Chaperone Suppression of Cellular Toxicity of Huntingtin Is Independent of Polyglutamine Aggregation*

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Polyglutamine protein aggregation is associated with eight inherited neurodegenerative disorders. In Huntington's disease, N-terminal fragments of mutant huntingtin form intracellular aggregates and mediate cellular toxicity. Recent studies have shown that chaperones inhibit polyglutamine-mediated aggregation and cellular toxicity. Because chaperones also inhibit caspase activation to protect cells from death, it remains unclear whether the protective effect of chaperones on polyglutamine-mediated cellular toxicity is dependent on their inhibition of protein aggregation. In this study, we show that several chaperones including HSP 40, HSP 70, and N-ethy maleimide-sensitive factor can inhibit cellular toxicity caused by N-terminal mutant huntingtin fragments. However, only HSP 40 is able to inhibit huntingtin aggregation. Furthermore, time-course study suggests that the protection of chaperones against huntingtin toxicity is not the result of their suppression of huntingtin aggregation. Chaperones inhibit caspase-3 and caspase-9 activation mediated by mutant huntingtin, and this inhibition is independent of huntingtin aggregation. We propose that the inhibition of caspase activity by chaperones is involved in their suppression of polyglutamine toxicity.

Eight inherited neurodegenerative disorders including Huntington's disease (HD)† are associated with polyglutamine expansion in the disease proteins (1). The N-terminal region of the HD protein huntingtin contains a glutamine repeat, which is often expanded to have more than 37 glutamines in HD (2). A common feature of polyglutamine diseases is that polyglutamine expansion causes the disease proteins to aggregate (1). The polyglutamine aggregates are found in both the nucleus and cytoplasm. Although their roles remain unclear, polyglutamine aggregates certainly represent a pathological event associated with polyglutamine expansion, as their formation is correlated with disease progression (3–7). In an attempt to develop a therapeutic means for polyglutamine diseases, considerable effects have been made to find drugs or chemicals that can inhibit polyglutamine aggregation (8–12).

Of the molecules that modulate protein aggregation, heat shock proteins (HSPs) are found to be able to reduce polyglutamine aggregation and protect against neurodegeneration caused by polyglutamine expansion (10, 11, 13–19). HSPs are molecular chaperones, which prevent protein misfolding and/or aggregation and thus help maintain normal protein structure and function (20, 21). Among them, HSP 40 is a cofactor for HSP 70, an ATPase whose expression is up-regulated by a variety of cellular stresses (21–23). The fact that chaperones inhibit both polyglutamine aggregation and toxicity logically raises the possibility that they protect against polyglutamine-mediated cellular toxicity by suppressing polyglutamine aggregation (10, 15, 18). However, recent studies indicate that chaperones also directly inhibit caspase activity and cytochrome c release (24–28), raising another possibility that the protective effects of HSPs may result from their suppression of caspase activity. Although the latter possibility explains the finding that in Drosophila polyglutamine models chaperones suppress neurodegeneration but not intranuclear aggregates (29, 30), it is still unclear whether the inhibition of aggregates in the cytoplasm is required for the protective effects of chaperones. Because understanding the mechanism of protection of the chaperone will help find targets for treating polyglutamine diseases, it is important to investigate how chaperones protect cells from polyglutamine-mediated toxicity.

In this study, we examined the relationship between huntingtin aggregation and the protective effects of several different chaperone molecules including N-ethylmaleimide-sensitive factor (NSF) that is an ATPase and enriched in neuronal cells (31, 32). The studies demonstrate that the suppression of cellular toxicity by chaperones is independent of huntingtin aggregation in either the nucleus or the cytoplasm. The protective effects of chaperones are commonly associated with their suppression of caspase activity. We propose that the protection of chaperones against polyglutamine toxicity is more likely to relate to their inhibition of caspase activity.

EXPERIMENTAL PROCEDURES

DNA Constructs—Reverse transcription-PCR using RNAs from human embryonic kidney (HEK) 293 cells was performed to isolate full-length cDNAs for human HSP 70, HSP 40, and NSF. HSP 70 cDNA was isolated with a sense nucleotide primer 5′-CAGAAGGATCCATGCGCGC-CAAAGC-3′ and an antisense primer 5′-GGATTCTATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′. HSP 40 cDNA was isolated using a sense primer 5′-GAGGGATCCATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′. NSF cDNA was isolated using a sense primer 5′-GGATCCATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′ and an antisense primer 5′-GAATTCTATGGGTTAAGACCTAC-3′. The PCR products were subcloned into the PRK-HA vector, which provides the HA epitope (YPYDVPDYA) at the C termini of the expressed proteins (33). Huntington DNA constructs in the PRK vectors were obtained from our previous studies (33). These DNA constructs encode the HD exon1

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† The abbreviations used are: HD, Huntington's disease; HSPs, heat shock proteins; NSF, N-ethylmaleimide-sensitive factor; NLS, nuclear localization sequence; MTS, a modified 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RFP, red fluorescence protein; HEK, human embryonic kidney; HA, hemagglutinin; Q, glutamines; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; SNARE, soluble NSF-attachment protein receptors.
proteins with 20 or 120–150 glutamines. The nuclear localization sequence (NLS) PKKKRRK of the SV large T antigen was placed at the N terminus of huntingtin to generate NLS-tagged huntingtin. Red fluorescence protein (RFP, CLONTECH) was also inserted into the PRK vector for expression in HEK293 cells. The DNA constructs were sequenced at the Emory DNA core facility.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37 °C in a humidified 5% CO₂ atmosphere. The subconfluent (80%) HEK293 cells in 6-well plates were transfected with 2 μg of plasmid DNA and 10 μg/ml LipofectAMINE (Invitrogen) per well. For co-transfection, huntingtin and chaperone or RFP cDNAs were mixed together to transfet HEK293 cells. Western blotting was used to find amounts of co-transfected cDNAs that gave rise to the protein expression level equivalent to that with transfection of only one type of cDNA. The same amounts of huntingtin cDNA and the PRK vector were used for expression of huntingtin alone in HEK293 cells. The control was PRK vector transfection alone. Primary neuronal culture was performed using the same method as described in our previous study (6). Neurons were isolated from the striatum of embryonic day 14–16 rats and cultured in neurobasal/B27 medium. For transfection, 7-day-striatal neurons were transfected with cDNAs encoding the HD exon1 protein containing 120 (120Q) glutamine repeats or co-transfected with huntingtin and chaperone for 24 or 48 h in 6-well plates. After cells were washed with phosphate-buffered saline, the cells were lysed in the lysis buffer (10 mM Tris-HCl, 10 mM NaPO₄/NaHPO₄, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate). To measure caspase activity, 200 μl of assay buffer (40 mM PIPES, pH 7.2, 200 mM NaCl, 20 mM dithiothreitol, 2 mM EDTA, 0.2% (v/v) CHAPS, 20% sucrose) were added to a tube with a final concentration of 10 ng/μl of the peptide substrate (Ac-DEVD-AFC) for caspase-3 and LEHD-7-amino-4-trifluoromethyl cumarin for caspase-9. Cell lysates (200 μg of protein) were added to the tube to start the reaction. When the caspase inhibitor (Z-VAD-Fmk) was used to measure the specificity of the assay, it was added to cell lysates at a concentration of 50 μM for 30 min before the addition of the specific caspase substrate. Background was obtained with the same assay buffer without cell lysates. The reaction was incubated at 37 °C for 1 h followed by the measurement of the caspase activity with a fluorescence plate reader (Fluostar Galaxy, BMG Labtechnologies, Durham, NC) set at 390 nm excitation and 460 nm emission. To examine the cleavage of caspase-9, a rabbit polyclonal antibody against human caspase-9 (35) was used for Western blotting.

Statistical Analysis—All values were obtained from 3–5 independent experiments and expressed as the mean ± S.D. Statistical significance was assessed by using Student’s t test. p < 0.05 was considered significant.

RESULTS

Different Effects of Chaperones on Huntington Aggregation—To study the effects of chaperones on the aggregation of mutant huntingtin, we transfected the HD exon1 protein containing 120Q in HEK293 cells. Using EM48, an antibody that specifically recognizes huntingtin aggregates (5, 6), we observed abundant aggregates in the cytoplasm of transfected cells. More huntingtin aggregates were seen at 48 h than at 24 h after transfection, indicating that the aggregation of mutant huntingtin increased over time (Fig. 1). Most huntingtin aggregates, however, were in the cytoplasm or the perinuclear region. When the mutant huntingtin (150Q) was tagged with

lum bromide (MTS) assay (Cell Titer 96, Promega) as described in our previous study (33). Briefly, transfected HEK293 cells in 6-well plates were transferred onto 96-well plates at a density of 20,000 cells/well in serum-free medium, and 20 μl of MTS reagent (2.1 mg/ml) was added to each well. The cells were then incubated for 45–60 min at 37 °C in a 5% CO₂ incubator. The reaction was stopped by adding 25 μl of 10% SDS. The plates were read with a microplate reader (SPECTRAMax PLUS, Molecular Devices, Palo Alto, CA) at 490 nm. Each data point was obtained using a triplet assay, and vector transfection served as a control.

Fluorometric assays of caspase-3 and caspase-9 activity (33, 34) were performed using kits obtained from Bio-Rad. Cultured HEK293 cells were transiently transfected with mutant huntingtin 120Q or NLS-150Q or co-transfected with huntingtin and chaperone for 24 or 48 h in 6-well plates. After cells were washed with phosphate-buffered saline, the cells were lysed in the lysis buffer (10 mM Tris-HCl, 10 mM NaHPO₄/NaHPO₄, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate). To measure caspase activity, 200 μl of assay buffer (40 mM PIPES, pH 7.2, 200 mM NaCl, 20 mM dithiothreitol, 2 mM EDTA, 0.2% (v/v) CHAPS, 20% sucrose) were added to a tube with a final concentration of 10 ng/μl of the peptide substrate (Ac-DEVDF-AFC) for caspase-3 and LEHD-7-amino-4-trifluoromethyl cumarin for caspase-9. Cell lysates (200 μg of protein) were added to the tube to start the reaction. When the caspase inhibitor (Z-VAD-Fmk) was used to measure the specificity of the assay, it was added to cell lysates at a concentration of 50 μM for 30 min before the addition of the specific caspase substrate. Background was obtained with the same assay buffer without cell lysates. The reaction was incubated at 37 °C for 1 h followed by the measurement of the caspase activity with a fluorescence plate reader (Fluostar Galaxy, BMG Labtechnologies, Durham, NC) set at 390 nm excitation and 460 nm emission. To examine the cleavage of caspase-9, a rabbit polyclonal antibody against human caspase-9 (35) was used for Western blotting.

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Because nuclear aggregation of polyglutamine proteins is not influenced by chaperones (29, 30), we focused on the effects of chaperones on the aggregation of the 120Q protein in the cytoplasm. HSP 40, HSP 70, and NSF were chosen for comparing the effects of different chaperones on huntingtin aggregation. Co-transfection of HSP 40 with 120Q dramatically inhibited the formation of huntingtin aggregates (Fig. 2A). In contrast, co-transfection of HSP 70 with 120Q did not significantly change the density of aggregates formed by 120Q. Similarly, the expression of NSF with 120Q also failed to suppress huntingtin aggregation in transfected cells. An examination of NLS-150Q transfected cells revealed similar results showing that HSP 40 inhibited the aggregation of nuclear huntingtin, whereas HSP 70 and NSF did not (data not shown). To confirm that these transfected cells expressed both transfected huntingtin and chaperones, double immunofluorescence labeling of these cells was performed using EM48 and 12CA5 antibody that reacts with the HA epitope in transfected chaperones. Cells expressing both HSP 40 and 120Q had much fewer aggregates than those expressing both HSP 70 and 120Q (Fig. 2B). Similarly, cells expressing both NSF and 120Q also displayed abundant aggregates in the cytoplasm (data not shown). Some aggregates were labeled by antibodies to HSP 40 and huntingtin, suggesting that HSP 40 was colocalized with aggregates.

To better examine the effects of chaperones on huntingtin aggregation, we used Western blots to detect aggregated proteins that usually remained in the stacking gel (Fig. 3). Co-transfection of huntingtin with chaperones into HEK293 cells gave rise to the protein products as expected (Fig. 3A). The majority of the 120Q protein without NLS displayed as aggregated proteins even after transfection for 24 h, reflecting the rapid aggregation of overexpressed mutant huntingtin in the cytoplasm. HSP 40, but not HSP 70 and NSF, could significantly reduce the amount of aggregated huntingtin and increase the level of the monomer form of the 120Q protein. This effect, however, was attenuated at 48 h when 120Q protein aggregation was more intensified, perhaps because a greater extent of aggregation of overexpressed mutant huntingtin at 48 h reduces the ability of HSP 40 to inhibit aggregation. To examine the expression level of chaperones, the same protein samples used for examining transfected huntingtin were also analyzed by Western blots with 12CA5 antibody (Fig. 3B). The expression levels of these chaperones were similar, also suggesting that the inhibition of huntingtin aggregation is specific to HSP 40 expression. To further confirm this observation, we used a filter trap assay, which is also able to detect polyglutamine protein...
aggregation (8). With this assay, we found that HSP 40, but not HSP 70 and NSF, significantly inhibited the aggregation of the 120Q protein (Fig. 3B). Thus, immunofluorescence labeling, Western blots, and filter trap assays all support the idea that only HSP 40 is able to significantly inhibit huntingtin aggregation in transfected HEK293 cells.

We have shown that mutant huntingtin also formed aggregates when it was directed to the nuclei by the NLS epitope (Fig. 1). To examine whether chaperones have different effects on the aggregation of nuclear huntingtin, we co-transfected NLS-150Q with chaperones into HEK293 cells. Transfection of NLS-150Q resulted in smear products on the blot with two major bands (64 and 54 kDa in Fig. 4). It is possible that NLS-150Q with a larger glutamine repeat might be unstable in the nucleus before it forms aggregates. Transfection for 24 h did not allow NLS-150Q proteins to aggregate in the nucleus to the same degree as the 120Q proteins in the cytoplasm. Consistent with the immunocytochemical result as shown in Fig. 1, the aggregation of the nuclear huntingtin became significant at 48 h after transfection. At this time point, HSP 70 or NSF expression did not affect the aggregation of NLS-150Q either. In contrast, HSP 40 expression was still able to inhibit the aggregation of nuclear huntingtin, leading to the increased amount of monomer forms of NLS-150Q (Fig. 4).

The Effect of Chaperones on Huntingtin-transfected Primary Neurons—The predominant distribution of 120Q in the cytoplasm allowed us to study the relationship between cytoplasmic aggregates and cytotoxicity. To examine the effect of chaperones on huntingtin aggregation in neurons, we expressed the 120Q protein in cultured striatal neurons. Similar to what we saw in HEK293 cells, 120Q forms cytoplasmic aggregates. In addition, it causes nuclear DNA fragmentation. Co-expression of HSP 40 with 120Q could prevent DNA fragmentation and reduce huntingtin aggregation (Fig. 5). As a result, fewer aggregates were visible in huntingtin-transfected cells. Like HSP 40, transfected HSP 70 and NSF could also reduce DNA fragmentation in 120Q-transfected cells. However, the amount of huntingtin aggregates was not significantly reduced by the overexpression of HSP 70 and NSF. Thus, the protection of DNA fragmentation by these chaperones seemed not to be associated with their ability to remove huntingtin aggregates. Some transfected neurons displayed huntingtin aggregates that also contained HSP 40 or HSP 70. More interestingly, these aggregates were localized in the neurites. Most of these aggregates were negative to the 12CA5 antibody that labeled the transfected chaperones. It is possible that aggregated huntingtin masked protein immunoreactivity, making chaperones in the aggregates unrecognizable by the antibody. Alternatively, the interaction of chaperones between the huntingtin and chaperones, if any, was not stable for aggregated huntingtin.

Inhibition of Huntingtin Toxicity by Chaperones—The experiments described above suggest that the prevention of cellular toxicity by chaperones is independent of polyglutamine aggregation. To quantitatively examine the effects of chaperones on huntingtin toxicity, we used an MTS assay, which detects early apoptotic events of transfected cells. HEK293 cells allowed for a similar reduction of cell viability was seen at both time points.
The decreased cell viability is specifically associated with expanded polyglutamine in the 120Q protein, because transfection of N-terminal huntingtin with a normal repeat (20Q) did not significantly affect cell viability. Co-expression of chaperones for 24 or 48 h significantly increased the viability of cells that were transfected with 120Q or treated with STS. 20Q or HSP 70 transfection alone did not significantly affect viability. **, p < 0.01 compared with 20Q transfection alone. B, the viability of HEK293 cells transfected with NLS-tagged huntingtin (NLS-150Q) and chaperones for 24 and 48 h. *, p < 0.05 and **, p < 0.01 compared with NLS-150Q transfection alone.

Chaperones are found to reduce cellular toxicity mediated by a variety of stresses and apoptotic stimuli (37). To confirm that our experiment conditions do allow chaperones to promote cell survival, we also examined the effects of chaperones on cells treated with staurosporine, an apoptotic agent that has been used to test the protective effects of the chaperone (38). As...
reported previously, a short time (4 h) of treatment of cultured N2a cells with staurosporine could significantly reduce cell viability and increase the susceptibility of cells to mutant huntingtin (39). We observed that a 6-h staurosporine treatment (2.5 \textmu M) could dramatically decrease the viability of HEK293 cells transfected with RFP. However, cells that have been co-transfected with HSP 70 for 24 or 48 h showed an obvious increase in their viability (Fig. 6). HSP 40 or NSF transfection generated similar protective effects (data not shown). Thus, the survival-promoting effects of chaperones on polyglutamine and other insult-induced toxicity could be mediated by the same protective mechanism.

Chaperones Inhibit Caspase Activity in Huntingtin-transfected Cells—Numerous studies have demonstrated that the protective effect of chaperones is at least mediated by the suppression of apoptosis (37). Also, a number of recent studies has shown that mutant huntingtin increases caspase activity (33, 34, 36, 40, 41). To study whether chaperones act on caspase pathways to protect against huntingtin toxicity, we first measured the activity of caspase-3, an executioner caspase that can be activated by various apoptotic stimuli. Mutant huntingtin transfection alone led to the increased caspase-3 activity as reported in the previous studies (33, 36, 40, 41). The specific effect of mutant huntingtin to increase caspase-3 activity was confirmed by transfecting HEK293 cells with 20Q or HSP 70 alone. Neither 20Q nor HSP 70 increased caspase-3 activity (Fig. 7A).

![A](https://via.placeholder.com/150)

**A**, caspase-3 activity was measured in HEK293 cells transfected with 120Q and chaperones. PRK vector transfection alone served as a control. The 120Q transfection for 24 or 48 h led to an increase of caspase-3 activity. Co-expression of chaperones significantly inhibited this increase. B, 20Q or HSP 70 transfection alone did not significantly affect caspase-3 activity. Co-transfection of 120Q with RFP still activated caspases-3. RFP or chaperone-transfected HEK293 cells (24 h post-transfection) were treated with staurosporine (STS, 2.5 \textmu M) for 6 h before examining caspase-3 activity. Values are expressed as % of control (PRK vector transfection alone). *, p < 0.05 and **, p < 0.01 compared with 20Q transfection alone.

was seen at 48 h (Fig. 3). Similar inhibition of chaperones on caspase-3 activity induced by NLS-150Q was also observed (data not shown). In addition, chaperones inhibited caspase activity augmented by staurosporine treatment (Fig. 7B) that has been shown to reduce cell viability at the same time (Fig. 6). Taken together, these data suggest that chaperone transfection in our experiments reduces caspase-3 activity mediated by mutant huntingtin and other insults.

Recent studies show that mutant huntingtin also promotes the activation of endogenous caspase-9 in transfected cells (36) and *in vitro* synthesized caspase-9 (34). Caspase-9 is an initiator caspase and activates caspase-3 and other caspases. A variety of chaperones was found to inhibit caspase-9 activation (24, 25, 27, 42). Using Western blot analysis, we found that 120Q transfection increased the cleavage of caspase-9 to the extent similar to that observed in other huntingtin-transfected cells (36). Co-transfection of chaperones, however, could inhibit the processing of caspase-9 in huntingtin-transfected cells (Fig. 8A). To more quantitatively measure caspase-9 activity, we performed a fluorometric assay to measure the production of the activated form of caspase-9. The result was consistent with the Western blot, showing that all three chaperones tested could inhibit the activation of caspase-9 (Fig. 8B).

DISCUSSION

Numerous studies have shown that chaperones inhibit both polyglutamine aggregation and cytotoxicity (9–19), leading to the hypothesis that the protection of the chaperone is found through its inhibition of polyglutamine aggregation. This study shows that the inhibition of huntingtin toxicity by chaperones is independent of their effects on polyglutamine aggregation. This inhibition is more likely to result from the inhibition of caspase activation. Thus, our study suggests that inhibition of cell death
processes may account for the protective effects of chaperones.

The finding that the inhibition of huntingtin toxicity by chaperones is independent of decreased huntingtin aggregation is supported by the following lines of evidence: 1) HSP 40, which is able to inhibit huntingtin aggregates, produces the protective effect that is not associated with the decrease in huntingtin aggregation. 2) Chaperones HSP 70 and NSF, which do not suppress huntingtin aggregation, can also reduce huntingtin toxicity. 3) All three chaperones inhibit caspase-3 and caspase-9 activity and improve cell viability regardless of their ability to inhibit huntingtin aggregation and different subcellular localization of transfected huntingtin. Thus, these findings suggest that inhibition of caspase activity by chaperones may be a common mechanism for the protective effects of chaperones.

These findings also support the idea that nuclear inclusions or aggregates of polyglutamine proteins are not the direct cause of cell death (43, 44). Although the role of nuclear aggregates remains controversial, these aggregates represent a pathological process caused by polyglutamine aggregation of polyglutamine proteins. Expanded polyglutamine stretch may form a β-sheet hairpin structure that causes the misfolding and aggregation of the protein (1). Scherzinger et al. (8) demonstrated that huntingtin polyglutamine aggregation is a self-driven process that occurs in a concentration-dependent and repeat length-dependent manner. It has been expected that molecular chaperones, which prevent protein misfolding, should suppress the aggregation of polyglutamine proteins and might reduce cellular toxicity caused by misfolded proteins. Indeed, many in vitro and in vivo studies have shown that chaperones can inhibit polyglutamine-induced cell death (11, 13–19). However, whether this protection results from the inhibition of polyglutamine aggregation is unclear. For example, in Drosophila polyglutamine disease models, the overexpression of chaperones HSP 70 and HSP 40 inhibits neurodegeneration in eyes but does not suppress nuclear polyglutamine aggregation (29, 30). Thus, it is interesting to know whether similar effects also occur with cytoplasmic huntingtin aggregates and whether chaperones protect against cell toxicity via a mechanism that is not related to polyglutamine aggregation.

These studies by focusing on the cytoplasmic mutant huntingtin and its aggregates show that three different chaperones can protect against cell death and inhibit caspase-3 and caspase-9 activity. It has been recently found that HSP 70 inhibits cytochrome c release and caspase activation (24–28). Because HSP 40 is a cofactor of HSP 70, HSP 40 may work with HSP 70 on the same pathway HSP 70 does. The protective effect of HSP 70 is required by the presence of the ATPase domain (24). NSF, which is involved in vesicular recycling, also carries an ATPase domain. NSF is thought to be a chaperone, because it disassembles the SNARE protein complex in a chaperone-like interaction driven by ATP hydrolysis (32, 45). NSF also binds to other proteins (46–49) and may be involved in refolding a variety of protein complexes. Although the role of NSF protection of polyglutamine-mediated cellular toxicity remains to be studied, this protection is not dependent on huntingtin aggregation. Thus, prevention of cell death by chaperones is not necessarily associated with the decrease in huntingtin aggregation. It is also probable that mutant huntingtin triggers apoptosis via multiple pathways. For example, cytoplasmic mutant huntingtin may activate caspase-9, which in turn activates caspase-3 (34). On the other hand, intranuclear huntingtin may alter gene expression to induce caspase-1 and caspase-3 activation (33). Because caspase-3 activation is a common process for various apoptotic stimuli and stresses, it is expected that the decrease in caspase-3 activity by different
chaperones could improve cell viability. Whether these chaperones directly or indirectly inhibit the same or different caspases remains to be further investigated.

Although this study demonstrates that suppression of cell death by chaperones is independent of huntingtin aggregation, it remains possible that chaperones suppress polyglutamine toxicity by altering biochemical properties of mutant huntingtin, and that their protection does not require complete refolding of mutant polyglutamine proteins to the native state. This possibility is suggested by polyglutamine Drosophila models in which transgenic Hsp 40 and Hsp 70 can significantly increase the amount of the monomer or soluble form of expanded polyglutamine proteins, although they cannot completely inhibit the aggregation of polyglutamine proteins (50).

In transfected cells, the rapid aggregation of overexpressed huntingtin might not allow us to see the effects of chaperones on soluble huntingtin. Also, refolding or degradation of misfolded and aggregated proteins requires the interactions between various chaperones and proteins in ubiquitin-proteasome pathways. The expression levels of these regulatory proteins may not be the same for in vitro and in vivo models. Moreover, huntingtin-induced cell death and chaperone-mediated protection may not be entirely through caspase cascades. For example, mutant huntingtin was found to induce cell death in a manner of non-classic apoptosis (51, 52). Thus, the role of chaperone inhibition of caspases in protecting huntingtin-induced cell death remains to be further characterized. Despite these findings, the dissociation of chaperone protection with huntingtin aggregation suggests that evaluation of therapeutic effects of drugs on HD pathology should not be solely based on their ability to inhibit huntingtin aggregation. Our findings also suggest that prevention of the downstream of apoptotic pathways without affecting highly insoluble huntingtin aggregates could efficiently reduce mutant huntingtin-induced cellular toxicity.

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