Experimental Neurology **155,** 195–203 (1999)
Article ID exnr.1998.6991, available online at http://www.idealibrary.com on IDEAL®

Expanded Polyglutamine Domain Proteins Bind Neurofilament and Alter the Neurofilament Network

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Received July 10, 1998; accepted October 30, 1998

Eight inherited neurodegenerative diseases are caused by genes with expanded CAG repeats coding for polyglutamine domains in the disease-producing proteins. The mechanism by which this expanded polyglutamine domain causes neurodegenerative disease is unknown, but nuclear and cytoplasmic polyglutamine protein aggregation is a common feature. In transfected COS7 cells, expanded polyglutamine proteins aggregate and disrupt the vimentin intermediate filament network. Since neurons have an intermediate filament network composed of neurofilament (NF) and NF abnormalities occur in neurodegenerative diseases, we examined whether pathologic-length polyglutamine domain proteins also interact with NF. We expressed varying lengths polyglutamine-green fluorescent protein fusion proteins in a neuroblast cell line, TR1. Pathologic-length polyglutamine–GFP fusion proteins formed large cytoplasmic aggregates surrounded by neurofilament. Immunoisolation of pathologic-length polyglutamine proteins coisolated 68-kDa NF protein demonstrating molecular interaction. These observations suggest that polyglutamine interaction with NF is important in the pathogenesis of the polyglutamine repeat diseases. © 1999 Academic Press

Key Words: **CAG-trinucleotide repeat diseases; Huntington's disease; protein aggregation; neurofilament.**

INTRODUCTION

Eight inherited neurodegenerative diseases are caused by expanded CAG repeats in the disease-producing genes. The CAG repeat encodes a polyglutamine domain in the expressed proteins (26, 43). These diseases, Huntington's disease (HD); dentatorubral-pallidoluysian atrophy (DRPLA); spinocerebellar atrophy 1, 2, 3 (Machado–Joseph disease (MJD)), 6, and 7 (SCA 1, 2, 3, 6, and 7); and spinobulbar muscular atrophy (SBMA) share common molecular, cellular, and clinical features.

The mechanism by which these disease genes cause cell death is unknown, but the expanded polyglutamine domain plays a critical role, since expression of proteins containing large glutamine domains is toxic to *Escherichia coli*, mammalian cells and transgenic mice (1, 4, 7, 8, 18, 19, 37, 39). The polyglutamine domain itself is responsible for cell death because cell death occurs whether the repeat domain is contained in a fragment, the entire native protein (4, 7, 8, 19), or fused to nonnative partners such as green fluorescent protein (GFP) or hypoxanthine phosphoribosyltransferase (37, 40).

Proteins containing pathologic-length polyglutamine domains form nuclear and cytoplasmic aggregates, while shorter polyglutamine domain proteins do not aggregate (1, 7, 8, 10, 18, 37, 42). The nuclear aggregate is well studied, but there is little information on the composition of the neuronal cytoplasmic aggregate (8, 35, 42). Polyglutamine binding proteins include glyceraldehyde-3-phosphate dehydrogenase (3, 25), huntington-associated protein (32), huntingtin-interacting protein (24), apopain (16), leucine-rich acidic nuclear protein (LANP) (35), cystathionine β-synthase (2), and vimentin (37), but none of these proteins are known to be involved in the pathologic cytoplasmic lesions.

We previously showed that expression of pathologic-length polyglutamine–GFP fusion protein in transfected COS7 cells causes cytoplasmic aggregation, disrupts the vimentin intermediate filament network, and causes cell death (37). Mature neurons do not, however, contain vimentin (28). Neurons contain the homologous proteins, neurofilaments (NF). Although NF and vimentin are homologous at the sequence and conformational level, they form the cytoskeleton by different mechanisms and with different groups of associated proteins (14). The NF distribution is altered in polyglutamine repeat and other neurodegenerative diseases (9, 10, 22, 31, 36, 45) supporting the hypothesis for interaction with polyglutamine-containing proteins, but there is no

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0014-4886/99 $30.00

direct evidence linking polyglutamine repeat containing proteins to NF.

To examine whether long-polyglutamine domain proteins interact with NF and alter the NF network, we transfected TR1 cells to express varying length polyglutamine domain–GFP fusion proteins. TR1 cells are a clonal murine cell line derived by sequential oncogenic retroviral infection of neocortical neuronal precursors (5). TR1 cells have neuronal-like features and express NF and neuron-specific enolase, but not glial fibrillary acidic protein (5) or vimentin (see Results).

In this study we demonstrate that pathologic-length (but not short) polyglutamine–GFP fusion proteins aggregate in a neuronal cell line (TR1) in a length- and time-dependent manner. Furthermore, we show that NF proteins accumulate around the cytoplasmic polyglutamine aggregate and that immunoisolation of expanded polyglutamine-containing proteins coisolates NF protein. Our data suggest that the interactions of polyglutamine with NF proteins is important in the pathogenesis of polyglutamine repeat diseases.

MATERIALS AND METHODS

Construction of Polyglutamine Clones

Plasmid vectors expressing polyglutamine–GFP fusion proteins containing a myc-epitope were synthesized as previously described (37). The PCR primers used were 5CEu, 5′TGATCTCGAGCGCCACCATGGTCTCAACACATCACCATCACCAC; and 3NEu3, 5′TGATGAATTCGAGGGGGCCCAGAGTTTCCGTG (38) and the product was subcloned into the *Xho*I and *Eco*RI site of the pEGFP-N1 vector (Clontech, Palo Alto, CA). The clones encoding 19, 56, and 80 glutamines were digested by *Xho*I and *BSRG*I and fragments cloned into *Xho*I and *Asp* 718 I site of pcDNA3.1(−)/Myc-His C vector (Invitrogen, San Diego, CA). The nucleotide sequences of all constructs were confirmed by the dideoxynucleotide chain terminator method. The amino acid sequence of each clone was MVSTHHHHH(Q)19-80HHGNSGPPRILQSTVPRARDPPVAT-GFP-Myc-His. The underlined sequence represents the sequence of the multicloning site of pEGFP-N1.

Cell Culture and Transfection

TR1 cells were cultured in Opti-MEM I (Gibco BRL, Gaithersburg, MD) containing 2.5% fetal calf serum, 20 mM D-glucose, 55 μM 2-mercaptoethanol, 1% (v/v) penicillin (500 U/ml), and streptomycin (5000 μg/ml) on poly-D-lysine or Cell-Tak (Becton-Dickinson Labware, Bedford, MA) coated tissue culture plates. Transfection was performed using lipofectamine reagent according to the protocol from the supplier (Gibco BRL). Cells were examined by phase, fluorescence, and confocal microscopy. The percentage of cells with visible fusion protein aggregates was determined by counting cells with visible aggregates and dividing by the total number of fluorescent cells in four independent experiments. In each series more than 500 transfected cells were counted.

Analysis of Fusion Protein Expression

Twenty-four or 72 hours after transfection, cells were scraped from six-well plates and centrifuged and the cell pellet was collected. The pellet was suspended in Laemmli buffer containing 2% SDS and 5% β-mercaptoethanol and boiled for 3 min. The samples were electrophoresed on a 10% polyacrylamide gel containing 2% SDS. Proteins were transferred to Immobilon P membrane (Milipore, Bedford, MA) by standard Western transfer techniques. After transfer, the membrane was incubated overnight with rabbit polyclonal anti-GFP antibody (Clontech) at 1:2000 dilution in blotto (TBS, pH 7.6, with 5% dried milk and 0.1% Tween 20) at 4°C. After repeated rinses in blotto, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 1:5000 dilution for 1 h at room temperature and then extensively rinsed in blotto. HRP was detected with an enhanced chemiluminescence detection kit (Amersham International, Buckinghamshire, England) and exposed to Hyperfilm ECL (Amersham International).

Immunofluorescence and Confocal Laser Microscopy Analysis

For immunofluorescent labeling, TR1 cells were grown on Lab-Tek 4 chamber slides (Nunc Inc., Naperville, IL) coated with Cell-Tak adhesion reagent. Twenty-four hours after transfection, cells were fixed in methanol at −20°C. Cells were washed in TBST (0.15 M NaCl, 0.02 M Tris–HCl, pH 7.4, 0.1% Triton X-100) and then incubated in 2% BSA at room temperature for 10 min to prevent nonspecific staining. Cells were incubated with primary antibodies at room temperature for 30 min, extensively rinsed in TBST, and exposed to secondary fluorescent antibodies at room temperature for 30 min. After repeated rinses in TBST, cells were treated with Vectashield (Vector Laboratories, Burlingame, CA) mounting medium to prevent photobleaching. The following immunoreagents were used: monoclonal antibodies to phosphorylated and nonphosphorylated neurofilament (SMI 31 and SMI 311, respectively) (Sternberger Monoclonals, Baltimore, MD); neurofilament 68 and actin (monoclonal AC40 and polyclonal rabbit) (Sigma BioSciences, St. Louis, MO); β-tubulin (Amersham International, Buckinghamshire, England); anti-vimentin (V9) (Boehringer-Mannheim); and Texas red-labeled horse anti-mouse IgG (H + L) and horse anti-rabbit IgG (H + L) (Vector Laboratories). Images were obtained using a Zeiss microscope equipped with

fluorescein and rhodamine filter sets and Zeiss 32× lens (Carl Zeiss, Inc., Thornwood, NY) and the images were photographed. Confocal images were obtained using Bio-Rad MRC-600 Confocal System (Bio-Rad Labs, Richmond, CA) with an argon laser and Nikon Diaphot microscope equipped with a Nikon 60× oil immersion objective. Hard copies of stored images were obtained from Adobe Photoshop files (Adobe Systems Inc., San Jose, CA).

Coimmunoisolation of Polyglutamine Fusion Proteins and Neurofilament

TR1 cells were transfected with Q19-GFP, Q56-GFP, or Q80-GFP expression plasmids in poly-D-lysine-coated six-well plates. Twenty-four hours after transfection, the cells were rinsed with PBS and then incubated with 0.5 ml of extraction buffer (27) (100 mM KCl, 10 mM 1,4-piperazinediethane sulfonic acid, pH 6.8, 300 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μg/ml aprotinin, and 1 mM PMSF) for 30 min at 4°C. After removing the supernatant the remaining material was incubated in 0.5 ml of cell lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μg/ml aprotinin, and 1 mM PMSF) for 60 min at 4°C. The cells were scraped with a rubber policeman, homogenized with a Teflon homogenizer, and centrifuged 5000 rpm for 3 min at 4°C. The supernatant was preincubated with 50 μl of Dynabeads (M-450) sheep anti-mouse IgG (Dynal, Lake Success, NY) at 4°C for 1 h to remove nonspecific binding. After separating the beads using a magnetic separation unit, the supernatant was incubated with 1 μl anti-Myc monoclonal antibody (Invitrogen) for 16 h at 4°C. Fifty microliters of Dynabeads (M-450) sheep anti-mouse IgG was suspended in 0.5 ml of binding buffer (50 mM Tris–HCl, pH 8.0, 0.5% Triton X-100, 2% dried milk, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μg/ml aprotinin, and 1 mM PMSF) and incubated with the supernatant for 2 h at 4°C. After incubation, the beads were separated using a magnetic separation unit and washed three times with washing buffer (50 mM Tris–HCl, pH 8.0, and 0.5% Triton X-100). Washed beads were boiled for 5 min in Laemmli buffer containing 2% SDS and 5% 2-mercaptoethanol and the supernatant was subjected to SDS–PAGE and Western blot analysis. NF protein was detected with SMI31 (Sternberger Monoclonals) at 1:1000 dilution, anti-NF160 at 1:400 dilution, and anti-NF68 (Sigma) at 1:5000 dilution.

RESULTS

Polyglutamine–GFP Fusion Proteins Aggregate in TR1 Cells in a Time- and Polyglutamine Length-Dependent Manner

Figure 1 shows TR1 cells transfected 24 h earlier with Q19-GFP (Fig. 1A) or Q80-GFP plasmid (Fig. 1B). Each fusion protein is denoted by Q followed by the number of uninterrupted glutamines and GFP for green fluorescent protein. The fluorescence in cells expressing Q19-GFP was diffusely distributed and remained unchanged 72 h after transfection. In contrast, Q56-GFP and Q80-GFP fluorescence was initially diffuse, but within 24 h formed single or multiple large cytoplasmic aggregates. Less frequently, aggregates were also seen in thin cellular processes (Fig. 4F).

Transfected cells produced approximately equal amounts of polyglutamine–GFP fusion protein as shown in Fig. 2. Cells expressing Q56 and Q80-GFP produced high molecular weight GFP fusion protein aggregates that did not enter the separating gel. These aggregates were not seen in TR1 cells expressing the Q19-GFP (Fig. 2, lane 2 versus lanes 3 and 4). The aggregates did not dissociate even when boiled in the presence of the reducing agent 2-mercaptoethanol.

The formation of high molecular weight protein aggregates increased with time after transfection (Fig. 2, lanes 3 and 4 (24 h) versus lanes 6 and 7 (72 h)). To examine the temporal formation of aggregates, we determined, by fluorescence microscopy, the percentage of transfected cells with aggregates at 24, 48, and 72 h (Fig. 3). Cells expressing Q19-GFP rarely produced aggregates, while cells expressing Q56-GFP and Q80-GFP frequently produced fluorescent protein aggregates. By 72 h after transfection, 49.1 ± 2.9% (SE) of the Q56-GFP-expressing cells contained fluorescent aggregate while 70.4 ± 3.1% (SE) of the Q80-GFP-expressing cells did.

Neurofilament Protein Accumulates around the Expanded Polyglutamine Aggregate

TR1 cells contain NF-L, NF-M, and NF-H protein, but not vimentin, as demonstrated by Western blot and immunocytochemistry (data not shown). To determine whether polyglutamine domain proteins interact with neurofilaments, TR1 cells expressing polyglutamine–GFP fusion protein were examined by confocal microscopy. Both in nontransfected cells and in cells expressing Q19-GFP the NF network extends throughout the cell. The normal appearance of the neurofilament, actin, and tubulin network in cells can be seen in the nontransfected cells shown in Fig. 4. In contrast, cells transfected with Q56- and Q80-GFP accumulate NF around the polyglutamine aggregates. Figure 4A shows the appearance of the polyglutamine–GFP aggregate using the FITC filter. Figure 4B is an image of the same cells as in Fig. 4A except that it shows the neurofilament accumulated in the same location as the polyglutamine aggregate (antineurofilament M and H antibody; Texas Red filter). Figure 4C shows the merged FITC and Texas red images from Figs. 4A and 4B. Figures 4D through 4I show only the merged FITC and Texas red images. The pattern of the accumulated NF network in the transfected cells appeared identical

with antibodies to phosphorylated NF-M and NF-H (Figs. 4C and 4D), nonphosphorylated NF-M and NF-H (Figs. 4E and 4F) and NF-L (Fig. 4G). The distribution of the microtubule and the actin network was unaffected by the presence of polyglutamine–GFP aggregates (Figs. 4H and 4I); there is no bright red halo surrounding the polyglutamine–GFP aggregate. The appearance of the NF, tubulin, and actin networks was identical in nontransfected cells shown in Fig. 4 and cultures untreated with lipofectamine and DNA (not shown).

NF-L Coimmunoisolates with Long Polyglutamine Fusion Proteins

The accumulation of NF protein around aggregated Q56- and Q80-GFP does not prove direct molecular interaction. To determine whether pathologic-length polyglutamine proteins bind NF protein we immunoisolated polyglutamine–GFP fusion proteins from TR1 cells transfected with Q19-GFP, Q56-GFP, or Q80-GFP. Figure 5 demonstrates that NF-L coimmunoisolated with the Q56- and Q80-GFP, but not with Q19-GFP. In contrast, actin, another cytoskeletal protein, did not coimmunoisolate (not shown). NF-M and NF-H did not coimmunoisolate with polyglutamine–GFP, perhaps due to the prior nonspecific adsorption of these proteins to the magnetic beads used for the immunoisolation procedure (data not shown). These data support the conclusion that expanded polyglutamine domain fusion proteins bind 68-kDa NF.

DISCUSSION

The mechanism by which expanded polyglutamine domain proteins cause neurodegenerative disease is unknown, but expansion of this domain causes protein aggregation and fibril formation both *in vitro* and *in vivo* (8, 10, 23, 37, 42). Much attention has focused on the nuclear aggregates, but the cytoplasmic aggregates have been less well studied. Several cellular proteins, including the intermediate filament protein vimentin, preferentially interact with long polyglutamine domains, but little evidence supports involvement in the pathologic cytoplasmic aggregate. In this paper we demonstrated that pathologic-length polyglutamine domain proteins bind NF and selectively alter the intracellular NF distribution. The interaction of polyglutamine and NF may be important in pathogenesis and explain cell-specific vulnerability.

Neurofilament Protein and Neurodegenerative Disease

NF proteins are members of the intermediate filament (IF) protein family and have sequence and structural homology with vimentin. The normal neurofilament structures is disrupted in polyglutamine-repeat disease neurons. In HD, the striatal neuron neurofila-

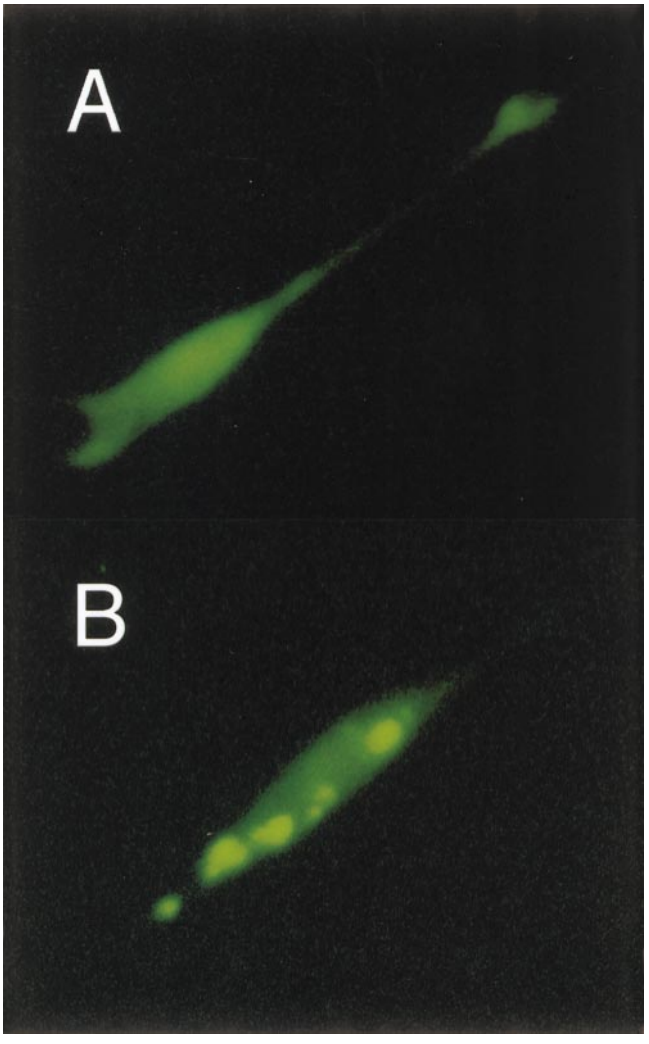
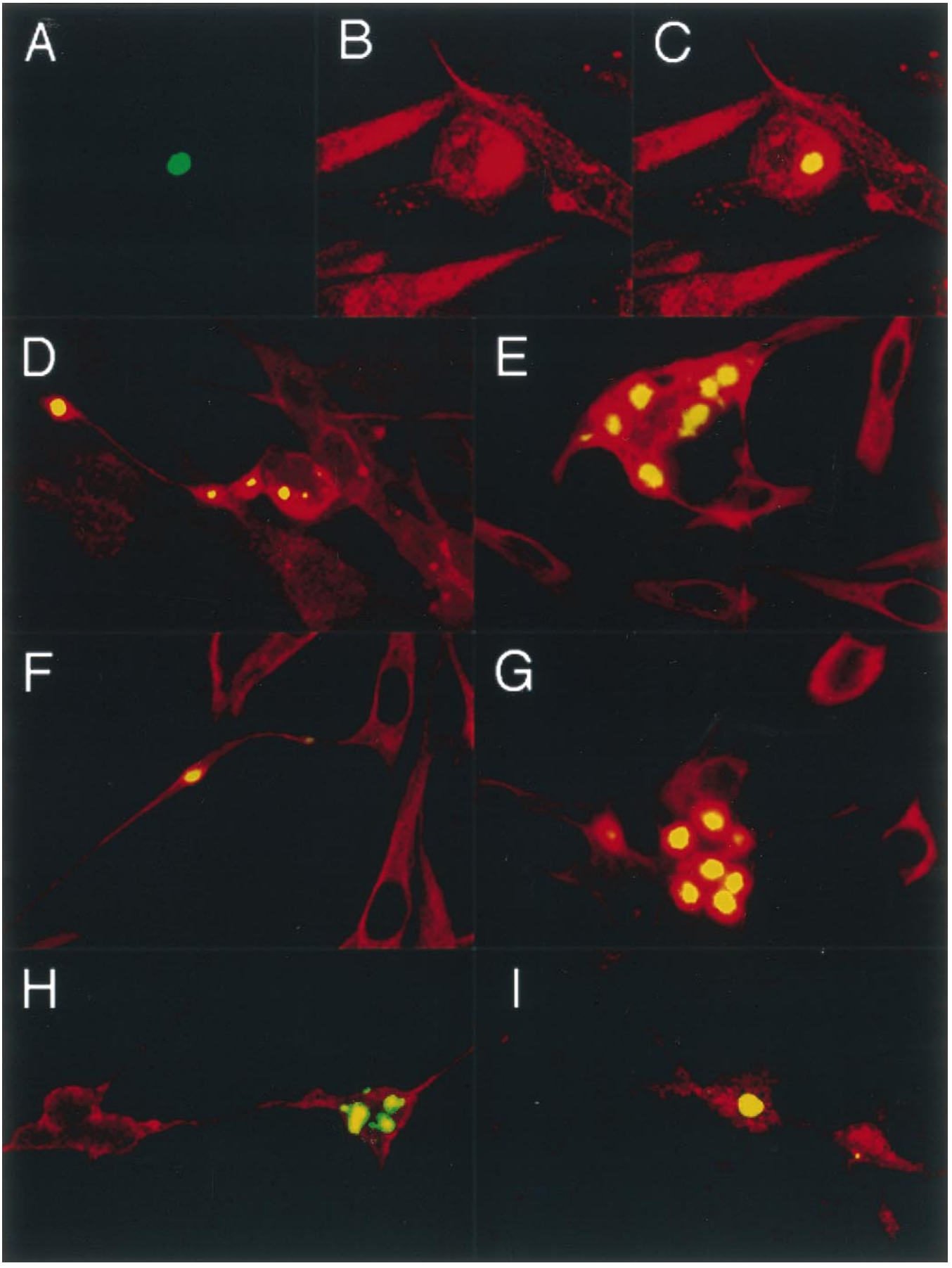


FIG. 1. Expanded, but not short, polyglutamine–GFP fusion proteins aggregate in transfected TR1 cells. Fluorescence micrograph demonstrating distribution of Q19-GFP (A) and Q80-GFP (B) 24 h after transfection in TR1 cells. Magnification ×320.

FIG. 4. Neurofilament protein accumulates around the polyglutamine–GFP fusion protein aggregate in TR1 cells. Cells expressing Q80-GFP were fixed 24 h after transfection and incubated with indicated antibody. GFP fluorescence (FITC optics) (A). Anti-phosphorylated neurofilament antibody (SMI 31) (Texas red optics) (B); merged image of A and B (C). (D through F) Merged FITC and Texas red images. Anti-phosphorylated neurofilament antibody (SMI 31) (D); anti-nonphosphorylated neurofilament antibody (SMI 311) (E and F); anti-NF-L 68 (G); anti-β tubulin (H) and anti-actin (AC40) (I). The cells were examined by confocal microscopy for GFP using FITC optics and for neurofilament or other cytoskeletal proteins using Texas red optics. The green signal is produced by polyglutamine–GFP fusion protein and the red signal represents neurofilament protein, actin, or tubulin (depending on the antibody used). The diffuse red color in the nontransfected cells is the normal appearance of neurofilament, actin, or tubulin (depending on the antibody). Magnification ×600.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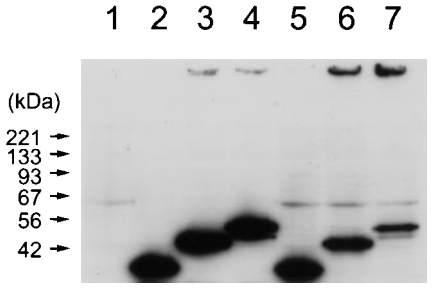

FIG. 2. Western blot analysis of polyglutamine–GFP fusion proteins in TR1 cells. Nontransfected TR1 cells (lane 1) and TR1 cells transfected with Q19-GFP (lanes 2, 5); Q56-GFP (lanes 3, 6); Q80-GFP (lanes 4, 7). Cells were harvested 24 h (lanes 2, 3, and 4) or 72 h (lanes 5, 6, and 7) after transfection. Fusion proteins were detected with anti-GFP antibody as described under Material and Methods.

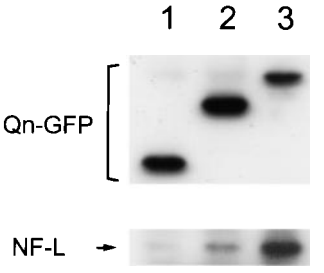

FIG. 5. Pathologic-length polyglutamine–GFP fusion proteins coimmunoisolate neurofilament. TR1 cells expressing Q19-GFP (lane 1); Q56-GFP (lane 2); or Q-80-GFP (lane 3) were lysed and immunoisolated with anti-Myc antibody. Immunoisolates were separated with secondary antibody-coated magnetic beads. Bound proteins were eluted and analysed on Western blots. (Top) Western blot probed with anti-GFP antibody. (Bottom) Western blot probed with anti-neurofilament 68-kDa antibody. Polyglutamine–GFP vector contains a myc-epitope. Qn-GFP, polyglutamine–GFP protein containing *n* glutamines.

ment network is condensed and fragmented (36). DiFiglia *et al.* reported that dystrophic neurites in HD contain filamentous aggregates associated with NF-positive axonal fibers (10). Cortical neurons in HD accumulate 10- to 15-nm fibrils which resemble NFs (23). Neurofilament aggregates (torpedoes and spheroids and neuroaxonal dystrophy) are also found in spinocerebellar ataxias and DRPLA (13, 17, 33).

Neurofilaments are the predominant IF protein in adult neurons and are the major determinant of neuronal size and axonal caliber (28, 50). Disruption of NF causes neuronal atrophy and reduction of axon caliber, pathologic features in HD, DRPLA, MJD, and SBMA (11, 21, 22, 31, 41). Iwabuchi *et al.* proposed that the characteristic neuropathologic feature of the CAG triplet repeat diseases is reduction of axonal diameter and cell volume (20–22). Neurofilament architecture is also disrupted in other neurodegenerative diseases, including Parkinson's disease (Lewy bodies), amyotrophic lateral sclerosis (motor neuron inclusions), corticobasal degeneration, and Pick's disease and may be a common mechanism in pathogenesis (9, 28, 45). Further support for a common mechanism involving NF in neurodegenerative disease is a recent report demonstrating that huntingtin is found in Pick bodies, which are composed of NF protein, in Pick's disease (44).

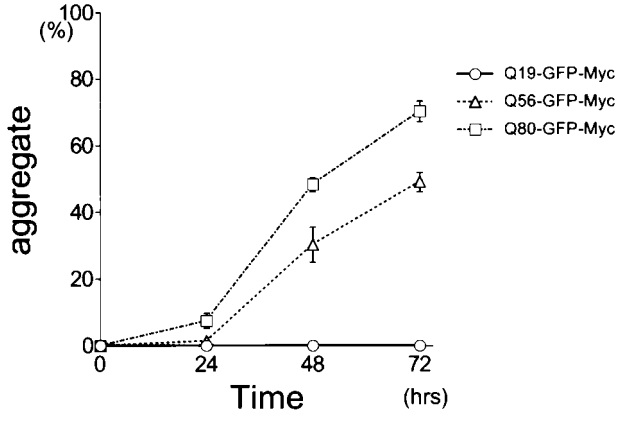

FIG. 3. Time and polyglutamine-length dependence of aggregates formation. Open circle, TR1 cells expressing Q19-GFP; open triangle, Q56-GFP; open square, Q80-GFP. The percentage of cells with visible aggregates of fusion protein were determined by dividing the number of cells with aggregates by the total number of cells expressing green fluorescence. Error bars, standard error.

Neurofilament Proteins Form a Fibrillar Network

The three major neurofilament proteins are NF light (NF-L; 68 kDa); NF medium (NF-M; 160 kDa); and NF heavy (NF-H; 200 kDa). NF fibrils are obligate heterooligomers requiring NF-L plus NF-M and/or NF-H for filament formation (30). Vimentin, in contrast, can form fibrils without other IF proteins. NF assembly can be altered by posttranslational modifications including phosphorylation and glycosylation (28). In our experiments we observed no difference in the appearance of the collapsed NF network whether the cells were probed with phosphorylated or nonphosphorylated NF antibodies. In addition to forming an elaborate three-dimensional network within the cell, NF proteins interact with other cytoskeletal proteins including the intermediate filament-associated proteins plectin and bullous pemphigoid antigen 1n (BPAG1n) (14).

Mechanisms of NF Accumulation

NF protein accumulates around the polyglutamine–GFP aggregates in TR1 cells. The mechanism by which expanded polyglutamine fusion protein aggregates cause NF accumulation is not known, but may involve alterations in synthesis, transport, or collapse of the NF network. The NF network is a stable cytoskeletal element but can be disrupted by intracytoplasmic injection of anti-neurofilament antibody (12), expression of mutated neurofilament protein (29, 48), and changes in

the relative expression levels of NF protein (15, 28, 49). IF networks can also be collapsed by expression of mutant IF-associated proteins (e.g., plectin, desmoplakin, and BPAG1n) (14). Expanded polyglutamine domain proteins, therefore, might alter the NF network by changing expression levels or directly interacting with NF blocking transport or causing collapse. Polyglutamine-containing proteins may also bind to NF indirectly via NF-associated proteins.

Specificity of Neuronal Degeneration

Normal and expanded polyglutamine domain proteins are expressed in virtually every cell type and tissue throughout the body, but the expanded polyglutamine domain proteins cause degeneration restricted to specific groups of neurons. The mechanism(s) by which proteins with expanded polyglutamine domains cause cell-specific neurodegeneration are unknown. The absence of pathology in nonneural cells in the CAG triplet repeat diseases may be due to several factors including lower levels of protein expression, the ability of nonneural cells to replicate and replace dying cells, molecular interactions imposed by other domains of the polyglutamine domain proteins, cell-specific processing, or to cell-specific interacting proteins (16, 32, 34, 35).

Interaction with NF could provide specificity since NF proteins are predominantly expressed in neurons and alterations in the NF network are known to cause neuron specific degeneration. Transgenic mice overexpressing NF-L or NF-H develop NF aggregates in motor neurons producing a pathology similar to motor neuron disease (amyotrophic lateral sclerosis) (6, 29, 49). Similarly, low levels of NF-M expression (2–25% of wild type) in transgenic mice cause age-dependent accumulation of neurofilament aggregates in selected neurons including the cerebral cortex and cerebellar Purkinje cells (47). Expression of truncated NF-H-lac-Z fusion protein in transgenic mice produces Lewy body-like inclusions in several types of neurons, but only Purkinje cells degenerate, a common finding in spinocerebellar ataxia (46). Selective binding of polyglutamine proteins to NF protein could, therefore, explain cytoplasmic aggregation and the specificity of neuronal degeneration seen in the polyglutamine repeat diseases.

ACKNOWLEDGEMENTS

We are grateful to Timothy Tucker for technical assistance, Shoji Tsuji, M.D., Ph.D. (Niigata University, Niigata, Japan), for providing the DRPLA cDNA from which the polyglutamine constructs were made, and Allelix Biopharmaceuticals (Mississaugh, Ontario, Canada) for supplying the TR1 cells. We thank Larry Hawkey for assistance with confocal microscopy and William Matthews for assistance with fluorescence microscopy. This work was supported by a Wills Foundation Grant (Y.N.), a Beeson Physician-Faculty Scholar Award from the American Federation for Aging Research (J.R.B.), and NIMH R29 MH51699 (J.C.).

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