Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells

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A number of observations point to the aggregation of expanded polyglutamine [poly(Q)]-containing proteins as playing a central role in the etiology of Huntington’s disease (HD) and other expanded CAG-repeat diseases. Transfected cell and transgenic animal models provide some of this support, but irrefutable data on the cytotoxicity of poly(Q) aggregates is lacking. This may be due in part to difficulties in observing all aggregated states in these models, and in part to the inability to conclusively rule out the role of monomeric states of the poly(Q) protein. To address these questions, we produced aggregates of simple poly(Q) peptides in vitro and introduced them to mammalian cells in culture. We find that Cos-7 and PC-12 cells in culture readily take up aggregates of chemically synthesized poly(Q) peptides. Simple poly(Q) aggregates are localized to the cytoplasm and have little impact on cell viability. Aggregates of poly(Q) peptides containing a nuclear localization signal, however, are localized to nuclei and lead to dramatic cell death. Amyloid fibrils of a non-poly(Q) peptide are non-toxic, whether localized to the cytoplasm or nucleus. Nuclear localization of an aggregate of a short, Q₂₀, poly(Q) peptide is just as toxic as that of a long poly(Q) peptide, supporting the notion that the influence of poly(Q) repeat length on disease risk and age of onset is at the level of aggregation efficiency. The results support a direct role for poly(Q) aggregates in HD-related neurotoxicity.

INTRODUCTION

There are eight known human genetic disorders, including Huntington’s disease (HD), associated with the expansion of a CAG repeat in an open reading frame (1). Expression of the encoded polyglutamine [poly(Q)] repeat is required for development of the disease. Significant data suggest that the pathology of poly(Q) expansion primarily involves a toxic gain of function that is centered in the poly(Q) sequence itself. There is as yet no consensus on the molecular basis of the toxicity of expanded poly(Q) sequences, however. The transition in poly(Q) sequence length from benign to pathological is quite sharp: lengths up to 36 glutamines are routinely not associated with disease, while lengths of ≥38 almost invariably lead to disease; the only exception to this trend is in the disease spinocerebellar ataxia type 6 (SCA6), where the threshold is in the 15–20 range (2). Increasing CAG length corresponds to a decrease in age of onset, with very long repeats, in the 80–100 range, often causing disease symptoms before the age of 10 years (2).

One hypothesis for the basis of poly(Q) toxicity is that expansion beyond 36 glutamines leads to a conformational change creating a misfolded structure with cytotoxic properties (reviewed in 1). Although some immunochemical data have been interpreted as being consistent with a structural difference between long and short poly(Q) molecules (3,4), circular dichroism (5–7) as well as other (7) measurements show that both long and short poly(Q) sequences exhibit random coil structure in monomeric poly(Q) sequences. The ability of some antibodies to discriminate long poly(Q) sequences from short can be attributed to the greater multiplicity of independent epitopes on longer poly(Q) sequences (7).

An alternative hypothesis is that the toxicity of long poly(Q) sequences is linked to the increased efficiency with which they can form insoluble, amyloid-like aggregates. A transgenic animal model of expanded CAG-repeat disease exhibits both neurological symptoms reminiscent of the human physiology and the accumulation of neuronal, intranuclear inclusions (NIIs) staining for poly(Q) and ubiquitin (8). Such inclusions are also seen in brain sections from patients with HD, Machado–Joseph disease (SCA3) and other expanded CAG-repeat diseases (9–11). In addition, expression of expanded CAG-containing gene fragments in cell models leads to both cell death and to the accumulation of inclusions (10). Consistent with the role of aggregation in the disease mechanism, genetic experiments in cell (12) and animal (13) models implicate certain molecular chaperones as suppressors of poly(Q) toxicity. In addition, in vitro studies suggest that the repeat-length dependence of disease risk and severity may be related to the increased efficiency with which long poly(Q)

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sequences form aggregates (4,5,14,15). In fact, the repeat-length dependence of aggregation nucleation appears to be sufficient to explain decreases of age of onset with increased poly(Q) repeat length (16).

A direct toxic role for poly(Q) aggregates has been questioned, however, because of the imperfect correlation between detectable inclusion burden and abnormal physiology observed in some studies (reviewed in 1,17–19). The subcellular location where aggregate toxicity is expressed is also open to debate. Although poly(Q) inclusions are most often observed in neuronal nuclei in animal and cellular models, they are more commonly found in the neuropil in HD brain material (20). Several studies have exploited nuclear import and export signals to achieve compartmental localization and aggregation of expressed poly(Q) proteins. In some (21,22), but not all (23), of these studies, nuclear localization has been shown to be important for cytotoxicity. The importance of the poly(Q) sequence for cytotoxicity, relative to other sequence elements in expanded CAG-repeat disease proteins, is also not always clear (24).

Technical factors limit the ability of many studies to conclusively test the role of poly(Q) aggregates in triggering disease pathology. Cell and animal models require over-expression of soluble, monomeric poly(Q) forms as necessary precursors to aggregate formation, making it difficult to draw conclusions about the relative toxicities of native monomer, misfolded monomer, aggregation intermediates or some form of mature aggregate. It is also difficult to detect and quantify the small aggregates that may be the most potent toxic forms of poly(Q) (5).

In the work described here, we examine the role of poly(Q) aggregates in the disease mechanism more directly by preparing protein aggregates in vitro from simple poly(Q) peptides and introducing them into the cytoplasm or nucleoplasm of cells in culture.

RESULTS

The experimental approach taken in this work was made possible by the recognition that even long poly(Q) peptides can be solubilized and rendered monomeric by a mild solvent treatment that produces poly(Q) peptides that from all appearances are chemically and conformationally identical to those produced in the cell as portions of larger proteins (25). Incubation of these peptides in vitro generates aggregates with kinetics and product morphologies similar to biosynthetic poly(Q)-containing protein fragments (5,26). A series of poly(Q) and other peptides, tagged with fluorescein and in some cases including nuclear localization signals (NLS), were used in this study (Table 1). Of the poly(Q) peptides, one (Q_{20}) is below and one (Q_{42}) above the pathological threshold of ~36 glutamines.

Poly(Q) aggregates of the kind used here exhibit many of the properties normally associated with amyloid, including a high content of β-sheet (5) and the ability to bind and alter the fluorescence properties of the dye thioflavin-T (26). In analogy to amyloid-like aggregates of poly(Q)-containing proteins expressed in cells, these in vitro produced aggregates of chemically synthesized poly(Q) peptides are highly resistant to dissolution in boiling aqueous SDS. A Coomasie Brilliant Blue-stained gel with individual lanes loaded with either aggregate or monomers of the Q_{20} and Q_{42} peptides showed that after 3 minutes in boiling SDS gel loading buffer, no detectable aggregate was released from a Q_{42} aggregate and only a small amount (<10%) of peptide was released from a Q_{20} aggregate (data not shown). Figure 1 shows electron micrographs of the aggregates used in this study. All the peptides form ordered aggregates of approximately the same width. However, the poly(Q) aggregates are short and curvilinear, while the peptides of the CspB-1 peptide used as a control (see below) are relatively long and straight.

Preliminary flow cytometry studies (Fig. 2) showed that it is possible to efficiently deliver aggregates of fluorescein-tagged Q_{42} poly(Q) peptides into both PC-12 and Cos-7 cells by first packaging them in liposomes. With both cell types, a 4-hour incubation with packaged poly(Q) aggregates results in >80% of the cells exhibiting fluorescein fluorescence. Surprisingly, however, controls with the same cells mixed with aggregates not formulated in liposomes also lead to a high percentage of fluorescent cells. Figure 2 shows that although aggregates mixed directly with cells are taken up more slowly, after 24–48 hours >80% of cells mixed with aggregates become fluorescent. We decided to conduct further experiments without liposome packaging, in order to reduce the possibility of artifacts associated with added lipids in the cell toxicity studies.

In order to confirm that aggregates were internalized within cells, we used fluorescence confocal microscopy. Figure 3A shows micrographs of interior focal planes of both PC-12 and Cos-7 cells that had been incubated with F-Q_{42} aggregates. Figure 3A clearly shows that these aggregates are located within cells, rather than adherent to the outside of the cells. In order to probe the dependence of aggregate toxicity on subcellular localization, we attempted to deliver them into the nuclei of cells. To do this, we added to some synthetic peptides a sequence consisting of the NLS sequence PKKKRKV with a fluorescein tag on the N terminus and a GG spacer on the C terminus (Table 1). Flow cytometry shows that aggregates of such peptides are taken up into cells about as efficiently as are aggregates without the NLS (not shown). When the nuclei of these cells are isolated and analyzed by confocal microscopy, fluorescent foci are clearly observed within the nuclear envelope (Fig. 3B). In contrast, aggregates of F-Q_{42} lacking an NLS accumulate on the nuclear membrane, but do not penetrate into the nucleus (Fig. 3B).

To improve the efficiency of cellular and nuclear uptake, we devised methods to produce a smaller effective particle size through sonication and membrane filtration (see Materials and Methods). Figure 3B shows that aggregates of F-NLS-Q_{42} become more efficient at crossing into the cell nucleus as the average particle size of the aggregates gets smaller. Decreasing the particle size of F-Q_{42} aggregates, in contrast, does not reverse their fundamental inability to penetrate the nucleus. Based on their filtration behavior, aggregates prepared in this way appear to have an average effective size of >100 nm, while the functional diameter of the nuclear pore is considered to be no larger than ~26 nm (27). Nonetheless, the dependence of aggregate uptake into the nucleus on the presence of an NLS strongly suggests that it occurs via the NLS-associated nuclear pore. Figure 3B also shows that fluorescein-tagged
NLS-CspB-1 aggregates are very efficient at getting into the nucleus. This shows that aggregate transport across the outer and nuclear membranes does not depend on the poly(Q) sequence.

In order to investigate the effect of cellular and nuclear uptake of poly(Q) aggregates on cell viability, we first used propidium iodide (PI) exclusion, a standard staining method for distinguishing viable from non-viable cells. Figure 4 shows that PC-12 cells efficiently take up F-Q42 aggregates but remain viable, despite their aggregate burden, with only 12% of the cells (essentially identical to the control) exhibiting PI staining. In contrast, 65% of the PC-12 cells treated with F-NLS-Q42 exhibit PI fluorescence and are hence non-viable. Figure 4 shows that similar results are obtained with Cos-7 cells. Although Q20 peptides form aggregates very sluggishly at 37°C (5), it is possible to produce Q20 aggregates by exploiting the process of freeze concentration (26). Figure 4 shows that aggregates of the peptide F-NLS-Q20 kill both PC-12 and Cos-7 cells about as effectively as do aggregates of F-NLS-Q42. Figure 4 also shows that aggregates of both Q20 and Q42 peptides lacking an NLS exhibit poor cytotoxicity.

As a control peptide for the cytotoxicity experiments, we added an NLS sequence to Csp-B1, a peptide fragment of a bacterial protein previously shown to make amyloid fibrils in vitro (28). This modified peptide remains capable of forming amyloid fibrils (Fig. 1) and the aggregates are as efficiently taken up and into cell nuclei as are poly(Q) aggregates (Fig. 3). Figure 4 shows that, in spite of this efficient nuclear uptake, Csp-B1 aggregates are not toxic to Cos-7 or PC-12 cells. Thus, the toxicity of intranuclear aggregates in these experiments seems to require the presence of the poly(Q) sequence.

Table 1. Synthetic peptides used in the experiments

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence</th>
<th>Molecular weight (Da)</th>
</tr>
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<tbody>
<tr>
<td>F-Q42</td>
<td>N(^\circ)-Fluor-KKQ42KKC(_{COOH})</td>
<td>6656</td>
</tr>
<tr>
<td>F-NLS-Q42</td>
<td>N(^\circ)-Fluor-PKKRKKVGG-Q42KKC(_{COOH})</td>
<td>7378</td>
</tr>
<tr>
<td>F-NES-Q42</td>
<td>N(^\circ)-Fluor-LPPLERLTLG-Q42KKC(_{COOH})</td>
<td>7662</td>
</tr>
<tr>
<td>F-Q20</td>
<td>N(^\circ)-Fluor-KKQ20KKC(_{COOH})</td>
<td>3452</td>
</tr>
<tr>
<td>F-NLS-Q20</td>
<td>N(^\circ)-Fluor-PKKRKKVGG-Q20KKC(_{COOH})</td>
<td>4175</td>
</tr>
<tr>
<td>F-NLS-CspB-1</td>
<td>N(^\circ)-Fluor-PKKRKKVGG-MLEGKVKWFNSEKGFIEVEGKKC(_{COOH})</td>
<td>4127</td>
</tr>
</tbody>
</table>

\(^\circ\)Fluor is a fluorescein group with the following structure:

Figure 4 summarizes the results of one experiment. We conducted three to eight (depending on the aggregates used) similar independent experiments, and confirmed that aggregates of both long and short poly(Q) peptides tagged with an NLS are highly cytotoxic, while cells treated with aggregates of identical peptides lacking the NLS exhibit essentially the same low amount of cell death as do untreated control cells (Fig. 5A). Aggregates of the NLS-tagged Csp-B-1 peptide are similarly non-toxic. Consistent with these results, we also found that that NLS–poly(Q) aggregates generate cell death responses in both Cos-7 and PC12 cells as measured by both the lactate dehydrogenase (LDH)-release assay (Fig. 5B) and in the MTT (MTS) reduction assay (Fig. 5C), two classic cytotoxicity tests (29).

Figure 6A shows that aggregates of both F-NLS-Q42 and F-NLS-Q20 exhibit similar dose–response curves in the LDH-release assay, with midpoints in the low micromolar range. At the same time, aggregates of poly(Q) peptides lacking the NLS, as well as the nuclear-targeted non-poly(Q) control F-NLS-CspB-1, are completely non-toxic up to 100 \(\mu\)M concentration or higher. Aggregates of a Q42 peptide containing a nuclear export signal, F-NES-Q42 (Table 1) were similarly non-toxic up to a 100 \(\mu\)M concentration (not shown). The lack of toxicity of these control peptides, even at very high concentrations, emphasizes the significance of the efficient cell killing observed for the nuclear-targeted aggregates. There are several possible explanations for the slightly higher potency of the F-NLS-Q42 aggregates compared with F-NLS-Q20 aggregates shown in Figure 6A. These include the 2-fold greater mass, a possible higher efficiency of cellular and/or nuclear uptake, and a lower tendency to disaggregate (but see below) of Q42 compared with Q20 aggregates.
Figure 6B shows the results of a similar dose–response study initiated by applying monomeric peptides to PC-12 cells in culture. All of these peptides, including F-NES-Q_{42} (not shown), are non-toxic up to 100 μM, with the exception of monomeric F-NLS-Q_{42}. The toxicity observed for monomeric F-NLS-Q_{42} is almost certainly due to the strong ability of this peptide to rapidly aggregate under the experimental conditions. In fact, at the end of the incubation period prior to LDH-release analysis, at toxic concentrations of the Q_{42} peptide, aggregates are clearly visible surrounding and covering the cells, and

Figure 1. Ultrastructure of in vitro aggregates. Aggregates were fixed to mica grids and negatively stained with 0.25% w/v potassium phosphotungstate solution. Micrographs were collected on a Hitachi H600 scanning electron microscope. Shown are aggregates of (A) F-NLS-Q_{42}; (B) F-Q_{42} (sonicated); (C) F-NLS-Q_{20}; (D) F-Q_{20}; (E) F-NLS-CspB-1.
fluorescent foci are observed in the cells by confocal microscopy (not shown). The lack of toxicity observed for monomeric F-NLS-Q20, in contrast to the toxicity observed for the aggregated version, again emphasizes that aggregation, which is very inefficient for short poly(Q) peptides at 37°C (5,26), is required for cytotoxicity.

To test whether significant monomer might be generated by aggregate dissociation, and thus contribute to cell or nuclear uptake of aggregates or to cytotoxicity, we conducted stability tests in PBS. In an experiment capable of detecting 2% dissociation, neither Q20 nor Q42 aggregates dissociated measurably after up to 5 days’ incubation at 37°C in PBS (Fig. 7). After five days, the Q20 aggregates begin to dissociate slightly, while Q42 aggregates show no measurable dissociation; this is consistent with their relative critical concentrations in aggregation (5). Csp-B1 aggregates dissociate slightly (5%) after incubation for 3 days under these conditions. It thus seems very unlikely that the observed ability of these aggregates to be transported across cell and nuclear membranes is due to a disassembly–reassembly pathway; this is especially true for F-NLS-Q20 aggregates, since Q20 peptides require high concentrations and long incubation times to aggregate (5). The unlikelihood of this mechanism is further supported by the lack of toxicity of F-NLS-Q20 as a monomer (Fig. 6B), contrasted with its toxicity as an aggregate (Fig. 6A).

The time course of cell death induced by toxic aggregates is consistent with the time course for aggregate internalization. Figure 8 shows the time course of cell death, as measured by the LDH-release assay, for cells treated with various aggregates (Fig. 8A) or monomeric peptides (Fig. 8B). In agreement with other results described here, nuclear-localized aggregates of both Q20 and Q42 peptides are toxic, while the only monomeric poly(Q) peptide to exhibit toxicity, presumably by virtue of contemporaneous aggregation, is F-NLS-Q42. Figure 2 shows that substantial uptake of poly(Q) aggregates occurs at some time between 4 and 24 hours. This is consistent with the results shown in Figure 8, which show an onset of cell killing between 15 and 18 hours after initiation of the experiment.

**DISCUSSION**

The results described in this paper suggest that the efficient and rapid development of cytotoxicity in response to poly(Q) sequences requires both their aggregation and their nuclear localization. In our model system, aggregates of poly(Q) peptides localized to the cytoplasm, including perinuclear inclusions, express little or no cytotoxicity. Likewise, aggregates of non-poly(Q) sequences delivered to the nucleus are not toxic. Monomeric poly(Q) sequences are toxic only to the extent that they can aggregate during the course of the experiment and are targeted to the nucleus. Nuclear-localized aggregates of poly(Q) peptides are toxic regardless of peptide length. This supports the hypothesis that the repeat-length dependence of disease risk in expanded CAG-repeat diseases is related to the length dependence of aggregation efficiency.
Once aggregates are formed, as governed by the biophysics of poly(Q) aggregation (16), and once these aggregates appear in the nucleus, any poly(Q) peptide aggregate is toxic.

The results described here speak against disease mechanisms that, on the one hand, invoke unique toxic features of special conformations of monomeric forms of expanded poly(Q) repeat proteins, and, on the other, postulate a generic protective role for aggregates in expanded CAG repeat-associated cytotoxicity. Our results show that poly(Q) aggregates themselves can be toxic to cells. It remains possible that the very large, easily visible super-aggregates known as inclusions (NIIs) may be protective to cells, to the extent that they consolidate, and thereby reduce the effective surface area (5), of smaller, toxic aggregates. The simplest explanation for the link between poly(Q) repeat length and disease risk and age of onset is that expanded poly(Q) sequences kill cells by virtue of their ability to efficiently form toxic aggregates.

These experiments are not focused on the cellular basis of aggregate toxicity. However, it is worth noting that were toxicity due to the saturation or inactivation of chaperones, proteasomes or other cellular machinery by accumulated aggregates, then it might be expected that nuclear aggregates of Csp-B1 would be as toxic as poly(Q) aggregates in our experiments. On the other hand, the observed toxicity of nuclear-localized poly(Q) aggregates, regardless of poly(Q) repeat length, is consistent with mechanisms by which poly(Q) aggregates specifically recruit, sequester, and thereby alter the activities of poly(Q)-containing proteins through a continuation of the aggregation process (30,31). Many transcription factors contain poly(Q) sequences of modest length (32), and several transcription factors have been implicated as mediators of poly(Q) toxicity by virtue of their abilities to be selectively recruited into NIIs (30,31,33–35). There is a significant body of data implicating transcriptional dysregulation as a component of expanded CAG-repeat diseases (36).

The cytotoxicity of nuclear, but not cytoplasmic, aggregates reported here is consistent with cell and animal experiments showing toxicity associated with aggregates localized to the nucleus. The observation of cytoplasmic aggregates in expanded CAG-repeat disease human tissue (20,37–39),
however, suggests a possible role for cytoplasmic aggregates in long-term toxicity. One unifying hypothesis is that aggregates remaining in the cytoplasm are benign, but that, by mechanisms as yet unknown, cytoplasmic aggregates occasionally migrate into the nucleus, with cataclysmic results. Alternatively, cytoplasmic poly(Q) aggregates may produce sublethal dystrophies in affected neurons. It may be possible to use the model system described here to examine more subtle effects of cytoplasmic poly(Q) aggregates on cell viability and function.

There is growing speculation that relatively small assembly intermediates may play key cytotoxic roles in a number of protein aggregation-related neurodegenerative diseases. Amyloid assembly intermediates of a number of proteins, including proteins not associated with disease, have been shown to have cytotoxic properties in cellular models (40). The identification of annular assembly intermediates in early aggregation timepoints in the amyloid formation of Aβ, α-synuclein and other proteins has drawn increasing support for early speculations (41–43) that amyloid-related toxicity is mediated by membrane insertion and depolarization (44,45). The toxicity reported in this paper appears to be fundamentally different from the above examples, however, in a number of ways. First, the toxicity of our aggregates requires the presence of a poly(Q) sequence, while amyloid-like aggregates of a similarly treated and targeted control peptide are benign. Second, toxicity of our aggregates, in parallel with toxicity in other cell models of HD, requires nuclear localization, whereas the target of the presumptive membrane depolarizing assembly intermediates prepared from other amyloidogenic proteins is presumably the outer membrane. Third, the poly(Q) aggregates used here appear to be mature products of aggregation, rather than metastable assembly intermediates. While it is an attractive model that all protein aggregates kill cells by a common mechanism, the weight of evidence to date is that poly(Q) aggregate toxicity is mediated by a set of protein–protein interactions that is very specific to the expanded CAG-repeat diseases.

It is of great interest why protein aggregates, despite their large sizes, are so efficiently taken up by cells and cell nuclei in the experiments described here. This surprising result is not restricted to poly(Q) sequences, since we observed similar or greater nuclear uptake of Csp-B1 aggregates. All of the aggregates described here carry a net positive charge at neutral pH; it is possible that this feature may contribute to their efficient membrane transport. Since multiple localization signals are thought to facilitate passage of large substrates through the nuclear pore (27), it is worth noting that the aggregates described here are multidentate, since each constituent peptide of the aggregate contains an NLS. Our results suggest that even relatively large aggregates of poly(Q) and other sequences can be transported across the nuclear membrane in a process mediated by an NLS. This suggests the possibility that, in the disease mechanism, poly(Q) aggregates containing cryptic NLS sequences [or co-aggregates between disease proteins and other poly(Q) proteins contain NLS sequences (46)] might form in the cytoplasm and then be transported into the nucleus.

The experiments described here confirm the intrinsic, sequence-specific cytotoxicity of intranuclear poly(Q) aggregates. The ability to deliver such aggregates into different cell types should make possible a variety of studies on the biochemical details of cytotoxicity pathways. Additional studies now underway should provide details on the mechanism by which cells die in this disease model. Our studies were designed to evaluate the fundamental toxic properties of the poly(Q) sequence itself. The surrounding polypeptide sequences of actual disease-associated proteins containing

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**Figure 4.** Cell viability by flow cytometry. PC-12 cells (top series) or Cos-7 cells (bottom series) were incubated with various aggregates for 24 h, then stained with propidium iodide and analyzed by flow cytometry. The individual panels show the result of one experiment each in which 1.2 μm filtered aggregates were mixed with cells and evaluated for both cell uptake of aggregates (FL-1) and cell death (FL-3). Thus, counts in the upper right quadrant indicate cells that have both taken up aggregates and lost membrane integrity. Panel labels indicate percentage cell death by PI staining.
Figure 5. Viability of aggregate-treated PC-12 (white bars) or Cos-7 (black bars) cells after treatment with various aggregates. (A) Propidium iodide staining. Summary of three to eight independent experiments of the type shown in Figure 4. (B) LDH release. Data show the mean and standard error of two to four experiments. (C) MTS reduction. Data show the mean and standard error of two to four experiments, except for F-NLS-CspB-1/Cos-7 which is from a single experiment. In each section, the asterisk indicates data in which $P \leq 0.001$ compared with control.
expanded poly(Q) may modulate disease features through a number of mechanisms. It may be possible to conduct further experiments on aggregates of disease proteins and their fragments to evaluate such effects.

MATERIALS AND METHODS

Peptides

The peptides used in these experiments were prepared by Fmoc chemistry at the Keck Biotechnology Center of Yale University. Poly(Q) peptides contain flanking pairs of lysine residues to improve solubility (5). All peptides were synthesized with a fluorescein group on the N terminus, and some peptides also included as NLS peptide sequence (47) separated from the poly(Q) by a pair of glycine residues. Peptides were used without purification and contained some glutamine-deletion peptides from inefficiencies in the chemistry (5). Peptides used in this study are listed in Table 1.

Preparation of monomers and aggregates

Peptides were processed by first dissolving and rigorously disaggregating the lyophilized solid-phase synthesis product

![Figure 6. Dose-dependent cytotoxicity of poly(Q) aggregates and monomers. PC-12 cells were incubated with various concentrations of poly(Q) aggregates (A) or monomers (B), and cell death was measured by the LDH-release assay, filled squares, F-NLS-Q42; open squares, F-Q42; filled diamonds, F-NLS-Q20; open diamonds, F-Q20; filled triangles, F-NLS-CspB-1. The data reflect the mean values of two to four independent repetitions, each conducted in triplicate; data were normalized to a value of 20% cell death for the untreated cells. The average EC_{50} for the F-NLS-Q42 aggregates is ~3–4 μM, and for F-NLS-Q20 aggregates 12–15 μM. The EC_{50} for monomeric F-NLS-Q42 is ~4–5 μM, while monomeric F-NLS-Q20 was non-toxic up to 100 μM.](https://academic.oup.com/hmg/article-abstract/11/23/2905/641229)
using an organic solvent treatment, followed by ultracentrifugation of a pH 3 aqueous solution (25,48). For toxicity experiments with monomers, this pH 3 solution was neutralized to pH 7 using a buffer concentrate (10/C2 PBS) and was immediately used to treat cells.

Aggregates were prepared by the C0/C14/C method as described previously (5). The aggregate suspension was centrifuged for 30 min at 14 000 g to collect the aggregates.

Aggregate size was modified by a two-step procedure involving sonication and membrane filtration. Aggregates were washed once with PBS using centrifugation at 14 000 g, then resuspended in PBS at a concentration of ~100 μg/ml. Sonication was accomplished with a probe sonicator at 0°C in six pulses of 30 s each. Sonicated aggregates were manually filtered through a 1.2 μm Acrodisc syringe filter (Gelman Sciences). In some cases, the filtrate was further filtered through a 0.45 μm filter and a 0.22 μm filter. The filtration yield was determined by comparing the fluorescence signal of a suspension of filtered aggregates with that of non-filtered aggregates. The aggregate suspensions were then adjusted to 10 μM concentration (monomer peptide equivalent) before applying them to cells.

Liposome encapsulation of aggregates was accomplished using a commercially available pre-liposome formula (L-α-phosphatidylcholine and b-oleoyl-g-palmitoyl cholesterol; Sigma L3906) according to the recommended procedure.

Cell culture
Cos-7 cells were purchased from the ATCC (CRL-1651) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine and antibiotics. PC-12 cells, kindly provided by Dr Erik Schweitzer, were maintained in DMEM high-glucose medium supplemented with 5% calf serum (Hyclone), 5% horse serum, and antibiotics. Medium was changed every other day; cells were plated at 100 000/cm² density onto a six-well plate the day before treatment with aggregates. For the LDH and MTS experiments, cells were plated in phenol red-free medium for 24 h before the assay.

For incorporation of unpackaged aggregates, cells were washed in 1× PBS and incubated with 10 μM aggregates for 24 h (or longer, for the time-course cytotoxicity study). For liposome-packaged aggregates, washed cells were mixed with liposome-encapsulated aggregates (see above) for 4 h, after which the liposome mixture was removed from the plastic-attached cells by several washes with PBS. Fresh culture medium was then added, and the cells incubated for another 20 h.

Isolation of cell nuclei
Nuclei were isolated by a modification of the published protocol (49). Cells were lysed in NP-40 lysis buffer (0.6% NP-40, 0.1% BSA and 0.15 mM NaCl) for 10 min on ice. Nuclei were collected by centrifugation at 500 g for 5 mins and washed twice, then resuspended in PBS. Isolated nuclei were stained for contrast with 50 μg/ml propidium iodide (PI) for 5 min before being examined by confocal microscopy.

Confocal microscopy and flow-cytometric analysis
Cells were detached with trypsin then suspended in serum-containing culture medium, then collected by centrifugation at 1000 g for 5 min and washed twice with PBS with centrifugation and resuspended in 1 ml PBS. Aliquots were analyzed by flow cytometry or plated on a chamber slide (Nalgene Nunc International, Lab-Tek II Chamber slide system) and analyzed within 4–6 h by confocal microscopy. Confocal microscopy was conducted using a Leica SP2 laser scanning confocal microscope.

To assess cell viability, cells were stained with 50 μg/ml PI for 5 min and then injected into a FACSScan (Becton-Dickinson, Figure 7. In vitro stability of aggregates. Different preparations of aggregates were incubated in PBS at a concentration of 10 μM (monomer equivalents) at 37°C, and aliquots were processed as described in Materials and Methods to measure aggregate dissociation. The ordinate shows the percentage of total aggregate-associated peptide released into the supernatant at various incubation times. Open squares, F-NLS-Q42; open diamonds, F-Q42; open circles, F-NLS-Q20; open triangles, F-Q42; filled squares, F-NLS-CspB-1.
San Jose CA). Excitation at 488 nm allowed observation of both the fluorescein-tagged aggregates (FL-1, emission 530 nm) and the PI-stained dead cells (FL-3, emission 650 nm). At least 10,000 events were collected for each sample and analyzed using Cell Quest software (Becton-Dickinson).

**Cell viability assays**

The LDH-release assay (50) was used to assess cell death by measuring the activity of the cytosolic enzyme LDH released into the cell medium (Promega CytoTox 96 assay kit, G1780).

After 24 h incubation of the cells with poly(Q) aggregates, 200 μl of medium was removed and centrifuged for 5 min at 14,000g, and aliquots of the supernatant were tested for LDH activity. Relative cytotoxicity was calculated by dividing LDH release for a particular experiment by the total LDH content of cells released by Triton X-100 lysis.

Cell death or dysfunction as indicated by diminished activity of mitochondrial dehydrogenases was assessed using the XTT assay, a modification of the standard MTT assay (51), using the Promega assay kit (G3582). After 24 h incubation of cells with aggregates, cells were incubated with 200 μl of assay solution

Figure 8. Time course of the cytotoxicity of poly(Q) aggregates and monomers. PC-12 cells were incubated with poly(Q) aggregates (A; 10 μM monomer equivalents) or monomers (B; 10 μM) and cell death was measured by the LDH-release assay at various time points. Filled squares, F-NLS-Q42; open squares, F-Q42; filled diamonds, F-NLS-Q20; open diamonds, F-Q20; filled triangles, F-NLS-CspB-1. Two independent experiments were conducted, each in triplicate; data were normalized by subtracting the amount of cell death at each time point in a control incubation of cells in medium alone. The percentage of cell death is calculated compared with a control generated by lysing an equivalent amount of cells and determining the amount of LDH released.
to 1 ml of cell culture, and the mixture was incubated in a 24-well sterile microplate for 4 h at 37°C, 5% CO2. The mixture was centrifuged at 14 000g for 5 min, and the absorbance of the supernatant was recorded. Relative cell survival was determined by dividing the corrected A490 for a particular experiment by the corrected A490 for untreated cells.

Statistics

Three to eight independent studies (depending on the aggregate tested), including aggregate preparation and cell treatment, were conducted for flow-cytometric analysis of cell viability. Two to four independent experiments were conducted for LDH and MTS assays. Bar-graph data are expressed as mean ± SEM. Statistical significance was analyzed by one-way ANOVA using the software package SPSS 10.0 (SPSS, Chicago IL). A P-value ≤ 0.001 is considered significant.

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REFERENCES


