Soluble polyglutamine oligomers formed prior to inclusion body formation are cytotoxic

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Expanded polyglutamine (polyQ) repeats cause neurodegenerative disorders, but their cytotoxic structures remain to be elucidated. Although soluble polyQ oligomers have been proposed as a cytotoxic structure, the cytotoxicity of soluble polyQ oligomers, not inclusion bodies (IBs), has not been proven in living cells. To clarify the cytotoxicity of soluble polyQ oligomers, we carried out fluorescence resonance energy transfer (FRET) confocal microscopy and distinguished oligomers from monomers and IBs in a single living cell. FRET signals were detected when donor and acceptor fluorescent proteins were attached to the same side, not the opposite side, of polyQ repeats, which agrees with a parallel β-sheet or a head-to-tail cylindrical β-sheet model. These FRET signals disappeared in semi-intact cells, indicating that these polyQ oligomers are soluble. PolyQ monomers assembled into soluble oligomers in a length-dependent manner, which was followed by the formation of IBs. Notably, survival assay of neuronally differentiated cells revealed that cells with soluble oligomers died faster than those with IBs or monomers. These results indicate that a length-dependent formation of oligomers is an essential mechanism underlying neurodegeneration in polyQ-mediated disorders.

INTRODUCTION

Expanded polyglutamine (polyQ) proteins cause nine inherited neurodegenerative disorders, whose severity correlates with the length of polyQ repeats: polyQ diseases (1). Since a causative protein with expanded polyQ repeats is incorporated into microscopic inclusion bodies (IBs) in a length-dependent manner, IBs have been suspected as the toxic structure associated with polyQ diseases (2–6). However, the onset of a neurological phenotype or cell dysfunction mediated by expanded polyQ proteins has been found to be independent of the formation of IBs (7–11). Hsp70/Hsp40 suppresses the cytotoxicity of expanded polyQ proteins without affecting the formation of IBs (8). Furthermore, the formation of IBs reduces the cytotoxicity mediated by expanded polyQ proteins (12,13). These findings indicate that IBs are not the toxic structure associated with polyQ diseases and may protect cells from the cytotoxicity mediated by expanded polyQ proteins.

Instead, soluble polyQ oligomers have been proposed as the cytotoxic structure of expanded polyQ proteins: monomers with expanded polyQ repeats assemble into a soluble oligomer in vitro (14–25). Molecules and antibodies that inhibit the formation of soluble polyQ oligomers reduce the cytotoxicity mediated by expanded polyQ proteins; however, these molecules and antibodies might prevent a conformational change of expanded polyQ monomers and also inhibit the formation of IBs (14–25). Therefore, it is unclear whether the cytoprotective effect of these molecules and antibodies results from the decrease in the cytotoxicity of monomers, oligomers or IBs. In addition, soluble polyQ oligomers are indistinguishable from polyQ monomers in living cells.

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To clarify the toxicity of soluble polyQ oligomers, it is crucial to distinguish soluble expanded polyQ oligomers from monomers and/or IBs in living cells.

To address this issue, a spatial resolution that exceeds the optical limits of conventional epifluorescence microscopy is required. Fluorescence resonance energy transfer (FRET) methods are unique in that they can overcome these constraints and enable the sensitive evaluation of protein–protein interactions in living cells (26,27). A FRET method has enabled the detection of insoluble IBs containing expanded polyQ proteins in vitro in cultured cells, and recently in neurons of Caenorhabditis elegans (24,28–31). Furthermore, a FRET method has been used to detect the intramolecular collapse and intermolecular association of polyQ proteins in vitro (15,16). However, in living cells, soluble polyQ oligomers have never been detected by a FRET method. In this study, we applied a FRET method to detect soluble polyQ oligomers in living cells at a single-cell resolution, and clarified the cytotoxicity of soluble polyQ oligomers.

RESULTS

Detection of polyQ oligomers by FRET microscopy

Several structures of oligomers of expanded polyQ proteins have been proposed, including antiparallel β-sheets, parallel β-sheets and head-to-tail cylindrical β-sheets (14,32–35). To determine whether expanded polyglutamine repeat monomers assemble into an oligomer in an orderly manner in living cells, monomeric variants of the CFP (monomeric cyan fluorescent protein: mCFP) gene or the YFP (monomeric yellow fluorescent protein: mYFP) gene were fused to a truncated atrophin-1 gene containing either 36 (trD-Q56) or 12 polyQ repeats (trD-Q12) at its N or C terminus (Fig. 1A) (27). We cotransfected these constructs into COS7 cells in various combinations (i.e. trD-Qn-mCFP or mCFP-trD-Qn together with trD-Qn-mYFP or mYFP-trD-Qn) and observed those cells by confocal microscopy. The corrected FRET signal intensity (cFRET) was calculated by correcting the signal intensity in the FRET channel from either contaminating CFP bleed-through or direct activation of YFP (Supplementary Material, Fig. S1 and Materials and Methods) (36). Then, FRET signal intensity (FRET-to-donor fluorescence ratio) was calculated by dividing cFRET signal intensity by CFP fluorescence intensity (ICFP) (36,37). A cell with a FRET signal intensity above 0.20 without microscopic IBs was defined as a ‘FRET-positive cell’.

FRET-positive cells were only those that expressed donor and acceptor fluorescent proteins attached to the same side, not the opposite side, of the polyQ repeats (Fig. 1B). In a cell without microscopic IBs, FRET signals were observed throughout the cell. FRET between these constructs was confirmed by acceptor-bleaching analysis (Supplementary Material, Fig. S2). In contrast, the cells cotransfected with either trD-Q12-mCFP with mYFP-trD-Q12, or mCFP-trD-Q56 with trD-Q56-mYFP, in which the donor and acceptor fluorescent proteins were attached to the opposite side of the polyQ repeats, failed to demonstrate FRET signals except in microscopic IBs (Fig. 1B). In microscopic IBs, both combinations, namely, trD-Q56-mCFP and mCFP-trD-Q56, showed FRET signals, suggesting that the polyQ proteins aggregated in IBs and showed FRET signals regardless of the side of attachment to polyQ proteins (Fig. 1B; arrow). The results indicate that in living cells the expanded polyQ monomers assemble into an oligomer in an orderly manner.

To confirm that the FRET signals arose from the direct interaction among polyQ repeats, not the interaction of the fluorescent protein or amino acid residues with surrounding polyQ repeats, we investigated soluble polyQ oligomer formation in the presence of QBP1. QBP1 is a peptide that preferentially binds to polyQ monomers of pathogenic length and reduces the cytotoxicity of expanded polyQ proteins (20,38). The number of FRET-positive cells significantly decreased when cells were transfected with (QBP1)$_2$-HcRed1 (19.1 ± 2.2%, mean ± SEM), rather than with (SCR)$_2$-HcRed1 (30.1 ± 0.9%, mean ± SEM), which is a randomly sequenced peptide of QBP1 (Fig. 1C) (38). Next, we investigated whether PGL-135, which inhibits the aggregation of huntingtin, prevents the formation of polyQ oligomers (39). The number of FRET-positive cells decreased when cells were incubated with PGL-135, whereas it did not change significantly when cells were incubated with D-KLVFA, an inhibitor of β-amyloid peptide fibrinogenesis (PGL-135; 22.9 ± 0.6%, d-KLVFA; 27.0 ± 0.1%, DMSO; 30.7 ± 2.9%, mean ± SEM) (Fig. 1D) (40). Taken together, we concluded that FRET signals arose from the direct interaction among polyQ repeats.

Next, we performed immunohistochemical analysis of FRET-positive cells to determine the structural properties of the polyQ oligomer formed using the A11 and 1C2 antibodies. The A11 antibody recognizes the common structure of soluble amyloid oligomers including expanded polyQ oligomers (15,20,23). Only a small number of FRET-positive cells immunoreacted with the A11 antibody; however, most of the FRET-positive cells did not immunoreact with the A11 antibody, and none of the FRET-negative cells immunoreacted with the A11 antibody (Fig. 1E). In the case of using the 1C2 antibody, which recognizes expanded polyQ monomers and not IBs, FRET-positive cells were weakly stained; in contrast, IBs clearly did not immunoreact with the 1C2 antibody, indicating that the structural properties of the polyQ oligomer formed were distinct from those of monomers and IBs (Fig. 1E).

PolyQ oligomer is soluble

The signals of FRET-positive cells were distributed throughout the nucleus and cytoplasm, suggesting that the polyQ oligomer is soluble (Fig. 1B and Supplementary Material, Fig. 2). To clarify the solubility and dynamics of a polyQ oligomer in living cells, we performed time-lapse FRET microscopy in a semi-intact cell system, in which cells are treated with a low concentration of digitonin and the plasma membrane is selectively permeabilized while the nuclear membrane is kept intact, thus releasing cytosolic soluble proteins from the cytoplasm while retaining insoluble proteins, organelles, cytoskeletons and large nuclear proteins in a cell (41). If the polyQ oligomer formed is soluble and/or dissociates to monomers, FRET signals will disappear under this condition. After the
cells were treated with a low concentration of digitonin, FRET signals in the cytoplasm rapidly disappeared, whereas the FRET signals in microscopic IBs remained intact (Fig. 2A and B). The disappearance of FRET signals was accompanied by a decrease in cytoplasmic polyQ-CFP fluorescence intensity, indicating that the polyQ proteins were released from the cytoplasm through the permeabilized cell membrane. In addition, the FRET signals in the nucleus also disappeared. The size of a polyQ oligomer is larger than the free-diffusion limit of a nuclear pore, which allows only molecules less than 60 kDa to pass, suggesting that the polyQ oligomers dissociated into monomers and translocated into the cytoplasm through the nuclear pore (Fig. 2A). Indeed, we detected a band corresponding to a high-molecular-weight protein from the supernatant of cells transfected with trD-Q56-mYFP or trD-Q80-mYFP after ultrasonication without detergent in native polyacrylamide gel electrophoresis analysis. The high-molecular-weight protein was detected in several detergents including digitonin at a low concentration (Fig. 2C). We detected no apparent dimers or oligomers in SDS gel electrophoresis analysis under reducing condition (Supplementary Material, Fig. S3). The observations support the hypothesis that the polyQ oligomers, which are detected as FRET signals, are soluble.

PolyQ monomers assemble into soluble oligomers in a length-dependent manner

The polyQ length dependence of cytotoxicity is a pivotal feature in polyQ diseases. If the orderly oligomerization of polyQ proteins is crucial for the pathogenesis of polyQ diseases, the oligomerization of polyQ proteins will occur in a length-dependent manner. To test this hypothesis, we compared the number of cells with soluble polyQ oligomers transfected with various lengths of polyQ repeats with that of cells with truncated atrophin-1 or huntingtin exon 1 (Fig. 1A). Cells with the truncated atrophin-1 or huntingtin exon 1 fusion proteins showed FRET signals throughout the cytoplasm and nucleus (Fig. 3A and Supplementary Material, Fig. S4). The number of FRET-positive cells increased in a length-dependent manner for polyQ repeats.

Figure 1. FRET microscopy shows polyQ oligomers in living cells. (A) Schematic representation of constructs of polyQ repeat proteins tagged with fluorescent proteins. A truncated atrophin-1 (trD) gene insert was fused to either mCFP or mYFP at the C terminus (top) or N terminus (middle). ‘Qn’ denotes glutamine repeats. The huntingtin exon 1 gene fragment (Htt<sup>ex1</sup>) was fused to either mCFP or mYFP at its C terminus (bottom). (B) PolyQ repeats show diffuse FRET signals. COS7 cells were cotransfected with trD-Q12-mCFP/mYFP, trD-Q56-mCFP/mYFP, trD-Q12-mCFP/trD-Q12 and trD-Q56-mCFP/trD-Q56. Corrected FRET signal intensity (cFRET) and the FRET-to-donor fluorescence ratio (cFRET/ICFP, FRET signal intensity) were calculated as described in Materials and Methods. The pseudocolor scale corresponds to the cFRET/ICFP values, i.e. FRET signal intensity, from 0.20 (blue) to 0.50 (red). Arrows indicate IBs. Scale bar, 10 μm. (C) QBP1 inhibits soluble polyQ oligomer formation. PolyQ binding peptide-1 (QBP1) and a random sequence (SCR) were oriented in tandem and tagged with HcRed1 at the C terminus and are denoted as (QBP1<sub>2</sub>–HcRed and (SCR)<sub>2</sub>–HcRed, respectively (38). COS7 cells were then cotransfected with trD-Q80-mCFP/mYFP and either (QBP1<sub>2</sub>–HcRed or (SCR)<sub>2</sub>–HcRed. The bar graph shows the average frequency of FRET-positive cells (n>100, three independent experiments). Error bars indicate SEM. Asterisks indicate P<0.01. (D) PG-135 inhibits soluble polyQ oligomer formation. COS7 cells expressing both trD-Q80-mCFP and trD-Q80-mYFP were incubated with PGL-135 (60 μM) or d-KLVFVA (20 μM). DMSO was used as the control (n=180, two to three experiments). Asterisk indicates P<0.05; n.s., not significant. Error bars indicate SEM. (E) Cells with or without FRET signals were stained with the antibody against oligomers (A11) or with that against expanded polyQ repeats (1C2) followed by the secondary Cy5-conjugated antibody (Cy5). Arrows indicate IBs. Scale bar, 10 μm. The pseudocolor scale corresponds to the cFRET/ICFP values, i.e. FRET signal intensities, from 0.20 (blue) to 0.50 (red).
COS7 cells (trD-Q12; 9.5 ± 0.9%, trD-Q36; 23.4 ± 3.3%, trD-Q56; 27.5 ± 2.2%, trD-Q80; 36.9 ± 4.9%, Htt-Q17; 10.5 ± 1.2%, Htt-Q36; 19.1 ± 4.3%, Htt-Q58; 29.6 ± 3.3%, mean ± SEM: Fig. 3B and C) and in SH-SY5Y human neuroblastoma cells (trD-Q12; 4.7 ± 0.9%, trD-Q80; 22.4 ± 6.8%, mean ± SEM: Fig. 3D). The observation that polyQ proteins form into oligomers in a length-dependent manner further confirms the importance of the formation of soluble polyQ oligomers in the pathogenesis of polyQ diseases.

FRET signal intensity represents the amount of polyQ–polyQ interaction (15,16,24,28–30); thus, we analyzed FRET signal intensity in each cell to determine whether the length of polyQ repeats affects the amount of polyQ–polyQ interaction. The FRET signal intensities of IBs were higher...
Figure 3. PolyQ monomers assemble into soluble oligomers in a length-dependent manner. (A) FRET signals were observed in cells transfected with either truncated atrophin-1 or huntingtin exon 1 harboring polyQ repeats of different lengths. COS7 cells were cotransfected with trD-Q36-mCFP/mYFP, trD-Q80-mCFP/mYFP, Htt\textsuperscript{ex1}-Q17-mCFP/mYFP, Htt\textsuperscript{ex1}-Q36-mCFP/mYFP or Htt\textsuperscript{ex1}-Q58-mCFP/mYFP. Arrows indicate FRET-positive cells and arrowheads indicate FRET-negative cells. Scale bar, 10 µm. (B and C) Number of FRET-positive cells transfected with polyQ repeats of different lengths: trD-Q12, trD-Q36, trD-Q56 and trD-Q80-mCFP/mYFP (B), and Htt\textsuperscript{ex1}-Q17, Htt\textsuperscript{ex1}-Q36 and Htt\textsuperscript{ex1}-Q58-mCFP/mYFP (C). Cells with a cFRET/ICFP value above the cutoff value of 0.20 are denoted as FRET-positive cells. Each bar indicates the mean frequency (%) of FRET-positive cells (n = 450 in three independent experiments). Error bars indicate the SEM. (D) FRET-positive cells among SH-SY5Y human neuroblastoma cells transfected either with trD-Q12 or trD-Q80-mCFP/mYFP. (n = 180 cells in three independent experiments.) Error bars indicate SEM. Single asterisks indicate P < 0.05. (E–I) The cFRET/ICFP values increase in a length-dependent manner. (E) IBs were punctate and showed markedly high cFRET/ICFP values. (F and G) Mean cFRET/ICFP values of FRET-positive cells transfected with different lengths of polyQ repeats: (F) trD-Q12, trD-Q36, trD-Q56 and trD-Q80-mCFP/mYFP, and (G) Htt\textsuperscript{ex1}-Q17, Htt\textsuperscript{ex1}-Q36 and Htt\textsuperscript{ex1}-Q58-mCFP/mYFP. Each bar indicates the mean cFRET/ICFP value from 20 cells per group. Error bars indicate SEM. Asterisks indicate P < 0.05. n.s., not significant. (H and I) Scatter diagram of cFRET/ICFP values from FRET-positive (red circles) and FRET-negative (black circles) cells: (H) trD-Q12, trD-Q36, trD-Q56 and trD-Q80-mCFP/mYFP, and (I) Htt\textsuperscript{ex1}-Q17, Htt\textsuperscript{ex1}-Q36 and Htt\textsuperscript{ex1}-Q58-mCFP/mYFP. We randomly selected 10 cells each from the FRET-positive and FRET-negative cells in each transfection.
Soluble polyQ oligomers are incorporated into IBs

It has been suggested that polyQ oligomers might be incorporated into IBs; however, the incorporation of soluble polyQ oligomers into IBs has never been observed in a living cell. To address this issue, we performed time-lapse FRET microscopy using COS7 cells cotransfected with Httex1-Q58-mCFP/mYFP. Initially, weak FRET signals, which indicate a weak polyQ–polyQ interaction, were homogeneously distributed throughout a cell (Fig. 4A). Subsequently, FRET signal intensity increased gradually from 50 to 75 min (Fig. 4B and C). At 80 min, a high-FRET-signal-intensity spot appeared (Fig. 4B), whereas no microscopic IBs were observed in the CFP channel or the phase contrast image (Fig. 4C and D; red circle). At 85 min, a microscopic IB was observed in the CFP channel, and the phase contrast image showed a spot with a high FRET signal intensity at 80 min (Fig. 4D). The spot with a high FRET signal intensity enlarged until 110 min accompanied by decreases in FRET and CFP signal intensities outside the IB, indicating that the decrease in FRET signal intensity was not simply due to the disruption of polyQ oligomers, but the incorporation of the oligomers into the IBs (Figs. 4B–D and Supplementary Material, Fig. 5). Thus, soluble polyQ oligomers are rapidly incorporated into IBs in a living cell.

Soluble polyQ oligomers induce cytotoxicity in neuronally differentiated SH-SY5Y cells

To determine whether soluble polyQ oligomers are more toxic to neuronal cells than monomers or IBs, we studied the survival of neuronally differentiated SH-SY5Y cells with or without soluble polyQ oligomers. After the transduction of lentiviral vectors containing trD-Q75-mCFP and trD-Q75-mYFP to SH-SY5Y, we established stable cell lines that expressed polyQ monomers or IBs. After 24 h, these neuronally differentiated SH-SY5Y cells were plated on a gridded glass-bottom dish, induced to differentiate into neuron-like cells with retinoic acid (RA) for 5 days, and then treated with brain-derived neurotrophic factor (BDNF). After 24 h, these neuronally differentiated SH-SY5Y cells were observed by FRET microscopy and classified into three groups according to the presence or absence of FRET signals and IBs: those with monomers (FRET-negative), oligomers (FRET-positive, IB-negative) and IBs (IB-positive) (Fig. 5A). These cells were observed by inverted microscopy every 24 h. Cells that lost their fluorescence or disappeared were defined as dead cells (Fig. 5B) (12). The cumulative risk of death of cells with the formed soluble polyQ oligomers was significantly higher than that of cells with polyQ monomers or IBs (Fig. 5C). In contrast, neurally differentiated SH-SY5Y cells with IBs survived significantly longer than the others (Fig. 5C). To investigate the nature of cell death, we performed immunohistochemical studies using an apoptotic marker, cleaved caspase-3. Neuronally differentiated SH-SY5Y cells with the formed soluble polyQ oligomers demonstrated a higher caspase-3 staining intensity than those with polyQ monomers or IBs (Fig. 5E and F). Similar results were obtained for neuronally differentiated cells transfected with huntingtin exon 1, Httex1-Q58-mCFP/mYFP; however, there was no apparent difference in the cumulative risk of death between cells with monomers and those with soluble polyQ oligomers (Fig. 5D).

DISCUSSION

We have shown that soluble polyQ oligomers induced greater cytotoxicity toward neuronally differentiated SH-SY5Y cells than polyQ monomers or IBs. This observation is consistent with previous observations that the oligomer-specific A11 antibody, which recognizes the common structure of amyloidogenic oligomers, reduces the cytotoxicity mediated by the soluble polyQ oligomers in culture medium (23), and that the presence of ~200 kDa soluble huntingtin exon 1 oligomers, not inclusions, closely correlates with cytotoxicity in yeast (15). Furthermore, Li et al. (42) observed soluble polyQ oligomers in vivo and showed their correlation with symptom onset in a mouse model. These findings support the hypothesis that polyQ oligomers are the cytotoxic structure associated with polyQ diseases (toxic oligomer hypothesis) (15,22,23,25).

Consistent with previous reports (12,13), we observed that the formation of IBs was protective against cytotoxicity mediated by expanded polyQ oligomers. The neurally differentiated SH-SY-5Y cells with IBs survived longer than those with monomers or oligomers. Furthermore, time-lapse FRET microscopy revealed that soluble polyQ oligomers were rapidly incorporated into IBs. These findings are similar to the observation that the formation of IBs is protective against cytotoxicity mediated by expanded polyQ proteins, which is accompanied by a reduction in the amount of intracellular soluble polyQ proteins (12). Therefore, we conclude that the formation of IBs leads to sequestration of cytotoxic polyQ oligomers into IBs and protects cells from cytotoxicity mediated by expanded polyQ proteins.

Several structures of oligomers of expanded polyQ repeats in vitro have been proposed, including antiparallel β-sheets, parallel β-sheets and head-to-tail cylindrical β-sheets (14,32–35). On the basis of our observation that polyQ monomers assembled into an oligomer when the fluorescent protein was attached to the same side of polyQ repeats, polyQ monomers might assemble into an oligomer with a head-to-head parallel or a head-to-tail cylindrical structure in a living cell.
We cannot exclude the possibility that we failed to detect FRET signals under head-to-tail antiparallel conditions owing to the distance of fluorescent proteins under these conditions even though polyQ monomers assemble into dimmers with a head-to-tail antiparallel structure. In addition, because we utilized fusions of truncated polyQ proteins and large fluorescent proteins, these fluorescent proteins may affect the oligomerization of expanded polyQ proteins.

The huntingtin exon 1 proteins, however, also produce FRET signals intensity in vitro when the donor and acceptor fluorophores, which are smaller than fluorescent proteins, are attached to the same side, not the opposite side, of polyQ repeats (16). The observation that the oligomer-specific antibody A11 detected a small number of FRET-positive cells suggests that the A11 antibody recognizes a specific species of polyQ oligomers (20,23). Taken together, these findings demonstrate that polyQ monomers assemble into an oligomer in an orderly manner (Fig. 5G) (16,32,33,35).

Although several previous studies showed FRET signals in insoluble IBs, this is the first study showing FRET signals corresponding to soluble polyQ oligomers in living cells (15,16,24,28–30). Our ability to detect FRET signals...
corresponding to soluble polyQ oligomers might be explained by the fact that we used the monomeric fluorescent proteins for analysis (27). In addition, we obtained CFP and YFP fluorescence images simultaneously utilizing an emission splitter and single-laser excitation at 405 nm to avoid the direct excitation of YFP.

The polyQ monomers assembled into an oligomer in a length-dependent manner. In addition, the disappearance of FRET signals in the nucleus of a semi-intact cell suggests that the polyQ oligomers dissociate into monomers in living cells. Interestingly, monomers with short polyQ repeats also assembled into an oligomer with a low FRET signal intensity.

Figure 5. Cytotoxicity of soluble polyQ oligomers in neuronally differentiated cells and model for soluble polyQ oligomer formation and FRET signal intensity. (A) Neuronally differentiated SH-SY5Y cells with soluble polyQ oligomers. The pseudocolor scale corresponds to the cFRET/ICFP values, i.e. FRET signal intensities, from 0.20 (blue) to 0.50 (red). (B) Longitudinal tracking of neuronally differentiated cells. Two neuronally differentiated cells with IB (arrow) and without IB. Neuronally differentiated cell without IBs died by day 7. (C and D) Hazard analysis demonstrates that soluble polyQ oligomers are significantly associated with an increased risk of death among neuronally differentiated cells transfected with trD-Q75 (C; n=172 neuronally differentiated cells, three independent experiments) and Htt-ex1-Q58 (D; n=117 neuronally differentiated cells, three independent experiments). Open triangles, FRET-positive cells without IBs; open circles, cells without FRET signals; closed circles, cells with IBs. Asterisk indicates P<0.002. Double asterisk indicates P<0.00001. (E) Neuronally differentiated cells with soluble polyQ oligomers are stained with a cleaved (activated) caspase-3 antibody. The pseudocolor scale corresponds to the cFRET/ICFP values, i.e. FRET signal intensities, from 0.20 (blue) to 0.50 (red). (F) Neuronally differentiated cells with soluble polyQ oligomers are significantly positive for cleaved (activated) caspase-3. Asterisk indicates P<0.001. Error bars indicate SEM. (G) Model for soluble polyQ oligomer formation and FRET signals. Non-toxic polyQ monomers transform into toxic β-sheet polyQ monomers and/or toxic cylindrical β-sheet monomers. The toxic β-sheet polyQ monomers assemble into oligomers in a parallel orientation. The cylindrical β-sheet monomers assemble into oligomers in a head-to-tail orientation. PolyQ proteins may shift between these two structures. When the fluorescent proteins are attached to the opposite side of the polyQ repeats, these large fluorescent proteins are too far to produce FRET signals. Soluble polyQ oligomers are incorporated into IBs. IBs sequester toxic polyQ monomers and oligomers. The dashed box indicates the cytotoxic structure. Blue balls, mCFP; yellow balls, mYFP; gray arrows, polyQ.
A transient interaction between short polyQ proteins has also been suggested by in vitro assay (16). In contrast, the long polyQ proteins assembled into an oligomer with a higher FRET intensity than an oligomer formed from the short polyQ proteins. The results raise the following possibilities: (i) long polyQ monomers assemble into oligomer more efficiently than the short polyQ monomers; (ii) short polyQ oligomers dissociate into monomer easier than long polyQ oligomers; and (iii) long polyQ oligomers are configured more tightly than short polyQ oligomers. Further experiments are necessary to clarify the dynamics and kinetics of oligomerization of polyQ proteins in living cells.

Notably, the analysis of average FRET signal intensities of cells without IBs revealed that the transfected cells were clearly separated into two different groups according to the average FRET signal intensity, namely, with and without soluble polyQ oligomers. If the transition from polyQ monomers to an oligomer slowly spreads throughout a cell and oligomers gradually accumulate in the cell, the average FRET signal intensities will be presented as continuous data, not as two distinct groups observed in this study. Thus, it seems likely that the transition from monomers to an oligomer, once it has occurred, rapidly extends throughout the cell. This hypothesis is consistent with an observation from in vitro assay of huntingtin with expanded polyQ repeats fused to GST, that is, oligomerization has reached a plateau within 1.5–2 h (16).

On the basis of these results, we propose a model for polyQ dynamics in a living cell, as depicted in Figure 5G: (i) polyQ monomers transform into β-strand polyQ monomers, which have the property for oligomerization; (ii) subsequently, β-strand polyQ monomers assemble into oligomers, and simultaneously, the formed polyQ oligomers dissociate into monomers; the transition between monomers and oligomers is bidirectional and dynamic; (iii) then polyQ oligomers are incorporated into IBs (Fig. 5G). The equilibrium constant of the direction of the transition between monomers and oligomers may depend on the length of polyQ repeats and amino acid sequences surrounding polyQ repeats. Thus, FRET-positive cells appear to contain both β-strand polyQ monomers and oligomers. In this study, we show clearly that expanded polyQ proteins induce the greatest cytotoxicity in this state, that is, FRET signal positivity without IBs.

We do not exclude the possibility that polyQ monomers with the β-sheet structure show cytotoxicity (20). Indeed, in Htr1-ex1-Q58-expressing neuronally differentiated cells, the cytotoxicities of polyQ monomers and soluble polyQ oligomers were similar (Fig. 5D and E). This observation may be partially explained by the cytotoxicity of β-sheet polyQ monomers. In addition, we speculate that β-sheet polyQ monomers and soluble oligomers may coexist in FRET-positive cells (Fig. 5G). Therefore, the cytotoxicity might not be due exclusively to any one species of toxic monomers and oligomers (25).

Our findings provide a new understanding of the dynamics of soluble polyQ oligomers and the cytotoxicity of expanded polyQ proteins. In many neurodegenerative disorders, including Alzheimer’s disease and Parkinson’s disease, soluble oligomers of causative proteins have been proposed to be the cytotoxic structure (23,43). This FRET microscopy assay has the potential to enable the identification of soluble cytotoxic oligomers in a living cell and substances that inhibit the formation of cytotoxic soluble oligomers.

**MATERIALS AND METHODS**

**Plasmids**

To generate the trD-Qn-mCFP/mYFP and exon1 HttQn-mCFP/mYFP constructs, the cDNA fragments of atrophin-1 and huntingtin exon 1 were generated by PCR using genomic DNA samples from affected patients as previously described (44). The truncated atrophin-1 gene insert contained eight amino acid residues at the N terminus and six amino acid residues at the C terminus of polyQ repeats. The PCR products were digested with EcoRI and XhoI, and subcloned into pEGFP-N1 (Clontech, Palo Alto, CA, USA). The EGFP insert was then substituted by either monomeric CFP (mCFP) or monomeric YFP (mYFP), which were generated by PCR using either pcDNA3.1/mCFP or mYFP as the template [provided by Tsien and coworkers (27)] and with the primers 5’-AAAGATCCAGTGACAGGCGAGGAGCT and 5’-AAACGCGCCGCTTACCTATTGTTACAGCTCGTCCA-T. The amplified products were then subcloned into the NotI and BamHI restriction sites of pEGFP-N1. To construct mCFP/mYFP-trD-Qn, the cDNA fragment of atrophin-1 was amplified from the genomic DNA of a patient using the following primers harboring the KpnI and BamHI sites: 5’-TGATGTTAGCGGTTCACACATACCAGTCA and 5’-TAATGGATCC-TTAAAGGAGGGGCCCAGATT. The resulting PCR products were subcloned into the corresponding restriction sites of either pcDNA3.1/mCFP or mYFP. PolyQ binding peptide-1 (QBP1) and a random sequence (SCR) were oriented in tandem and tagged with HcRed1 at the C terminus and are denoted as (QBP1)2–HcRed and (SCR)2–HcRed, respectively (38). The (QBP1)2–HcRed1 and (SCR)2–HcRed1 constructs were generated by subcloning the HcRed insert from pHcRed-N1 (Clontech) into the BamHI/NotI site of p(QBP1)2-CFP and p(SCR)2-CFP, respectively (38).

**Cell culture and transfection and biochemical analysis**

COS7 or SH-SY5Y neuroblastoma cells were cultured in a 35 mm 1-lysine-coated glass-bottom dish (Mattek, Ashland, MA, USA) as previously described (45). Transient transfections were performed using lipofectamine 2000 (Invitrogen). PGL-135 and D-KLVFFA were purchased from Sigma.

**Immunofluorescence microscopy**

Cells grown on Lab-Tek chamber slides (Nunc Inc., Naperville, IL, USA) precoated with cell-tak (Becton Dickinson, Bedford, MA, USA) were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature and washed three times in PBS. After treatment with 0.2% Triton X-100 in PBS for 15 min, cells were incubated with blocking buffer (1% BSA, 0.1% cold water fish skin gelatin, and 0.1% Tween 20 in PBS) for 120 min. Cells were incubated with a rabbit anti-cleaved caspase-3 antibody (1:200, Asp175, Cell Signaling Technology, Beverly, MA, USA), an anti-polyQ mouse monoclonal antibody (1C2) (1:4000, Chemicon, Temecula, CA, USA), and a rabbit
anti-oligomer polyclonal antibody (A11) (1:200, Chemicon) and then incubated for 120 min at room temperature with a Cy5-conjugated goat anti-rabbit (1:400, Chemicon) or an antimouse secondary antibody (1:500, Chemicon). Images were obtained using an inverted microscope (TE-300NT; Nikon, Tokyo, Japan) and a confocal microscope (CSU-10; Yokogawa Electric Corp., Tokyo, Japan) equipped with a 40× objective (NA 0.80; Olympus, Tokyo, Japan).

Cell permeabilization
COS7 cells were grown on a 35 mm 1-lysine-coated glass-bottom dish (Matsunami Glass Industries) and treated with transport buffer [TB; 25 mM HEPES (pH7.4), 115 mM potassium acetate, 5 mM sodium acetate, 5 mM MgCl2, 2 mM DTT, 2 mM EGTA] containing 40 μg/mL digitonin (Sigma Aldrich, Milwaukee, WI, USA) for 5 min (41). The permeabilized cells were washed with ice-cold TB and then incubated in TB at 37°C for 60 min.

FRET microscopy
All images were acquired using an inverted microscope (TE-300NT; Nikon) and a confocal microscope (CSU-10; Yokogawa Electric Corp.) equipped with a 40× objective (NA 0.80; Olympus). Donor and acceptor fluorescence images were captured using the following filter sets: donor (mCFP) filter set, 405 nm excitation (iFLEX-2000; Point Source, Hamble, UK) and 450–510 nm emission filters; acceptor (mYFP) filter set, 405 nm excitation and 510–570 nm emission filters. Each emission spectrum was separated through the W-view system (Hamamatsu Photonics), acquired using a digital camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan), and analyzed using AQUACOSMOS software (Hamamatsu Photonics). Forty-eight hours after transfection, the culture medium in each sample was replaced with serum-free DMEM without phenol red (GIBCO) to reduce background fluorescence intensity. Cells were then subjected to FRET analysis. The atmosphere in the chamber was maintained at 37°C and 5% CO2 in air (Sankei, Tokyo, Japan). Time-lapse video images were then recorded every 5 min for 2 h.

FRET signal intensity was calculated by correcting the signal intensity in the FRET channel for any contaminating CFP bleed-through or YFP direct activation. Relative FRET signal intensity was calculated using the formula $cFRET = IFRET − a \times ICFP − b \times IYFP$, essentially as described previously (36), where cFRET is the corrected FRET value, and IFRET, ICFP and IYFP are the signal intensities measured using FRET, CFP and YFP filters, respectively. The norms a and b were determined using cells expressing only mCFP and those expressing only mYFP, respectively. By quantifying the IFRET/ICFP or IFRET/IYFP ratios, we obtained an average a value of 0.46, indicating that 46% of CFP signals had contaminated FRET signals (Supplementary Material, Fig.1). Under our experimental conditions, we did not detect any direct excitation of mYFP in the FRET channel at 405 nm excitation. We calculated the FRET-to-donor fluorescence ratio by dividing cFRET by the measured ICFP (cFRET/ICFP: FRET signal intensity), which yielded a value representing the efficiency of energy transfer from CFP to YFP (36,37).

To determine the number of FRET-positive cells, we measured the FRET signal intensity in cells transfected with trD-Q56-mCFP, a fusion of mCFP to the C terminus of a truncated atrophin-1 insert with 56 polyQ repeats (Fig. 1A). The average FRET signal intensity was determined to be 0.026 ± 0.023 (Fig. 1B). A numeric cutoff was then calculated on the basis of the mean and 95% confidence interval for the FRET signal intensities in COS7 cells, in which mCFP and mYFP were coexpressed. Only cells showing FRET signal intensities above the cutoff value of 0.20 were considered to be FRET-positive cells. We classify FRET signal intensities by color-scale imaging in which red denotes a strong signal and blue indicates a weak signal. We analyzed the mean FRET signal intensity of 150 cells per polyQ length. One-way analysis of variance (ANOVA) and the Tukey post hoc test were used for the statistical analysis of the results.

FRET analysis using an acceptor bleaching method was performed by bleaching at 505–545 nm for 2 min. We tested COS7 cells expressing trD-Q56-mCFP and trD-Q56-mYFP (prebleach) and acquired a prebleach donor (mCFP) image first. After bleaching the acceptor (mYFP), we acquired a postbleach donor (mCFP) fluorescence image. We then analyzed whether the pre-to postbleach donor intensity ratio increased following acceptor bleaching.

Native polyacrylamide gel analysis
Cell pellets transfected with trD-Q12-mYFP, trD-Q56-mYFP and trD-Q80-mYFP were treated by ultrasonication in a lysis buffer [25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA] and centrifuged at 13 000 g for 10 min at 4°C. The supernatants were incubated with or without a detergent [2.5% digitonin, 5% dodecylmaltoside (DDM), 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1% Triton X-100 or 1% sodium dodecyl sulfate (SDS)] at room temperature for 15 min then diluted with 5× sample buffer [310 mM Tris–HCl (pH 7.5), 0.05% bromophenol blue, 50% glycerol] and subjected to electrophoresis through 10% polyacrylamide gel. Separated products were visualized using Typhoon 9400 scanner (GE Healthcare).

Lentivirus particle preparation and transduction for stable SH-SY5Y cell lines
The trD-Q75-mCFP/mYFP and Httex1-Q58-mCFP/mYFP constructs were TOPo-cloned into pLenti6-V5, and the lentivirus particles were prepared using the Virapower Expression system (Invitrogen, Carlsbad, NY, USA). For in vitro transduction, SH-SY5Y cells were plated on 6-well plates to 30–50% confluence. After 12 h, virus stocks were added to a medium containing 6 μg/mL polybrene (Sigma Aldrich). After 24 h, the culture medium was washed twice with PBS and replaced with a fresh medium. On day 4, the culture medium was replaced with a fresh medium with 10 mg/ml blasticidin. The medium was replaced every 3–4 days. After 20 days, the cells were seeded on a 4×4 mm grided glass-bottom dish (Matsunami Glass Industries, Tokyo, Japan) to a confluency of 5000–10 000 cells/cm². All-trans RA at...
10 μM (Sigma) was added 1 day after plating. After incubation for 5 days in the presence of all-trans RA, cells were washed three times and incubated with 50 ng/ml human BDNF (Sigma) in a serum-free medium. Survival was analyzed using Kaplan–Meier plots and a log-rank test between subgroups.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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