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Insoluble detergent-resistant aggregates form between pathological and nonpathological lengths of polyglutamine in mammalian cells

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ABSTRACT **Pathological degeneration of neurons in Huntington's disease and associated neurodegenerative disorders is directly correlated with the expansion of CAG repeats encoding polyglutamines of extended length. The physical properties of extended polyglutamines and the intracellular consequences of expression of polyglutamine expansion have been the object of intensive investigation. We have extended the range of lengths of polyglutamine produced by recombinant DNA methodology by constructing a library of CAG/CAA repeats coding for a range of 25–300 glutamine residues. We have investigated the subcellular localization, interaction with other polyglutamine-containing polypeptides, and the physical properties of aggregated forms of polyglutamine in the cell. Extended polyQ aggregated in the cytoplasm and was only transported to the nucleus when a strong nuclear localization signal was present. Polyglutamine below pathological lengths could be captured in aggregates and transported to ectopic cell locations. The CREB-binding protein (CBP), containing a homopolymeric stretch of 19 glutamines, was likewise found to coaggregate in a polyglutamine-dependent manner, suggesting that pathology in polyglutamine disease may result from cellular depletion of normal proteins containing polyglutamine. We have observed a striking detergent resistance in aggregates produced from polyglutamine of pathological length. This observation has led to the development of a fluorescence-based assay exploiting the detergent resistance of polyglutamine aggregates that should facilitate high-throughput screening for agents that suppress polyglutamine aggregation in cells.**

Eight autosomal dominant progressive neurodegenerative disorders, including Huntington's disease (HD) are known to be caused by the expansion of CAG repeats in the coding sequence of a diverse set of unrelated proteins (1, 2). In HD, the length of CAG expansion correlates with age of onset and severity of neuronal degeneration. The CAG expansion does not appear to interfere with the normal function of the huntingtin gene product and is the only identified mutation implicated in the disease (2–4). An ectopically expressed small N-terminal fragment of human HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice which is associated with the presence of polyQ aggregation in the form of neuronal intranuclear inclusions and neuropil aggregates (5–7). Neuronal intranuclear inclusions have become the neuropathological signature of polyglutamine disorders (8), and in HD insoluble polyQ aggregates have also been detected outside the nucleus in the form of dystrophic neurites (9) and neuropil aggregates (10).

Experiments with ectopically expressed polyglutamines in mammalian cell culture demonstrate that efficiency of aggregation increases with the length of the polyQ segment, whereas polypeptides with less than 40 glutamine residues fail to aggregate at all (11–14). As reported by different groups, polyQ aggregates are variously found in the nucleus, in the cytoplasm, or in both subcellular locations. With time some polyglutamine inclusions become ubiquitinated and colocalize with cellular proteasomes (15).

Extended polyglutamines aggregate as amyloid-like protein *in vitro* and, at least in this case, aggregation appears to depend on hydrogen-bonded polar zipper formation of polyQ molecules (16, 17). Abnormal interaction of proteins containing extended polyQ, or products of such interactions in the form of insoluble aggregates, have been implicated as a common cytotoxic ''gain-of-function'' factor in the pathogenesis of neurodegenerative disorders (3).

In this report, we investigate the atypical properties of extended polyQ, following the formation of aggregates in mammalian cells as an indicator of these interactions. We show that polyglutamines of normal length can form insoluble detergent-resistant aggregates when coexpressed with extended polyQ tracts. The affinity of interaction between extended polyQ is sufficient to translocate polypeptides to a novel subcellular location. A normal cellular protein which contains a homopolymeric stretch of 19 glutamines can coaggregate with extended polyglutamine in a polyglutamine-dependent manner. Once the process of aggregation is initiated, an expanded length of the polyQ tract is no longer required for joining the aggregate. Thus the depletion from the cell pool by sequestration in aggregates of any protein with a significant polyQ segment may represent a potential mechanism for the cytoxicity of polyglutamine aggregates. Furthermore, our data indicate that the process of aggregation does not require dramatic conformational changes of proteins, and that proteins can exist in their native form even in the core of inclusions. Yet the interactions between extended polyQ in aggregates protect proteins inside from dissolution and denaturation by high concentrations of detergents.

METHODS

Synthesis of Alternating CAG/CAA Triplet Repeats. The CAA CAG CAG CAA CAG CAA and complementary TTG TTG CTG TTG CTG CTG oligonucleotides were annealed to generate double-strand duplex DNA with trinucleotide extensions. Short duplex DNA molecules were used as starting material for two consecutive ligations to obtain alternating CAG/CAA repeats (CAA CAG CAG CAA CAG CAA)n of

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Abbreviations: HD, Huntington's disease; EGFP, enhanced green fluorescent protein; TBP, TATA-binding protein; NLS, nuclear localization signal; CBP, CREB-binding protein.

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different lengths. The second ligation reaction was terminated by addition of double-stranded DNA linkers that included 5′ trinucleotide extensions and the restriction sites *Hin*dIII at 5′ and *Pst*I at 3′ with respect to the CAG/CAA DNA strand. Alternating CAG/CAA repeats of different lengths were subcloned into Bluescript-KS vector and maintained in XL-1 Blue (Stratagene). Repetitive CAA CAG CAG CAA CAG CAA consensus was verified by two-strand sequence analyses, and clones containing 25–300 alternating CAG/CAA repeats were selected to generate mammalian expression constructs.

Polyglutamine Mammalian Expression Constructs. The N-terminal HD cDNA fragment, including the Kozak box, the start codon, and the first 16 amino acids, was amplified by PCR (Amplitaq; Perkin–Elmer), ligated with various polyglutamine repeats (25 Q, 104 Q, 191 Q, 230 Q, 250 Q, and 300 Q), and subcloned into pcDNA 3.1 (Invitrogen). Constructs were tagged at the 3′ end with c-*myc* or enhanced green fluorescent protein (EGFP; Clontech). The first methionine of EGFP sequence was replaced by lysine in polyQ/EGFP constructs. Nucleolin cDNA cloned into pMAMNEO (Clontech) was generously provided by M. Srivastava (National Institutes of Health) and was previously described (18). Nucleolin sequence was amplified by PCR and inserted between polyglutamine (25Q, 104Q, 300Q) and EGFP sequences in HD polyQ EGFP constructs. The first methionine of nucleolin cDNA was changed to lysine in polyQ/nucleolin/EGFP constructs. Wild-type TATA-binding protein (TBP) cDNA was amplified from genomic DNA extracted from HeLa LTRα3 (generous gift from P. Sharp, Massachusetts Institute of Technology). To replace native 38Q homopolymeric stretch in TBP sequence, N-terminal and C-terminal fragments of TBP (GenBank accession no. M55654) were amplified by PCR with primers introducing novel internal *Hin*dIII and *Pst*I restriction sites at the nucleotide positions 412 and 521, respectively. DNA fragments encoding 25Q, 42Q, 65Q, and 104Q were ligated with N-terminal and C-terminal TBP fragments and subcloned into pcDNA3.1. Final TBP fusion constructs were tagged with c-*myc* at the carboxy terminus.

Full-length CBP cDNA cloned in pcDNA 3.1 was a generous gift from J. Borrow, Massachusetts Institute of Technology (GenBank accession no. U47741). A polyglutamine-rich domain near the C terminus was removed by digesting with *Sac*II and *Xba*I, amplifying the C-terminal fragment with primers containing *Sac*II and *Xba*I sites, then fusing with the N-terminal fragment. This results in an internal ≈200-aa deletion (6652–7228 nt) at the C terminus which removes a polyglutamine-rich fragment, including a 19Q homopolymeric stretch. Both full-length and deletion constructs are tagged with c-*myc* at the C terminus.

Polyglutamine Aggregation in Ecdysone-Inducible Expression System. EGFP and polyQ/EGFP fusions with 25Q, 104Q, and 300Q were subcloned into pIND DNA vector (Invitrogen). EcR-293 cells (Invitrogen) were transfected with pIND DNA by using Transfectam reagent (Promega). Stable integrants were selected in 0.4 mg/ml G418 and 0.4 mg/ml Zeocin. PolyQ expression and aggregation were tested in isolated cell lines by induction with 0–5 μM Muristerone A and Ponasterone A. (Invitrogen).

Fluorescent Analyses of Transfected Cells. Polyglutamine aggregation was assayed in COS-1, COS-7, NIH 3T3, 293, EcR-293, HeLa LTRα3, NT-2, and PC-12 cell lines. Cells were grown on coverslips to 50% confluence and lipofected for 2 hr with Transfectam reagent (Promega). Polyglutamine aggregation was assayed from 16 to 72 hr after transfection. Cells were fixed in 2% formaldehyde/0.1% Triton X-100 for 10 min and incubated with primary mouse monoclonal anti-c-*myc* (Invitrogen) Ab (1:500) and secondary FluoroLink Cy3 (Amersham Life Science) Ab (1:2000). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Epifluorescent microscopy was performed on a Zeiss Axioplan II equipped with a Quantix CCD camera (Photometrics, Tucson, AZ) and IPLAB Spectrum imaging software (Scanalytics, Billerica, MA).

***In Situ* Detergent-Resistant Aggregate Method.** Transfected or induced cells expressing polyQ were harvested from Petri dishes, washed twice with PBS, and lysed with 0.3% Nonidet P-40, followed by wash with 0.1% Triton X-100/PBS. Simultaneously, floating aggregates from dead cells were pelleted from culturing medium by high-speed centrifugation, washed with PBS and 0.1% Triton X-100/PBS, and combined with cell lysates. Aggregates were washed twice with 1% Triton X-100/PBS for 1 hr (overnight) and washed in 0.1% SDS/PBS. Aggregates were pelleted after each wash by centrifugation. Finally, semipurified aggregates were incubated with 2–5% SDS/Triton X-100 mix for 1–48 hr at room temperature (37°C). Alternatively, live cells expressing polyQ/EGFP fusions were lysed *in situ* with 2–5% SDS/2–5% Triton X-100. EGFP fluorescence in polyQ aggregates was detectable as long as after 48 hr of incubation at room temperature (37°C).

RESULTS

Generating PolyQ of Lengths Ranging from Benign to Well Beyond Pathological Range. To circumvent difficulties associated with the propagation of long CAG repeats in bacteria, we developed a cloning strategy that used alternating CAG/CAA repeats encoding 25 glutamine residues (normal in HD), extended 104 (pathological in HD), and extended 191, 230, and 300 glutamine residues (elongated beyond pathological range). All lengths of alternating CAG/CAA repeats were quite stable in bacteria, in contrast to native extended CAG repeats. A short huntingtin N-terminal cDNA fragment, including the Kozak box and the first 17 aa, was ligated to polyglutamine repeats of different lengths (25Q–300Q). To monitor the formation of aggregates in cells, we fused polyglutamines at the C terminus with either a 28-aa c-*myc* tag or with a 230-aa EGFP tag.

Extended PolyQ Peptides Form Cytoplasmic and Perinuclear Aggregates. We tested the ability of synthetic extended polyQ peptides to form aggregates in cells. Normal length (25Q) polyglutamines always showed diffuse cytoplasmic expression by fluorescence microscopy (Fig. 1*A*). We observed aggregation of extended polyglutamines (104Q, 191Q, 230Q, 300Q) in seven mammalian cell lines tested, including PC-12

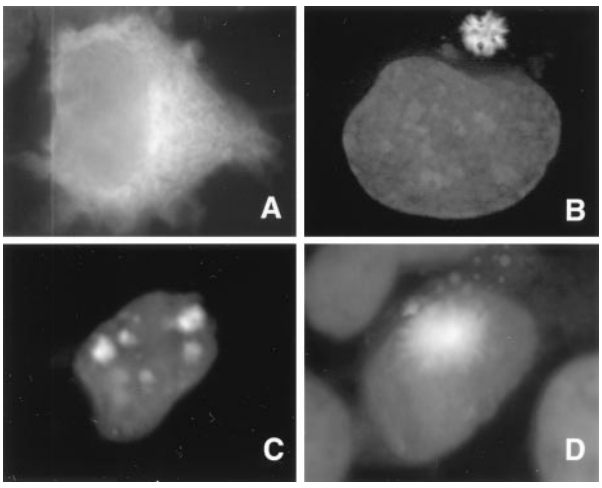

FIG. 1. Expression of normal length (25Q) and extended (104Q) polyglutamine constructs in single COS-1 cells shown at high magnification. (*A*) Normal length (25Q) naked construct shows diffuse cytoplasmic expression pattern. (*B*) Extended polyglutamine construct (104Q) aggregates in the cytoplasm. Diffuse, presumably soluble material is not seen in cells with aggregates. (*C*) 104Q/TBP shows multiple aggregates within the nucleus. (*D*) 104Q/nucleolin shows a single nucleolar aggregate.

and NT-2. Early in the time course of precipitation, extended polyQ formed small, star-like aggregates, which were detected in COS-1 cells as early as 16 hr after transfection. Within 36 hr after transfection, polyQ aggregates grew into dense, brilliantly fluorescent spherical structures, which could be as large as 4–5 microns (Fig. 1*B*). We found polyglutamine aggregates to be located exclusively in the cytoplasm, often in the perinuclear space or associated with the nuclear membrane. Although we did not detect any significant difference in number or size of aggregates by using either the c-*myc* or the EGFP-tagged extended polyQ constructs (19), the polyQ/c-*myc* aggregates that were detected by means of fluorescent Ab stained with an intense peripheral rim. In the intrinsically fluorescent polyQ/EGFP aggregates, the most intense fluorescence typically came from inside the core of the aggregate (see Fig. 3 *B*, *C*, and *E* and Fig. 2). We reasoned that polyQ aggregates form a very dense structure, which is impenetrable to antibody. Moreover, because denatured EGFP lacks fluorescence, this observation suggests that polypeptides inside the aggregate are at least partially in native form.

Nuclear Localization of Aggregates Depends on Flanking Sequence of Extended PolyQ. Polyglutamine aggregates have been found in HD brain in dystrophic neurites in the cortex and white matter and as nuclear inclusions in the striatum. In our cell culture models, we always found the extended polyQ aggregates in the cytoplasm. However, our constructs included only a small N-terminal fragment of huntingtin. Therefore, it is conceivable that the C terminus of the huntingtin sequence has a nuclear localization signal (NLS). Such a putative NLS would confer slow accumulation of mutant huntingtin in the nucleus and eventual formation of nuclear inclusions. To test the effect of a strong NLS on the subcellular localization of aggregates, we chose the nucleolin protein (650 aa), which has strong nuclear and nucleolus localization signals. We generated expression constructs (polyQ/nucleolin/EGFP) by inserting nucleolin cDNA between polyQ (25Q, 104Q, 300Q) and EGFP sequences. When we expressed normal length poly25Q/nucleolin/EGFP and extended polyQ/nucleolin/EGFP fusion proteins in cells, we found that all polypeptides were located in the nucleus and particularly in the nucleoli. No fluorescent signal was seen in the cytoplasm. We also sought to target aggregate formation to the nucleus by extending the naturally occurring stretch of 38 polyglutamines in the TBP to 104Q. Extended polyQ/nucleolin/EGFP constructs gave nucleolar aggregates (Fig. 1*D*). Moreover, the length of the fusion protein, which included extended polyglutamines and nucleolin sequence for a total of up to 1200 aa did not limit nuclear translocation and aggregation. As predicted, TBP/104Q formed multiple aggregates, which were clearly located within the nucleoplasm (Fig. 1*C*). Our results suggest that the subcellular localization of polyQ aggregates is determined by polyQ flanking sequences. Additionally, we demonstrate that at least 1,000 aa can be translocated into the nucleus by active transport and aggregated without cleavage.

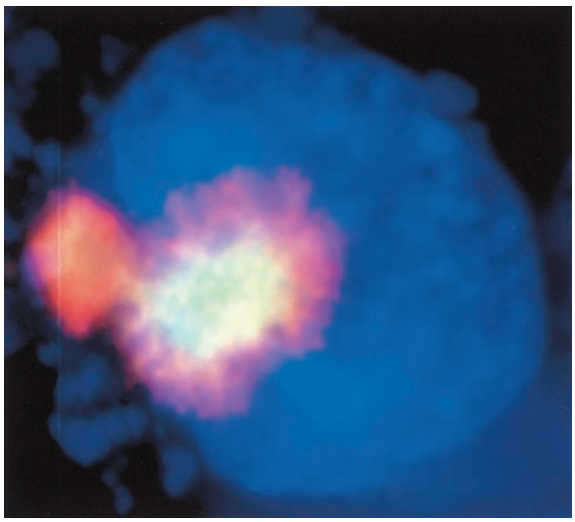

FIG. 2. Coexpression of 104Q/nucleolin/EGFP (green) and 104Q/c-*myc* (red) at high magnification. Extended polyglutamines are colocalized (yellow) in the nucleus (blue) and the cytoplasm (not stained) of a single cell. Interactions between polyglutamines are sufficiently strong to relocate 104Q/c-*myc* into the nucleus.

Extended PolyQ with a NLS Can Translocate Polyglutamine-Containing Proteins to the Nucleus Through PolyQ Interactions. In previous experiments we found polyQ aggregates exclusively in the cytoplasm, unless the extended polyQ construct included nucleolin. To determine whether the strong NLS could also function *in trans*, we coexpressed extended poly104Q/nucleolin/EGFP fusion with polyQ constructs lacking nucleolin. Strikingly, we found that extended polyQ/c-*myc*, which lacks an NLS, was now detected in heterogeneous aggregates in the nucleus (Fig. 2). Nuclear localization was strictly dependent on coaggregation with poly104Q/nucleolin/EGFP fusion protein. Despite the presence of the strong nuclear localization signal *in cis*, we found that polyQ/nucleolin/EGFP also aggregated in some cells with extended polyQ in the cytoplasm, and thus was excluded from the nucleus (Fig. 2). Thus, subcellular localization of aggregation depends in general on the functional characteristics of the protein in which the polyQ is embedded. Nonetheless, strong intermolecular interactions mediated by polyQ domains can in some cases be sufficient to override the effects of such intrinsic localization signals.

Certain Polyglutamine-Containing Cellular Proteins Can Coaggregate with Extended PolyQ. Huntingtin with unexpanded polyQ tracts of from 8 to 39 glutamine residues is expressed from the unaffected allele in HD. A number of cellular proteins have been identified with naturally occurring homopolymeric polyQ segments ranging in length from 6 to 38 glutamine residues. We sought to establish whether normal length polyglutamine peptides of no more than 40 glutamine residues could interact with and perhaps aggregate with extended polyglutamines when coexpressed in cells. To test this hypothesis we cotransfected normal length poly25Q/EGFP and extended poly104Q/c-*myc*. Normal length polyglutamines showed a diffuse pattern of expression when transfected alone. Remarkably, these same normal length polyglutamines were recruited into cellular aggregates when they were coexpressed with extended polyglutamine constructs. In contrast, when EGFP lacking a polyQ segment was coexpressed with extended polyQ/c-*myc*, EGFP fluorescence was not detected in aggregates. Coexpression experiments by using poly25Q/nucleolin/EGFP and extended polyQ/c-*myc* yielded coaggregates in nucleoli, whereas EGFP/nucleolin cotransfected with extended polyQ/c-*myc* gave cytoplasmic aggregates, which had no EGFP signal. These results demonstrate the strict polyglutamine-dependent nature of the coaggregation phenomenon (19).

Our results suggest that intermolecular interactions between mutant extended polyQ and normal cellular proteins with significant glutamine stretches (below a threshold of 38) may play a role in the cellular pathology of the polyglutamine neurodegenerative disorders. To investigate this possibility in our cell culture system, we tested the nuclear transcriptional coactivator CREB-binding protein (CBP), which, like several such proteins, has a glutamine-rich C terminus within which is a homopolymeric stretch of 19 glutamine residues. Coexpression of our 25Q/EGFP construct with CBP showed a diffuse cytoplasmic localization (Fig. 3*A*). In contrast, when we coexpressed c-*myc*-tagged CBP along with our extended polyQ constructs, CBP was detected within cytoplasmic aggregates formed by naked extended polyQ in the vast majority of

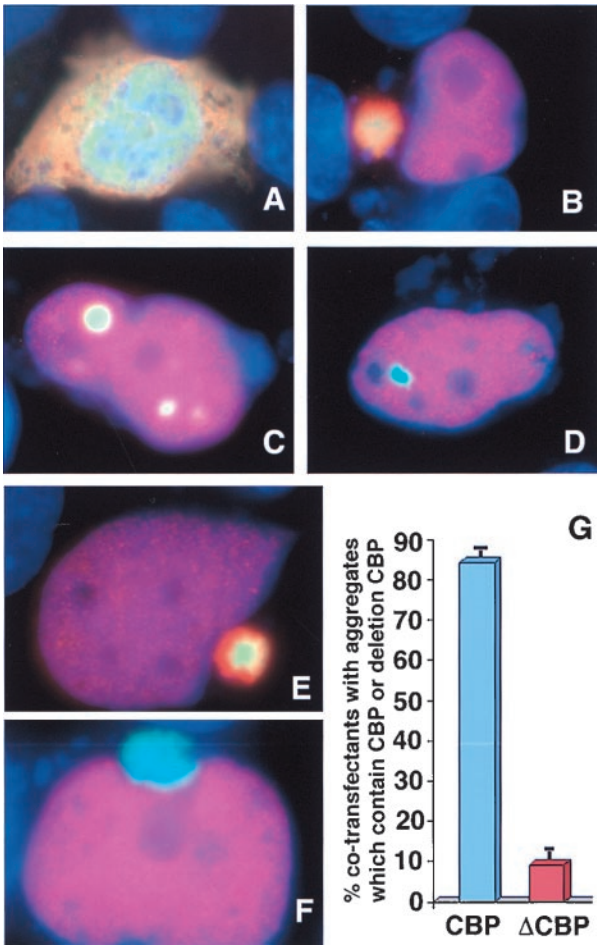

FIG. 3. Cotransfection of polyglutamine/EGFP constructs (green) and CBP/c-*myc* (red) at high magnification. (*A*) 25Q/EGFP (green) and CBP/c-*myc* (red) show diffuse cytoplasmic colocalization (yellow). (*B*) 104Q/EGFP (green) and full-length CBP/c-*myc* (red) colocalized in a single cytoplasmic aggregate. (*C*) 104Q/TBP/EGFP (green) and CBP/c-*myc* (red) colocalized in nuclear aggregates (yellow). (*D*) CBP/c-*myc* (red) is not included in nucleolar aggregates formed by 104Q/nucleolin/EGFP (green). (*E*) Full-length CBP/c-*myc* (red) colocalized (yellow) with 104Q/EGFP (green). (*F*) CBP/c-*myc* (red) with deletion of 19 glutamines and surrounding glutamine-rich region fails to coaggregate with 104Q/EGFP (green). (*G*) CBP deletion construct shows greater than 8-fold reduction of coaggregation compared with full-length CBP.

cotransfected cells with aggregates (Fig. 3*B*). As predicted, when we coexpressed CBP with 104QTBP, which forms aggregates in the nucleus, we detected CBP in these nuclear aggregates (Fig. 3*C*). In sharp contrast, however, when CBP was coexpressed with 104Q/nucleolin, we did not detect CBP in the nucleolar aggregates (Fig. 3*D*). This failure to detect CBP in the nucleolar aggregates can be explained by the subcellular and subnuclear location of CBP itself. Although transfected CBP is seen strongly in the nucleus and weakly in the cytoplasm, it is clearly excluded from nucleolar bodies. These results suggest that the likelihood that an endogenous cellular polyQ containing protein will be found in a polyQ aggregate may depend on the interplay between a number of factors including local concentration within the cell. In particular, the ability of mutant extended polyQ to recruit cellular proteins into aggregates may be exquisitely dependent on colocalization within precise subcellular compartments. To determine whether coaggregation of CBP and 104Q/EGFP (Fig. 3*E*) is mediated by the homopolymeric polyQ domain in the CBP molecule, we made an ≈200-aa deletion construct which excludes that domain as well as the surrounding glutamine-rich region. By using this polyQ-deleted CBP construct in coaggregation experiments, we did not detect CBP signal in the majority of cotransfected cells with aggregates (Fig. 3 *F* and *G*). Thus we have clearly demonstrated that recruitment of cellular proteins into polyQ aggregates depends on interactions between polyQ domains.

Insoluble PolyQ Aggregates Shield Entrapped Polypeptides and Protect Them from Denaturation, Even Under Harsh Conditions. The unusual microscopic appearance of our extended polyglutamine-containing aggregates led us to consider alternative experimental approaches that might reveal key properties of their structure and its effects on cellular processes. To investigate further the nature of these very insoluble aggregates, we took advantage of the difference in fluorescence property between native and denatured EGFP to develop a novel *in situ* assay to test the resistance of cellular aggregates to high concentrations of detergents. Cells were transfected with normal length poly25Q/EGFP, and with extended poly104Q/EGFP. Forty hours later cells were treated *in situ* with SDS/Triton X-100 at various concentrations as high as 5%/5% overnight at room temperature. High-intensity EGFP fluorescence was detected in surviving aggregates formed by extended polyQ/EGFP polypeptides

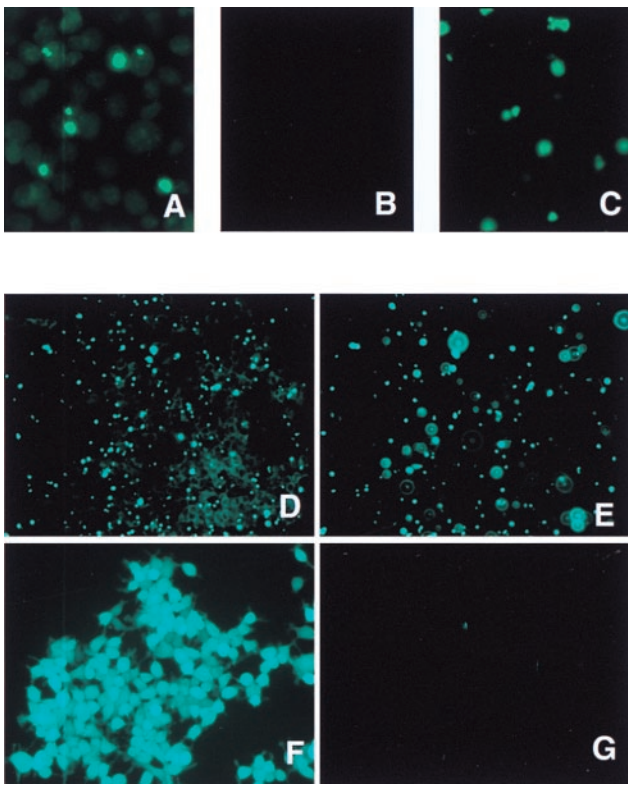

FIG. 4. *In situ* assay demonstrates detergent-resistant insolubility of extended polyQ aggregates and coaggregates. (*A*) Low-power view of 25Q/EGFP cotransfected with 104Q (not stained), demonstrating aggregation of normal length polyQ by extended polyQ. (*B*) 25Q/EGFP transfected alone and treated with 2.5% SDS/2.5% Triton X-100 for 2 min. Normal length polyQ alone does not aggregate and EGFP fluorescence is not preserved. (*C*) 25Q/EGFP cotransfected with 104Q (not stained), treated with 2.5% SDS/2.5% Triton X-100. Aggregates remained fluorescent for up to 24 hr of detergent treatment. (*D*) Lower-power view of stable inducible live culture expressing 104Q/EGFP (green) and forming aggregates. (*E*) Same field as *A* after 2-min treatment with 2.5% SDS/2.5% Triton X-100. Aggregates were similar in size, number, and fluorescent intensity even after 24 hr of treatment at 37°C. (*F*) Low-power view of stable inducible live culture expressing 25Q/EGFP. (*G*) Same field as *C* after 2-min treatment with detergent as above. No remaining fluorescence is detectable.

after treatment with detergents (data not shown, see Fig. 4). In contrast, soluble, nonaggregated EGFP and poly25Q/EGFP were completely denatured by detergents, and EGFP fluorescence was no longer detected (data not shown, see Fig. 4). The results show that high concentrations of detergents are unable to destroy polyQ aggregates or denature aggregated native EGFP structure.

Extended PolyQ Peptides Trap Soluble, Normal Length PolyQ Peptides into Insoluble Detergent-Resistant Aggregates in Cells. To demonstrate directly that extended polyQ peptides are able to trap normal length polyQ peptides into insoluble aggregates in the cell and are not simply loosely associated or colocalized, we treated coaggregates with high concentrations of detergents as described above. Cells were cotransfected with poly25Q/EGFP and extended polyQ104/c-*myc* (Fig. 4*A*). These coaggregates were extracted from the cells and were shown also to be resistant to high concentrations of detergents (Fig. 4*C*). The fluorescence due to protection, by the coaggregation with extended polyQ, of native normal length poly25Q/EGFP was identical to the fluorescence in aggregates formed by extended poly104Q/EGFP alone. In control experiments cells were cotransfected with EGFP (lacking a polyQ segment) and extended polyQ/c-*myc*. Detergents denatured soluble poly25Q/EGFP and EGFP lacking a polyQ segment, and EGFP fluorescence was no longer detected (Fig. 4*B*). Thus detergent-resistant insolubility was shown to be dependent on interactions between the polyQ stretches themselves.

System for High-Throughput Screening for Agents to Suppress Polyglutamine Aggregation. Our results showing strong interactions between polyglutamines led us to consider a search for those molecules that might interact with extended polyglutamines and yet suppress aggregation. Identification of such molecules in a cell culture system would require the reliable induction of extended polyglutamine aggregates. As a first step toward this goal, we generated ecdysone-inducible mammalian cell lines that expressed EGFP-tagged 25Q, 104Q, and 300Q in a ligand-dependent manner. Cells from selected clones showed uniform fluorescence after induction. Polyglutamine expression level and number of aggregates formed was dose-dependent. Aggregates were detected as early as 24 hr after induction, with maximum appearance at 48–72 hr. Typically we observed 2–3 aggregates per colony of 12–16 cells 48 hr after induction with 10 mM Muristerone A. When treated *in situ* with 5% SDS/5% Triton X-100, EGFP fluorescence was protected in polyQ aggregates even after 48 hr of lysis at 37°C (Fig. 4 *D* and *E*). In contrast, soluble nonaggregated EGFP-tagged material was denatured instantly and completely, and fluorescence no longer detected (Fig. 4 *F* and *G*). This fluorescence quenching effect to assess solubility is simple to generate, highly reproducible, and straightforward to score, because of the very intense fluorescent signal generated by the aggregated polyglutamines compared with the total absence of fluorescence seen with soluble molecules. Moreover, such an approach to a suppressor screen should be quite amenable to automation. Thus, our fluorescence-based solubility assay in combination with our inducible aggregate-producing cell lines represent the components necessary for high-throughput screening for agents to suppress polyglutamine aggregation.

DISCUSSION

We synthesized alternating CAG/CAA triplet repeats, ranging from 25 to 300 glutamine residues. All repeats were highly stable in bacteria and were easy to manipulate by using conventional methods of molecular cloning. We generated DNA constructs expressing essentially naked polyQ of different lengths. To direct the subcellular localization of polyQ in cells, we fused alternating CAG/CAA triplet repeats with nucleolin cDNA. Likewise, we expanded the naturally occurring 38Q domain in the TBP molecule to 104Q to directly study the effects of polyQ aggregation in the nucleus.

Our results highlight a critical property of polyglutamine aggregate formation. We show that relatively short, soluble peptides with short polyQ tracts can coprecipitate in cells in the presence of extended polyQ tracts. Moreover, we have clearly demonstrated in our experiments that coaggregation is strictly dependent on the presence of a polyglutamine segment in both molecules, as has been suggested by Perutz *et al.* (17). Therefore, this interaction is directly mediated through the polyglutamine domains.

We propose that extended mutant polyQ has the potential to interact with any normal polyQ containing protein in the cell. A possible pathogenic mechanism in the trinucleotide neurodegenerative diseases might involve depletion or sequestration of normal cellular proteins that contain short homopolymeric polyQ domains. The identification of TBP in nuclear inclusions supports this notion (20). Our results with CBP have shown that the recruitment of such cellular proteins is likely to depend on the coexistence of the extended polyQ and such cellular proteins within narrowly defined cellular compartments.

Protein database searches reveal that hundreds of polyQ containing proteins have been identified to date. An interesting class of nuclear proteins that contain glutamine-rich regions and often homopolymeric glutamine stretches are transcription factors and transcriptional coactivators. It is unlikely, however, that a neurodegenerative disease that appears to selectively involve such specific cell populations as the medium spiny projection neurons of the striatum, as in Huntington's disease, or the cerebellar Purkinje cells, as in spino-cerebellar ataxia, would be caused by sequestration of ubiquitously expressed components of the transcriptional machinery such as TBP or CBP. But certainly the expression of many transcriptional activators (or repressors) can and must be exquisitely specific with respect to cell type and developmental stage. Most likely the vulnerability to polyglutamine expansion of specific neuronal populations in these diseases is governed not simply by properties of the mutant proteins themselves. In fact, it has been shown that an expanded polyQ repeat inserted into hypoxanthine phosphoribosyltransferase gene (*Hprt*) caused a neurological phenotype and intranuclear neuronal inclusions in transgenic mice (21). There are certain to be cell-type-specific cofactors for pathogenesis that remain to be elucidated. Among such cell-type-specific cofactors could be polyQ-containing molecules that could interact with expanded mutant polyglutamines within specific cellular compartments or at particular developmental stages.

Regardless of the mechanism by which polyQ expansion causes neuronal dysfunction and cell death, the tight correlation between the length of a polyQ tract capable of aggregation and the pathogenic threshold of HD suggest that the initiation of the aggregation pathway is the molecular trigger that causes this disease (22). Therefore, the prevention of polyQ aggregation presents a rational target for the development of therapeutics. We have found that aggregates formed by poly104Q/EGFP show dramatic resistance to high concentrations of detergent and that EGFP maintains at least some native conformation within the inclusion as detected by fluorescence. We have exploited these properties to develop an inducible cell based assay that is particularly suitable for conversion to a high-throughput screen for the identification of compounds that can suppress polyQ aggregation.

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