Detection of Polyglutamine Protein Oligomers in Cells by Fluorescence Correlation Spectroscopy

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Abnormal aggregation and deposition of misfolded proteins in the brain have been implicated as a common molecular pathogenesis of neurodegenerative diseases including Alzheimer, Parkinson, and the polyglutamine (poly(Q)) diseases, which are collectively called the conformational diseases. The poly(Q) diseases, including Huntington disease and various types of spinocerebellar ataxia, are caused by abnormal expansions of the poly(Q) stretch within disease-causing proteins, which triggers the disease-causing proteins to aggregate into insoluble β-sheet-rich amyloid fibrils. Although oligomeric structures formed in vitro are believed to be more toxic than mature amyloid fibrils in these diseases, the existence of oligomers in vivo has remained controversial. To explore oligomer formation in cells, we employed fluorescence correlation spectroscopy (FCS), which is a highly sensitive technique for investigating the dynamics of fluorescent molecules in solution. Here we demonstrate direct evidence for oligomer formation of poly(Q)-green fluorescent protein (GFP) fusion proteins expressed in cultured cells, by showing a time-dependent increase in their diffusion time and particle size by FCS. We show that the poly(Q)-binding peptide QBP1 inhibits poly(Q)-GFP oligomer formation, whereas Congo red only inhibits the growth of oligomers, but not the initial formation of the poly(Q)-GFP oligomers, suggesting that FCS is capable of identifying poly(Q) oligomer inhibitors. We therefore conclude that FCS is a useful technique to monitor the oligomerization of disease-causing proteins in cells as well as its inhibition in the conformational diseases.

Abnormal aggregation and deposition of misfolded proteins in the brain have been implicated as a common molecular pathogenesis of neurodegenerative diseases including Alzheimer disease, Parkinson disease, and the polyglutamine (poly(Q))² diseases, which are collectively called the conformational diseases (1–3). The poly(Q) diseases are a group of at least nine inherited neurodegenerative diseases including Huntington disease and various types of spinocerebellar ataxia, which are caused by abnormal expansions of the poly(Q) stretch to above 40 glutamines within each unrelated disease-causing protein (4, 5). In the pathogenesis of the poly(Q) diseases, expansions of the poly(Q) stretch in disease-causing proteins are believed to cause alterations in the protein conformation, resulting in assembly of the proteins into insoluble β-sheet-rich amyloid-like fibrillar aggregates, and eventually their deposition as inclusion bodies inside affected neurons. However, Finkbeiner and colleagues (6) demonstrated that neuronal cells with poly(Q) protein inclusions have a decreased risk of death, suggesting that the diffuse poly(Q) protein rather than inclusion bodies causes cytotoxicity. Inclusion bodies, which are large intracellular deposits of aggregated proteins, are believed to be formed as a cytoprotective response against the overproduction of misfolded/aggregated proteins (7), whereas oligomers/aggregates are formed by the intrinsic property of misfolded proteins to misassemble. In addition, recent accumulating evidence strongly implies that soluble oligomers are more toxic than mature amyloid fibrils in the conformational diseases in general (8–11). However, importantly, there has been little evidence on oligomer formation of the amyloidogenic proteins in vivo so far, although their oligomer formation in vitro has been well characterized.

In this study, we employed fluorescence correlation spectroscopy (FCS) to explore oligomerization of the poly(Q) proteins in cells. FCS is known to be a powerful tool for investigating the dynamics of fluorescent probes and molecules in homogeneous solution at single molecule sensitivity, which cannot be analyzed by conventional microscopy (12, 13). Sev-

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Polyglutamine Oligomers Detected by FCS

**EXPERIMENTAL PROCEDURES**

**Cell Culture, DNA Transfection, and Microscopy**—COS-7 cells were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a 5% CO₂ humidified atmosphere at 37 °C. Cells were transfected with expression vectors for various lengths of poly(Q) stretches or poly(Q) stretches with proline insertions fused with enhanced green/yellow/cyan fluorescent protein (poly(Q)GFP/YFP/CFP; n = 19, 45, or 81) or GFP alone as a control, using FuGENE6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. To evaluate the inhibitory effects of the poly(Q)-binding peptide QBP1 (SNWKWWPGIFD) (17) on poly(Q) protein oligomerization/ aggregation, expression vectors for a tandem repeat of QBP1 fused with CFP (QBP1) or a scrambled sequence of QBP1 fused with CFP (SCR), used as a control, were co-transfected with poly(Q)-GFP or GFP. To evaluate the effects of Congo red, a poly(Q) aggregate inhibitor, on poly(Q) protein oligomerization/ aggregation, COS-7 cells transfected with poly(Q)-GFP or GFP expression vectors were treated with 25 μM Congo red. Cells were examined at 12, 36, 48, 60, and 72 h after transfection under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) or a laser scanning microscope (FV1000, Olympus, Tokyo, Japan). The percentage of cells with inclusion bodies was calculated by dividing the number of cells with inclusion bodies by the total number of fluorescent cells. At least 200 transfected cells were counted in each experiment, and experiments were repeated at least three times. Data are expressed as the mean ± S.E.

**Fluorescence Resonance Energy Transfer (FRET) Analysis**—At 48 h after transfection, COS-7 cells co-expressing poly(Q)-YFP and poly(Q)-CFP were resuspended in phosphate-buffered saline containing protease inhibitors, and lysed by gentle sonication. The poly(Q)-YFP/CFP inclusion bodies were then removed by centrifugation at 14,000 × g for 15 min. The resulting supernatants, which were confirmed to contain no visible inclusion bodies, were then subjected to FRET analyses. The soluble supernatants of the cell lysates (200 μl) each containing the same amount of poly(Q)-YFP or poly(Q)-CFP proteins were placed into a 96-well plate, and then excitation and emission scans were performed using a fluorescence plate reader (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA). Buffer baselines were subtracted and data were plotted against wavelength.

**FCS Measurement and Data Analysis**—COS-7 cells expressing poly(Q)-GFP or GFP with or without QBP1 or SCR, were lysed at 12, 36, 60, and 72 h after transfection with lysis buffer (10 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were gently sonicated and centrifuged at 10,000 × g for 30 min to remove insoluble poly(Q)-GFP inclusion bodies. The resulting supernatants containing no visible inclusion bodies were subjected to FCS measurements. FCS measurements were carried out using the MP-FCS/FCCS system with a ×40 objective (Olympus, NA = 1.15) (19). Cell lysate samples (10 μl) were set on an inverted microscope. GFP was excited at the 488 nm laser line of a CW Ar+ laser through a water immersion objective, and the emission was detected at 510–610 nm. FCS measurements were performed for 32 s three times consecutively at room temperature. The intensity fluctuations were detected by an avalanche photodiode. The calculation of the autocorrelation functions was performed during the measurement. After the measurement, the autocorrelation function, G(τ) was fitted to the model as follows (Equation 1),

\[
G(\tau) = \frac{\langle |I(t)|^2 \rangle}{\langle |I(t)| \rangle^2} = 1 + \frac{1}{N} \sum_{i=1}^{2} \left(1 + \frac{\tau}{\tau_i} \right)^{-1/2} \left(1 + \frac{\tau}{\tau_s} \right)^{-1/2}
\]

(Eq. 1)

where \(<|I(t)|>(\text{counts per second}), N\) denotes the number of fluorescent particles in the volume element with the radius \(w_{xy}\) and length \(2w_{z}\), \(\tau_i\) denotes the diffusion time of the fraction \(F_i\) (\(i = 1, 2\)), and \(s\) denotes the axial ratio of the volume element (\(w_{z}/w_{xy}\)). The diffusion time (\(\tau_s\)) is proportional to the average time for diffusion of fluorescent particles across the detection area, which reflects the size of the particles. The counts per particle (CPP) is calculated by dividing the average fluorescence intensity (\(\langle |I(t)| \rangle\)) by the number (\(N\)), and hence directly reflects the number of the fluorescent molecules per particle (Equation 2).

\[
\text{CPP} = \frac{\langle |I(t)| \rangle}{N}
\]

(Eq. 2)

We employed a simple two-component fitting analysis (\(i = 1, 2\)) using a Gaussian function, where two kinds of molecules distribute in the detection area. Two-component fitting analyses were performed for (Q)19- and (Q)81-GFP because their autocorrelation curves fitted well with a two-component model, whereas one-component fitting analyses (\(i = 1\)) were performed for GFP and (Q)19-GFP (Fig. 4, A and B).

**Western Blot Analysis**—COS-7 cells expressing poly(Q)-GFP or co-expressing poly(Q)-GFP with QBP1 or SCR were lysed 48 h after transfection, and separated by 7.5% PAGE under non-denaturing conditions or 12.5% PAGE containing SDS. Proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) by standard Western transfer techniques, and then incubated overnight at 4 °C with a rabbit polyclonal anti-GFP antibody (MBL, Nagoya, Japan) at a 1:1,000 dilution followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (DakoCytoama-
circles}

that we previously showed to bind the poly(Q) stretch, against poly(Q) inclusion formation, and confirmed significant suppression of poly(Q) inclusion formation by co-expression of QBP1 with poly(Q)-GFP as described previously (17) (Fig. 1B).

Although it is reported that soluble oligomers are more toxic than mature amyloid fibrils, and that the inclusion body itself is even cytoprotective in the poly(Q) diseases (2, 6, 20), there has been little evidence of oligomer formation of the poly(Q) protein in vivo so far. To analyze intracellular interactions of the soluble poly(Q) proteins that are distinct from formation of insoluble and microscopically visible inclusion bodies, we performed FRET analyses of poly(Q)-YFP and poly(Q)-CFP fusion proteins co-expressed in COS-7 cells. FRET occurs when two poly(Q) proteins interact with each other and exist in close proximity (<100 Å) such that the excited poly(Q)-CFP (donor) can transfer its energy to poly(Q)-YFP (acceptor) resulting in emission from the acceptor molecule (21). As shown in poly(Q)-GFP expressing cells, (Q)_{81}YFP and (Q)_{81}CFP proteins were colocalized into inclusion bodies in COS-7 cells, whereas (Q)_{19}YFP and (Q)_{19}CFP proteins remained diffusely distributed (Fig. 2A and data not shown).

To analyze the interaction of the soluble (Q)_{81}YFP and (Q)_{81}CFP proteins, cells co-expressing (Q)_{81}YFP and (Q)_{81}CFP were lysed at 48 h after transfection, centrifuged to remove insoluble inclusions, and the soluble fraction of the lysates containing no microscopically visible inclusions were subjected to FRET analyses. Emission scans of either (Q)_{81}CFP or (Q)_{81}YFP alone excited at 436 nm revealed that (Q)_{81}CFP exhibits a
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full emission with a maximum at 480–500 nm, whereas (Q)81-YFP exhibits a slight crossover emission with a maximum at 530 nm (Fig. 2B). Notably, we found that the cells co-expressing (Q)81-YFP and (Q)81-CFP exhibit a dramatic increase in the emission with a maximum at 530 nm upon excitation at 436 nm. Moreover, we found a corresponding decrease in the emission with a maximum at 480–500 nm, although the amount of (Q)81-CFP proteins used for the FRET experiments was the same as that of (Q)81-CFP alone. These results indicate that FRET occurs between soluble (Q)81-YFP and (Q)81-CFP proteins. We did not observe any significant FRET between (Q)19-YFP and (Q)19-CFP proteins in the soluble cell lysates (data not shown), confirming that this interaction between (Q)81-YFP and (Q)81-CFP is specific. These results provide the first evidence for assembly of the soluble expanded poly(Q) protein in cultured cells.

In earlier studies, FRET has been applied to monitor inclusion body formation of poly(Q) proteins in cell culture (22, 23). However, these studies only detected gathering of the poly(Q) proteins into insoluble inclusion bodies, and not the molecular interactions of the soluble poly(Q) proteins. Because inclusion body formation itself is considered to be a cytoprotective response (6, 7), monitoring molecular interactions of the soluble poly(Q) proteins in vivo is critical for understanding cytotoxicity of the poly(Q) proteins and its suppression.

Polyglutamine Protein Oligomer Formation in Cells Detected by FCS—Although we clearly demonstrated molecular interactions of expanded poly(Q) proteins in the soluble fraction using FRET, FRET does not provide quantitative information such as the size of the assemblies; i.e. whether they are dimers, trimers, or larger oligomers. In addition, the efficiency of FRET does not always correlate with the quantity of interactions because it also depends on the distance between fluorescent probes (24). Therefore, to further characterize and quantify the assemblies, and to explore intracellular oligomer formation of the soluble expanded poly(Q) protein in cells, we employed FCS analyses, which is a highly sensitive technique for investigating the dynamics of fluorescent molecules in solution at single molecule sensitivity (12, 13) (supplementary materials Fig. S1). Several dynamic properties of the fluorophore, such as the number, diffusion time, and size of the oligomerizing molecules can be analyzed by FCS (14–16).

COS-7 cells expressing poly(Q)-GFP were lysed at certain intervals after transfection and centrifuged to remove inclusions, and the soluble fractions of the lysates containing no microscopically visible inclusions were subjected to FCS measurements. Autocorrelation curves of GFP and poly(Q)-GFP, which indicate the relative distribution of molecules with various diffusion times, are shown in Fig. 3, A and B. The autocorrelation curves of both GFP and (Q)19-GFP overlapped with each other until at least 72 h after transfection, suggesting that the mobility of GFP and (Q)19-GFP molecules remained unchanged during the experimental period (Fig. 3A and data not shown). However, we found that the autocorrelation curves of (Q)45- and (Q)81-GFP at 60 h after transfection were shifted toward the right with increasing length of the poly(Q) stretch. The degree of the rightward shift of the autocorrelation curves of (Q)45- and (Q)81-GFP increased in a time-dependent manner (Fig. 3B and data not shown). These results suggest that the mobility of (Q)45- and (Q)81-GFP gradually decreased and the size of the (Q)45- and (Q)81-GFP particles gradually became larger as compared with GFP and (Q)19-GFP, implying formation of slowly moving oligomeric species.

We next analyzed time-dependent changes of the CPP of poly(Q)-GFP, which is the average fluorescence intensity of the fluorescent particles measured, and therefore directly reflects the number of poly(Q)-GFP monomers per oligomeraggregate. Both the CPPs of GFP and (Q)19-GFP were unchanged until at least 72 h after transfection, suggesting that GFP and (Q)19-GFP molecules remained as a monomer during the experimental period (Fig. 3C). However, importantly, we found that the CPPs of (Q)45- and (Q)81-GFP significantly increase in a time- and poly(Q) length-dependent manner, indicating that these poly(Q)-GFP molecules gradually assemble into oligomers (Fig. 3C). These results provide the first evidence for soluble oligomer formation of poly(Q) proteins in cultured cells, in which the soluble poly(Q) proteins are diffusely distributed, and not accumulated as inclusion bodies. The CPP value of (Q)81-GFP at 60 h after transfection (58.3) was about 3.5-fold larger than that of (Q)45-GFP at 12 h (16.5), indicating that (Q)81-GFP oligomers at 60 h consist of 3.5 monomers on average. Because the CPP value is simply an average fluorescence intensity of the fluorescent particles, and the (Q)81-GFP particles present in cells at 60 h are assumed to be a mixture of monomers and oligomers of various sizes, the actual (Q)81-GFP oligomers at this time point are expected to be much larger than the CPP value.

To further confirm oligomer formation of poly(Q)-GFP proteins by an alternative biophysical method, we performed Western blot analyses of poly(Q)-GFP proteins to see if we could detect any oligomeric species as a ladder pattern. The soluble cell lysates of COS-7 cells expressing poly(Q)-GFP were separated by native PAGE followed by Western blot analysis with an anti-GFP antibody. We found intense bands corresponding to poly(Q)-GFP monomers in all samples (Fig. 3D, arrows), and also considerable amounts of (Q)45- and (Q)81-GFP proteins remaining at the gel top, probably due to their assembly into oligomers/aggregates (Fig. 3D, black arrowheads). Notably, upon long exposure, we successfully detected faint ladder bands in addition to the monomer bands in the lysates of (Q)45- and (Q)81-GFP expressing cells (Fig. 3D, red arrowheads). Such a ladder pattern was not evident in the (Q)19-GFP cell lysate. These faint ladder bands probably correspond to dimers, trimers, etc., which were detected as increases in the CPP values by FCS. Larger oligomers were not detected probably because they were not separated by native PAGE. Thus, FCS has an advantage over Western blot analysis in detecting these oligomers quantitatively in solution.

Quantitative Analyses of Polyglutamine Protein Oligomer Formation by a Two-component Fitting Analysis—We next performed quantitative analyses on the diffusion times of poly(Q)-GFP proteins obtained by FCS, which reflect the size of the poly(Q)-GFP particles. Whereas GFP and (Q)19-GFP showed a simple decay of autocorrelation curves, which fitted with a one-component model, the autocorrelation curves of (Q)45- and (Q)81-GFP fitted well with a two-component model, suggesting
that at least two kinds of fluorescent particles with different mobility exist in the samples (Fig. 4, A and B). We therefore employed a two-component fitting analysis to evaluate the diffusion times of (Q)\textsubscript{45}-GFP and (Q)\textsubscript{81}-GFP. The autocorrelation curves of (Q)\textsubscript{45}-GFP were shifted toward the right in a time-dependent manner. These results suggest that the mobility of (Q)\textsubscript{45}- and (Q)\textsubscript{81}-GFP gradually decreased and the size of the (Q)\textsubscript{45}- and (Q)\textsubscript{81}-GFP particles gradually became larger, implying formation of slowly moving oligomeric species. C, time-dependent changes of the CPP of poly(Q)-GFP proteins expressed in COS-7 cells. Data are expressed as mean ± S.E. (error bars). *, p < 0.05; **, p < 0.01 (Student’s t test). The CPPs of (Q)\textsubscript{45}-GFP (open triangles) and (Q)\textsubscript{81}-GFP (open circles) significantly increased in a time- and poly(Q) length-dependent manner, indicating that these poly(Q)-GFP molecules gradually assemble into oligomers. Data are expressed as mean ± S.E. (error bars). D, Western blot analyses of poly(Q)-GFP proteins expressed in COS-7 cells. The soluble cell lysates were separated by native PAGE followed by Western blot analysis with an anti-GFP antibody. Faint ladder bands probably corresponding to dimers, trimers, etc. (red arrowheads), in addition to monomer bands (arrows) were detected in (Q)\textsubscript{45}- and (Q)\textsubscript{81}-GFP expressing cells. Considerable amounts of (Q)\textsubscript{45}- and (Q)\textsubscript{81}-GFP proteins also remained at the gel top, probably due to their assembly into larger oligomers/aggregates (black arrowhead).
particles are therefore likely to be large soluble aggregates. These results indicate the remarkable ability of FCS to define monomers, small oligomers, and large aggregates formed in cells at once.

We further evaluated the ratio of the slow diffusion component fraction ($F_2$) to total fractions ($F_1 + F_2$), which indicates the proportion of large soluble aggregates to total fluorescent particles. We found that the ratio of $F_2$ of (Q)$_{45}$- and (Q)$_{81}$-GFP increased in a time-dependent manner during the process of aggregate formation in cells (Fig. 4F). The rate of increase in the $F_2$ ratio of (Q)$_{81}$-GFP was much faster than that of (Q)$_{45}$-GFP, reflecting that (Q)$_{81}$-GFP forms large aggregates much faster than (Q)$_{45}$-GFP. We could detect a significant increase in the ratio of $F_2$ of (Q)$_{81}$-GFP as early as 36 h after transfection, indicating that the increase in the ratio of $F_2$ is the earliest parameter for detection of poly(Q) protein oligomerization/aggregation by FCS.

**QBP1 Inhibits Polyglutamine Oligomer Formation in Cells**—From a therapeutic point of view, we previously identified the poly(Q)-binding peptide QBP1, and showed that QBP1 inhibits poly(Q) protein aggregation in vitro, and suppresses poly(Q) inclusion formation and cytotoxicity in cell culture as well as poly(Q)-induced neurodegeneration in Drosophila (17, 25, 26). However, whether QBP1 inhibits poly(Q) oligomer formation in vivo has not been clarified so far. To address this issue, we performed FCS measurements of poly(Q)-GFP co-
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A typical normalized autocorrelation curves of (Q)45-GFP co-expressed with QBP1 (gray) or SCR (black) extracted from transfected COS-7 cells at 36 (open circles) and 60 h (closed circles) after transfection. Co-expression of QBP1 with (Q)45-GFP significantly inhibited the rightward shifts of the curves at both 36 and 60 h after transfection, suggesting that the decrease in the mobility of (Q)45-GFP was suppressed by QBP1. B-D, suppression of time-dependent increases in the CPP (B), DT1 (C), and ratio of F2 (D) of (Q)45-GFP by co-expression of QBP1 (closed columns). SCR was used as a control (open columns). Data represent mean ± S.E. of at least three independent experiments. *, p < 0.05; **, p < 0.01 (Student’s t test). Co-expression of QBP1 significantly suppressed time-dependent increases in the CPP, DT1, and F2 ratio of (Q)45-GFP, indicating that QBP1 inhibits oligomerization/aggregation of the poly(Q) protein at an early stage in cells. Suppression of the increase in the DT1 of (Q)45-GFP by QBP1 was evident only at 60 h (C), whereas the increase in the F2 ratio of (Q)45-GFP was significantly suppressed as early as 36 h by QBP1 (D), suggesting that the F2 ratio is the most sensitive parameter for detection of the inhibitory effect of QBP1. E, Western blot analyses of (Q)45-GFP proteins co-expressed with QBP1 or SCR in COS-7 cells. The soluble cell lysates were separated by SDS-PAGE followed by Western blot analysis with an anti-GFP antibody. The expression levels of (Q)45-GFP were similar in both cells co-expressing QBP1 or SCR, F; suppression of the increase in the CPP of poly(Q)-GFP by co-expression of QBP1 (closed columns) at 60 h after transfection. SCR was used as a control (open columns). Data represent mean ± S.E. of at least three independent experiments. **, p < 0.01 (Student’s t test). Significant suppression of the increase in the CPP by QBP1 was evident only for (Q)45-GFP, and not for (Q)45-GFP, probably because the longer poly(Q) stretch has a higher propensity to form oligomers/aggregates. B-D and F, data are expressed as mean ± S.E. (error bars).

We also found that the effect of QBP1 on inhibition of poly(Q) oligomerization was greater for (Q)45-GFP than for (Q)81-GFP (Fig. 5F), probably because the longer poly(Q) stretch has a higher propensity to form oligomers/aggregates, consistent with our previous observation (17). We therefore conclude that FCS is a remarkably useful technique to detect the earliest effects of poly(Q) aggregate inhibitors in cells.

Congo Red Prevents the Growth of Polyglutamine Oligomers/Aggregates, but Not the Initial Formation of Oligomers—To validate the usefulness of FCS for evaluating aggregate inhibitors for their inhibitory effects on oligomer formation of the poly(Q)
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A

Q45-GFP

Q45-GFP+Congo red

B

C

D

FIGURE 6. Congo red inhibits the growth of polyglutamine-GFP oligomers/aggregates, but not the formation of oligomers. A, fluorescence microscopic images of (Q)19-GFP expressing COS-7 cells treated with 25 μM Congo red, at 48 h after transfection. Modest suppression of poly(Q) inclusion formation was confirmed. Scale bars indicate a length of 100 μm. B–D, the CPP (B), DT (C), and ratio of F2 (D) of (Q)45-GFP expressing cells either treated with Congo red (closed columns) or left untreated (open columns) measured at 60 h after transfection. Data represent mean ± S.E. of at least five independent experiments. *, p < 0.05 (Student’s t test). Congo red significantly suppressed the increase in the CPP, but neither the increase in the DT, nor the increase in the F2 ratio of (Q)45-GFP, indicating that Congo red does not inhibit the initial formation of oligomers, but inhibits the growth of poly(Q) oligomers/aggregates. B–D, data are expressed as mean ± S.E. (error bars).

In this study, we successfully detected time- and poly(Q) length-dependent oligomer formation of expanded poly(Q)-GFP proteins in cell lysates. Although FCS is applied for investigating biological molecules in living cells, the interaction of fluorescent probes with cellular elements such as organelles and the cytoskeletal network are known to interfere with free diffusion of fluorescent probes (31). High viscosity of the cytosol compared with diluted aqueous buffer solutions would also result in slower diffusion of fluorescent probes. Indeed, we failed to characterize oligomer formation of poly(Q)-GFP proteins in intact living cells, probably because misfolded poly(Q) proteins are transported by microtubule-dependent transport (7), and not by free diffusion. It is also possible that interactions between poly(Q)-GFP proteins with various cellular proteins such as molecular chaperones, cytoskeletal proteins, or even endogenous poly(Q)-containing proteins, may affect the diffusion of poly(Q)-GFP proteins (32, 33). These possible interactions would affect only the diffusion time but not the fluorescence intensity in the FCS measurements, and hence may account for the discrepancy between the average size of

protein, we examined the effect of Congo red, a known poly(Q) aggregate inhibitor, on poly(Q) oligomerization in cells. We treated COS-7 cells expressing (Q)45-GFP with Congo red, and confirmed modest suppression of poly(Q) inclusion formation as described previously (29) (Fig. 6A). FCS analyses revealed that Congo red treatment significantly suppresses the increase in the CPP of (Q)45-GFP, suggesting that Congo red inhibits oligomerization of the poly(Q) protein (Fig. 6B). However, we could not detect significant suppression of the increase in the DT of (Q)45-GFP, indicating that Congo red does not inhibit formation of small oligomers of the poly(Q) protein (Fig. 6C). This observation is consistent with a previous in vitro study showing that Congo red prevents the assembly of mutant huntingtin into mature fibrils, but not the initial formation of protofibrils (30), which is in contrast with QBP1 (Fig. 5C). We also could not detect significant suppression of the increase in the F2 ratio of (Q)45-GFP, but detected suppression of the increase in the CPP, further supporting that Congo red only inhibits the growth of poly(Q) oligomers/aggregates (Fig. 6D). Taken together, these results indicate that Congo red does not inhibit the initial oligomer formation, but binds poly(Q) protein oligomers/aggregates, and prevents the further growth of poly(Q) oligomers/aggregates into larger aggregates, resulting in inhibition of aggregation of the poly(Q) protein. We therefore conclude that FCS is capable of distinguishing poly(Q) aggregate inhibitors for their ability to inhibit the initial formation of oligomers or the growth of oligomers/aggregates.

Disruption of the Conformation of the Polyglutamine Stretch Leads to Suppression of Oligomer Formation—The difference between the effects of QBP1 and Congo red on oligomerization of the poly(Q) protein probably results from the difference in their effects on the conformation of the poly(Q) protein. In fact, we recently demonstrated that QBP1 prevents the conformational transition of the poly(Q) protein monomer (27), whereas Congo red has been reported to prevent the growth of poly(Q) protein oligomers/aggregates that are formed after the conformational transition (30). Accordingly, we evaluated the effect of disruption of the conformation of the poly(Q) stretch on oligomer formation, by introducing proline insertions into the poly(Q) stretch. We previously demonstrated that proline insertions indeed disrupt the conformation of the poly(Q) stretch and reduce aggregation and cytotoxicity in cell culture (18). We expressed poly(Q)-GFP-Myc proteins with various proline insertions in COS-7 cells and performed FCS analyses (Fig. 7A). We found that (Q)73P3-GFP-Myc, which has three proline insertions in the poly(Q) stretch separating 18–19 successive glutamines, showed a considerably lower CPP value (37.0) as compared with (Q)81-GFP-Myc (67.7), which has an uninterrupted poly(Q) stretch (Fig. 7B). (Q)73P7-GFP-Myc, which has seven proline insertions, exhibited a more drastic reduction in the CPP value (13.1), which is a similar level to (Q)19-GFP (16.5, Fig. 3C), suggesting that proline insertions into the poly(Q) stretch reduce oligomer formation in correlation with the number of inserted prolines. We could not perform a two-component fitting analysis with these samples because the autocorrelation curve of (Q)73P7-GFP-Myc fitted well with a one-component model, like as (Q)19-GFP. Taken together, we conclude that disrupting the conformation of the poly(Q) stretch suppresses oligomer formation in cells.

DISCUSSION

In this study, we successfully detected time- and poly(Q) length-dependent oligomer formation of expanded poly(Q)-GFP proteins in cell lysates. Although FCS is applied for investigating biological molecules in living cells, the interaction of fluorescent probes with cellular elements such as organelles and the cytoskeletal network are known to interfere with free diffusion of fluorescent probes (31). High viscosity of the cytosol compared with diluted aqueous buffer solutions would also result in slower diffusion of fluorescent probes. Indeed, we failed to characterize oligomer formation of poly(Q)-GFP proteins in intact living cells, probably because misfolded poly(Q) proteins are transported by microtubule-dependent transport (7), and not by free diffusion. It is also possible that interactions between poly(Q)-GFP proteins with various cellular proteins such as molecular chaperones, cytoskeletal proteins, or even endogenous poly(Q)-containing proteins, may affect the diffusion of poly(Q)-GFP proteins (32, 33). These possible interactions would affect only the diffusion time but not the fluorescence intensity in the FCS measurements, and hence may account for the discrepancy between the average size of
Aggregation of misfolded proteins into β-sheet-rich amyloid fibrils, despite the distinct primary amino acid sequences of the proteins, has been recognized as a common feature among the conformational neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and the poly(Q) diseases (1–3). The conformational neurodegenerative diseases such as Alzheimer proteins, has been recognized as a common feature among the fibrils, despite the distinct primary amino acid sequences of the fibrils.

Proline insertions lead to suppression of oligomer formation. A, schematic representation of the poly(Q)-GFP constructs with proline insertions into the poly(Q) stretch. Inserted prolines are indicated as closed boxes. B, suppression of the increase in the CPP of poly(Q)-GFP by introducing proline insertions into the poly(Q) stretch, in correlation with the number of inserted prolines. The FCS measurements were performed at 60 h after transfection. Data represent mean ± S.E. of at least three independent experiments. **, p < 0.01 (Student’s t test). Disrupting the conformation of the poly(Q) stretch by proline insertions dramatically suppresses oligomer formation in cells. Data are expressed as mean ± S.E. (error bars).

Poly(Q)-GFP oligomers estimated from the CPP (3.5 for (Q)₈₁-GFP at 60 h) and that estimated from the DT₅ (5.7 for (Q)₈₁-GFP at 60 h).

Aggregation of misfolded proteins into β-sheet-rich amyloid fibrils, despite the distinct primary amino acid sequences of the proteins, has been recognized as a common feature among the conformational neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and the poly(Q) diseases (1–3). The aggregates of amyloid β-protein (Aβ) as well as poly(Q) proteins formed in vitro were shown to cause toxicity in cell culture when incubated in the culture medium (34, 35). Recently, however, soluble oligomers of Aβ, rather than mature amyloid fibrils, are thought to play an important role in the pathogenesis of Alzheimer disease (8–11). The small diffusible Aβ oligomers formed in vitro, referred to as Aβ-derived diffusible ligands, were shown to be toxic to neurons in organotypic mouse brain slice cultures (36). Microinjection of naturally secreted Aβ oligomers has been shown to inhibit long-term potentiation in the rat hippocampus, suggesting toxicity of Aβ oligomers in vivo (37).

Soluble oligomers composed not only of various amyloidogenic proteins but also of non-disease-associated proteins have been reported to cause cytotoxicity, suggesting intrinsic toxicity of the pre-fibrillar structures, regardless of their primary amino acid sequences (20, 38). Formation of poly(Q) protein oligomers in vitro has also been shown by atomic force microscopy analyses (30, 39). In this study, we successfully demonstrated soluble oligomer formation of the poly(Q) protein in cultured cells for the first time by FCS, which is implicated in the disease pathogenesis (6, 40).

Because it has been believed that poly(Q) protein oligomers/aggregates formed during the aggregation process cause neurotoxicity, molecules that inhibit poly(Q) aggregation have been extensively investigated so far. These include small peptides binding to the poly(Q) stretch (17, 25, 41), intrabodies against the poly(Q) proteins (42, 43), molecular chaperones targeting the misfolded proteins (32, 44, 45), and small chemical compounds inhibiting poly(Q) aggregation (29, 46–48). However, none of these inhibitors have been shown to inhibit poly(Q) oligomer formation in cells except for QBP1, as shown in this study. Recently, CCT, a cytosolic chaperonin, has been reported to prevent large aggregate formation (F₂) of the poly(Q) protein by FCS, but not small oligomer formation (F₁) (49). In this study, we clearly demonstrated that QBP1 inhibits oligomer formation of the soluble poly(Q) protein in cultured cells by FCS, which was shown to suppress poly(Q)-induced neurodegeneration in vivo (25, 26). We also showed that Congo red only inhibits the growth of oligomers/aggregates, but not the initial oligomer formation of the poly(Q) protein. The therapeutic effects of Congo red on poly(Q)-induced neurodegeneration have remained controversial, probably because of the lack of its ability to suppress poly(Q) oligomer formation (47, 50). Because soluble oligomers are thought to be the neurotoxic species, monitoring oligomer formation of the soluble poly(Q) proteins in cells by FCS is indispensable for developing therapies for the poly(Q) diseases. Therefore, FCS can be applied for screening for inhibitors specifically of poly(Q) oligomerization, rather than aggregation, as therapeutic candidates for the poly(Q) diseases.

In conclusion, we applied the FCS technique to detect poly(Q) oligomer formation in cells. We successfully detected a time-dependent increase in the diffusion time and particle size of the poly(Q) protein, indicating oligomer formation at an early stage in cells. We also detected large soluble aggregates with a much slower diffusion time after insoluble poly(Q) inclusions became evident, implying that FCS can detect heterogeneous poly(Q) protein assemblies quantitatively and simultaneously in cells. We further demonstrated that QBP1 inhibits poly(Q) oligomer formation, whereas Congo red only inhibits the growth of oligomers, but not the initial formation of the poly(Q) oligomers, suggesting that FCS is useful for identifying poly(Q) oligomer inhibitors. We further show that disruption of the conformation of the poly(Q) stretch by proline insertions suppresses oligomer formation. We therefore conclude that FCS is a useful technique to monitor the oligomerization of poly(Q) protein monomers in cells as well as inhibition of oligomerization in the poly(Q) diseases, and is also applicable to the other conformational neurodegenerative diseases.

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