

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