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# Intracellular Aggregate Formation of Dentatorubral-Pallidoluysian Atrophy (DRPLA) Protein with the Extended Polyglutamine

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**Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder caused by the abnormal CAG triplet-repeat expansion resulting in an elongated polyglutamine (polyQ) stretch. We have recently showed that the DRPLA protein is cleaved during apoptosis by caspase-3, one of the cysteine protease family members known to be activated during apoptosis. We report here the subcellular localization of the DRPLA protein by fusing the green fluorescent protein as a tag. The full length DRPLA protein is localized predominantly but not exclusively in the nucleus regardless of the length of the polyQ stretch. In contrast, an N-terminal-deleted fragment containing polyQ produced by the proteolytic cleavage with caspase-3 is found both in the nucleus and the cytoplasm. Moreover, the same fragment with the elongated polyQ showed aggregation when overexpressed. Some cells with aggregate formation showed apoptotic phenotype. These findings raise the possibility that the DRPLA protein processed by caspase-3 may lead to aggregation of the protein resulting in the development of neurodegeneration.** 

***Key Words:*** **apoptosis; confocal microscopy; cysteine proteases; Huntington's disease; spinocerebellar ataxias.**

Apoptosis or programmed cell death is essential for the development and maintenance of multicellular organisms. Due to extensive efforts, several protein families have been discovered which are crucial for the regulation or execution of apoptosis. Among them, the importance of cysteine proteases belonging to the caspase family is underscored by the following observations. First, caspases are activated by proteolysis during apoptosis triggered by various stimuli (1,2). Second, inhibitors of the caspases are capable of interfering with most (if not all) types of apoptosis (3). Third, mice deficient in caspase-3, which is believed to play a central role in mediating apoptosis, show lethal defects resulting from the dysregulation of apoptosis (4). Based on these findings, proteolytic cleavage of the key substrates for the caspase family should play essential roles in the execution of apoptosis. Although more than ten substrates have been reported to date, no single substrate has yet been proven to be sufficient for the execution. Therefore, it is more likely that the coordinate degradation of several key proteins is necessary for apoptosis.

We recently reported the dentatorubral-pallidoluysian atrophy (DRPLA) gene product (DRPLA protein) as a novel substrate of caspase-3, which is cleaved at a site near the N-terminus ($^{106}$DSLG$^{110}$) (5). DRPLA is an autosomal dominant neurodegenerative disorder characterized by progressive dementia, myoclonic epilepsy, cerebellar ataxia and choreoathetotic movements. We and others previously identified the expansion of unstable CAG repeats of the DRPLA gene in the patients resulting in the elongation of a polyglutamine (polyQ) stretch (6,7). CAG/polyQ expansion has been found in eight neurodegenerative disorders including Huntington's disease (HD), Machado-Joseph disease (MJD), spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxia (SCA) types 1, 2, 6 and 7. Although the molecular mechanism responsible for the onset of these disorders remains to be identified, it is

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Abbreviations used: DRPLA, dentatorubral-pallidoluysian atrophy; GFP, green fluorescent protein; Huntington's disease, HD; polyQ, polyglutamine; MJD, Machado-Joseph disease; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; NLS, nuclear localization signal.

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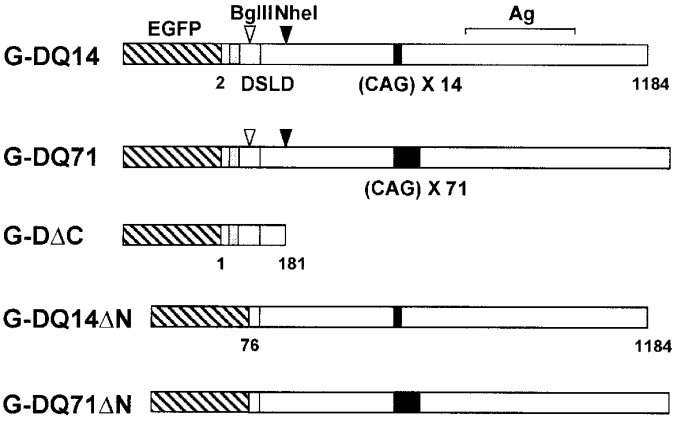


**FIG. 1.** Schematic structures of the expressed fusion proteins. The expressions were driven by the cytomegalovirus early promoter/enhancer. The regions encoding GFP, a putative nuclear localization signal and polyQs are hatched, dotted and filled, respectively. The region used as an antigen to raise specific antiserum is represented as Ag. The numbers below the boxes represent the position of the amino acid counted from the initiation methionine of the DRPLA protein.

likely that a similar gain-of-function mechanism can explain the etiology of all these diseases (8).

Our initial observation of the proteolytic cleavage of the DRPLA protein during apoptosis prompted us to investigate the biological role of this phenomenon. In this paper, using an anti-DRPLA antibody and green fluorescent protein (GFP)-tagged fusion proteins, we have shown that DRPLA protein is distributed predominantly in the nucleus. In contrast, the N-terminal-deleted fragment (DRPLAΔN) produced by caspase-3 is localized both in the nucleus and the cytoplasm. In addition, DRPLAΔN with the elongated polyQ track showed remarkable intracellular aggregation. This finding may shed light on the etiology of neurodegenerative diseases due to polyQ repeat expansion.

## METHODS

*Cells and culture conditions.* HeLa cells and mouse neuronal SN49 cells (9) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C under a humidified atmosphere of 5% $CO_2$.

*Transient transfections.* HeLa cells were seeded at the density of $7 \times 10^5$/100 mm plate on the day before transfection. Cells were transfected with 10 $\mu$g of DNA using 50 $\mu$g of lipofectin (GIBCO BRL, Rockville, MD) and 10 ml OPTI-MEM I (GIBCO BRL). After 6 hrs of transfection, the cells were grown in the usual medium for 48 hrs, collected and then resuspended in the lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X 100, and 1% NP-40). For some experiments, 100 $\mu$M of a caspase-3 inhibitor, Ac-DEVD-CHO (Peptide institute, Inc., Osaka, Japan) was added to the medium after 6 hrs of lipofection.

*Cell staining.* Cells growing on a chamber slide (Becton Dickinson, Franklin Lakes, NJ) were fixed with acetone at −20 °C for 2 min. After rinsing with PBS, the slides were preincubated with a preblock solution [10 mM Tris (pH 7.6), 0.15 M NaCl, 5% skim milk, 2% BSA, and 0.1% Tween 20] for 2 hrs. The slides were then incubated at 4 °C for 16 hrs with either a 1:500 dilution of anti-DRPLA antibody (5), a 1:500 dilution of preimmune serum or a 1:1000 dilution of anti-$\beta$-galactosidase ($\beta$-gal) antibody (10). After three 5 min washes with PBS, a 1:350 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was applied to the specimens. The slides were washed as above and the antibodies detected using an avidin-biotin-complex reagent containing horseradish peroxidase (Vector Laboratories), followed by diaminobenzidine containing 0.01% $H_2O_2$ for color development. Preblock solution was used for all antibody dilutions.

*Plasmid construction.* All GFP-DRPLA fusion proteins were driven by the cytomegalovirus early promoter/enhancer and are depicted in Fig. 1. To generate the following expression constructs, either pEGFP-C1 or C2 (Clontech, Palo Alto, CA) was used.

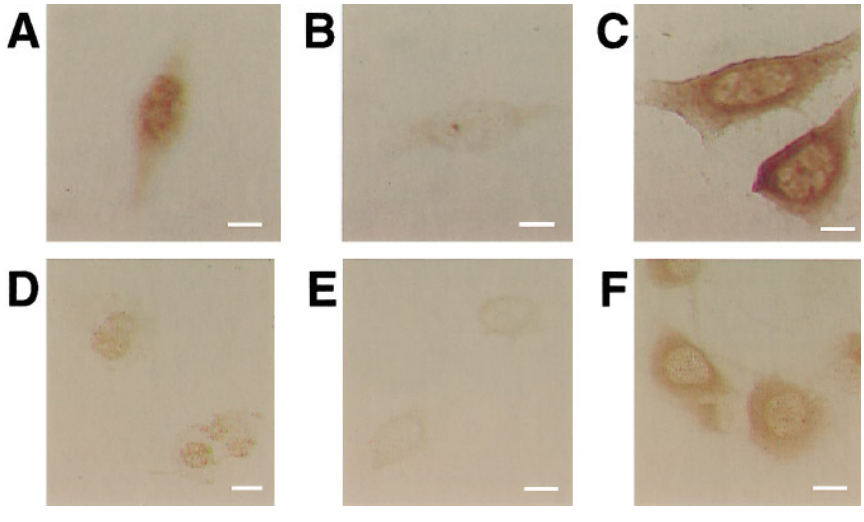


**FIG. 2.** Subcellular localization of the DRPLA protein. HeLa cells (A, B, C) and SN49 cells (D, E, F) were immunostained with anti-DRPLA (A, D), anti-$\beta$-gal (C, F) and preimmune serum (B, E). Bars, 10 $\mu$m.

pGD(Q14) contains a full length DRPLA coding sequence (11) (residues 2 to 1184) including 14 CAG repeats. pGD(Q71) is the same plasmid but with 71 CAG repeats. pGDΔC and pGD(Q14)ΔN contain sequences of DRPLAΔC (residues 1 to 181) and DRPLAΔN (residues 76 to 1184), respectively. pGD(Q71)ΔN has the same deletion as pGD(Q14)ΔN but with 71 CAG repeats. All constructs described above have GFP tags at the N-terminus. The in-frame junctional sequence between GFP and DRPLA was confirmed in each construct.

*Immunoblotting.* Western analysis was performed as described previously (5). In brief, 30 $\mu$g of cell lysate were loaded onto 6% or 12% SDS-PAGE gels. Resolved proteins were electrophoretically transferred to a nitrocellulose membrane. A rabbit anti-GFP (Clontech) antiserum was used as a primary antibody. Horse radish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) was used as a secondary antibody, and proteins were visualized by ECL (Amersham, Buckinghamshire, UK).

*Laser-scanning microscopy.* HeLa cells were transfected as described above. After 6 hrs of lipofection, the trypsinized cells were seeded onto a glass-bottomed culture dish (MatTek corp, Ashland, MA) and grown in DMEM medium for 48 hrs. A fluorescent image was obtained using a confocal laser scanning microscope (Model GB-200, OLYMPUS, Tokyo, Japan) equipped with a triple line Kr/Ar laser with excitation at 488 nm and detection at 500 to 530 nm bandpass. For some experiments, cells were incubated with 5 $\mu$M of Hoechst33342 (Calbiochem, La Jolla, CA) for 10 min prior to confocal microscopy with excitation at 400 nm and detection at 400 to 420 nm bandpass.

## RESULTS

*DRPLA protein is predominantly localized in the nucleus of cultured cells.* Firstly, we analyzed the subcellular localization of the DRPLA protein using a rabbit antiserum raised against the GST-DRPLA fusion protein (5). As shown in Fig.2, the DRPLA protein in both HeLa and SN49 cells was predominantly localized in the nucleus. Cytoplasmic staining of $\beta$-gal and negative nuclear staining using preimmune serum confirmed the specificity of the experiment.

*Transient expression of GFP-DRPLA fusion proteins.* In order to investigate the subcellular localization of cleaved proteins in alive cells, we fused DRPLA protein fragments as well as the full length protein to GFP.

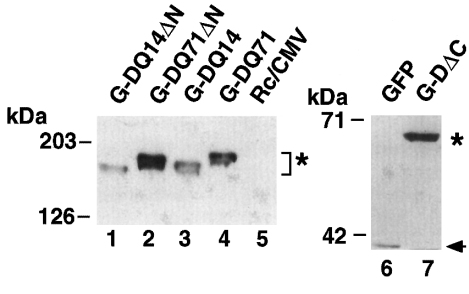


**FIG. 3.** Immunoblot analysis of the expressed fusion proteins. HeLa cells were transfected with the plasmids described at the top and cell lysates were subjected to Western blotting using anti-GFP antibody. Asterisks and an arrow indicate expected GFP-DRPLA fusion proteins and GFP non-fusion protein, respectively.

**TABLE 1**

Subcellular Distribution of GST-DRPLA Fusion Proteins

| Fusion proteins | N > C | N ≈ C | N > C* | N ≈ C* |
|---|---|---|---|---|
| G-DQ14 | 52.1 | 44.2 | 0.9 | 2.8 |
| G-DQ71 | 50.0 | 43.0 | 5.6 | 1.4 |
| G-DQ14ΔN | 4.7 | 92.9 | 0 | 2.4 |
| G-DQ71ΔN | 1.9 | 64.1 | 1.0 | 33.0 |
| G-DΔC | 94.3 | 5.7 | 0 | 0 |

*Note.* Each number indicates the percentage of fluorescent cells falling into each distribution category for each plasmid transfected. At least 200 fluorescent cells were counted for each transfection. Ac-DEVD-CHO, an inhibitor of caspase-3-like proteases, was added to the medium after transfection to avoid cleavage of the fusion proteins which may affect the interpretation of the results. Cells were counted 24 hrs after transfections.

N > C: mainly localized in the nucleus, N ≈ C: localized almost equally in the nucleus and the cytoplasm, N > C*: same as N > C with aggregates, N ≈ C*: same as N ≈ C with aggregates.

We utilized NheI and BglII sites, both of which are situated very close to the cleavage site, to make GFP-DRPLAΔC and GFP-DRPLAΔN, respectively, which simulate the cleaved products. HeLa cells were transiently transfected with these constructs and subjected to immunoblotting. Using the anti-GFP antiserum, we detected a prominent band from each sample corresponding to the expected fusion protein (GFP-DQ14: 175 kDa. GFP-DQ71: 185 kDa, GFP-DΔC: 63 kDa, GFP-DQ14ΔN: 170 kDa, GFP-DQ71ΔN: 180 kDa) (Fig. 3). Except for GFP-DΔC, the sizes of the fusion proteins, all of which contain polyQ, were much larger than the predicted ones based on their amino acid compositions as described previously for non-fusion DRPLA proteins (5,12).

*Subcellular localization of fusion proteins.* To address the question of whether the proteolysis of the DRPLA protein has any effect on its subcellular localization, we transiently expressed GFP-DRPLA fusion proteins in HeLa cells and observed them under the fluorescent microscope (Fig. 4 and Table 1). In 52.1% of fluorescent cells, the full length DRPLA protein (G-DQ14) was localized mainly in nucleus, while in 44.2% it was localized both in the nucleus and the cytoplasm. The distribution pattern was not affected by the length of polyQ. The distribution was diffuse without any small structures regardless of the distribution pattern. In contrast, DRPLAΔN (G-DQ14ΔN) was localized both in the nucleus and the cytoplasm in 92.9% of fluorescent cells. Remarkably, 33% of cells expressing DRPLAΔN (G-DQ71ΔN) containing the elongated polyQ showed aggregation both in the nucleus and the cytoplasm. The number of aggregates per cell ranged from 3 to 13 ( 6.9 ± 3.3; mean ± SD). They were 1 to 9 $\mu$m in diameter, roughly 5 to 45% of that of the nu-

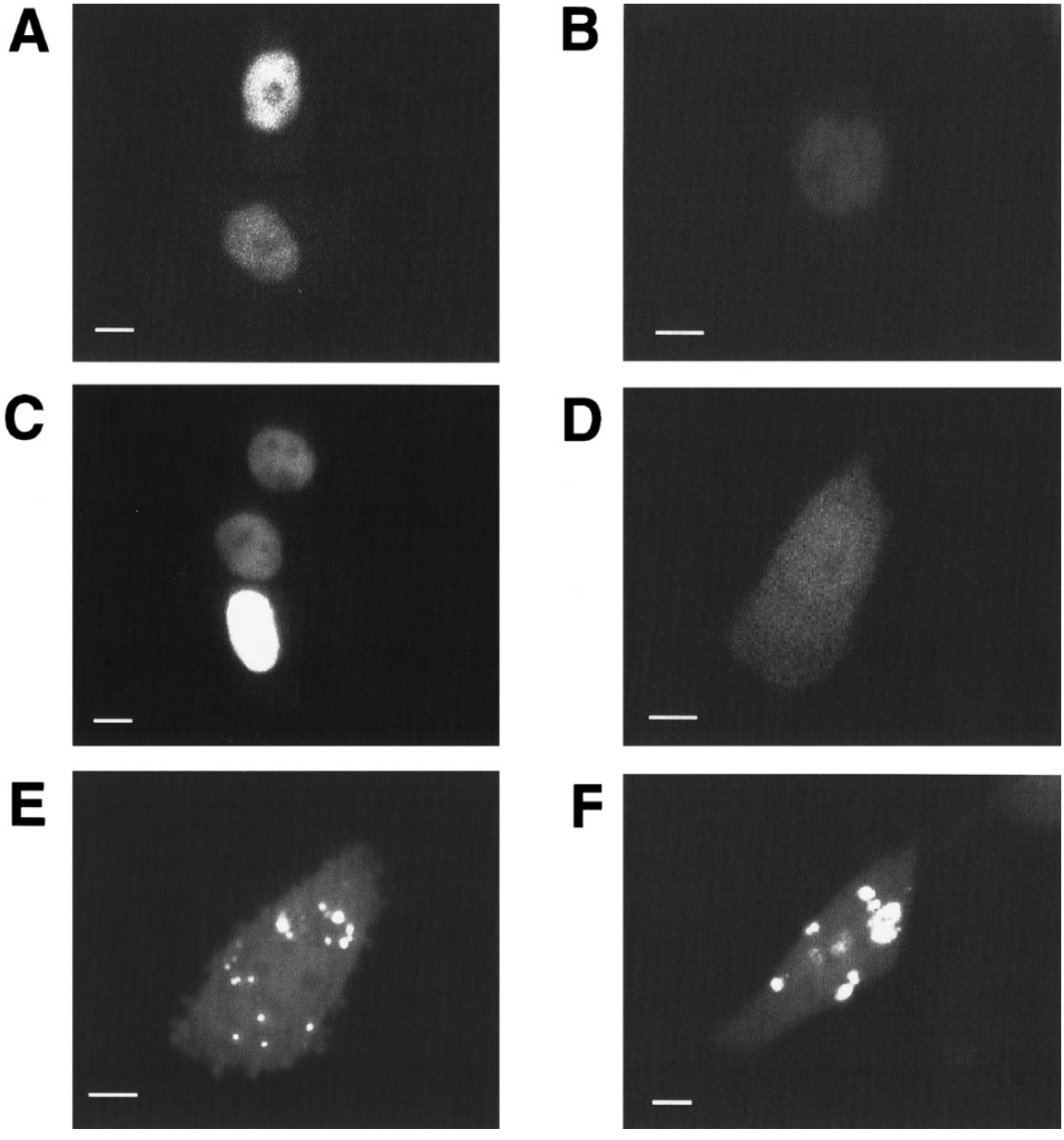


**FIG. 4.** Confocal microscopy of GFP-DRPLA fusion proteins. HeLa cells were transfected with G-DQ14 (A), G-DQ71 (B), G-DΔC (C), G-DQ14ΔN (D), and G-DQ71ΔN (E and F). After 48 hrs of cultivation in DMEM, the cells were subjected to laser-scanning microscopy. Bars, 10 $\mu$m.

cleus. In addition, a considerable number of cells with aggregates were floating and thus were not counted in Table 1, suggesting that the aggregation seemed to be toxic to the cells. Although a small number of cells transfected with G-DQ14 and G-DQ14ΔN showed similar aggregation (3.7%, 2.4%, respectively), both the number and size of the aggregates were smaller than those observed in cells expressing G-DQ71ΔN (2.6 ± 1.1; mean ± SD, 0.6 to 3 $\mu$m, respectively). Of cells expressing DRPLAΔC, 94.3% showed nuclear distribution and none of them had aggregates, although 7.0% of fluorescent cells transfected with G-DQ71 showed aggregation mainly in the nucleus.

*Cells with aggregates show apoptotic phenotype.* To investigate whether intracellular aggregation causes cell death via apoptotic mechanism, nuclei of transfected cells were stained with Hoechst33342 and fluorescent images were overlaid with those of EGFP fusion proteins. As shown in Fig. 5, when transfected with G-DQ71ΔN, condensed or fragmented nuclei were

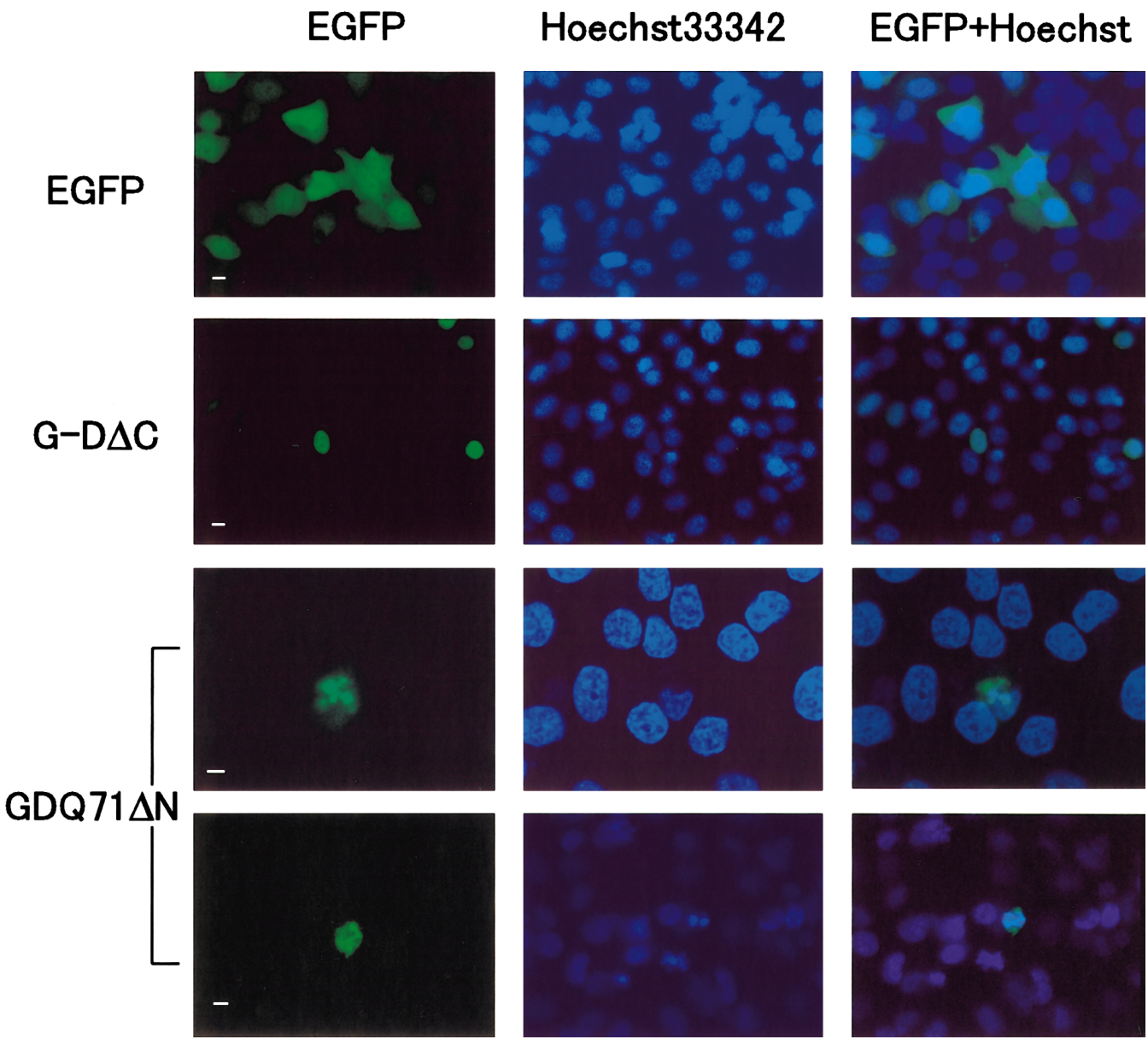


**FIG. 5.** Aggregate formation induces apoptotic phenotype of the nucleus. HeLa cells were transfected with the plasmids described at the left. After 48 hrs of cultivation, the cells were stained with Hoechst33342 and subjected to laser-scanning microscopy. Left panels: fluorescent images of EGFP fusion or non-fusion proteins expression. Middle panels: fluorescent staining of the nuclei. Right panels: overlaid views of the left and the middle panels. Bars, 10 $\mu$m.

observed in cells with aggregates. Very little, if any, number of cells transfected with other constructs showed similar phenotype.

## DISCUSSION

A growing number of neurodegenerative diseases are now known to be caused by the expansion of CAG triplet repeats coding for polyQ. They all involve neuronal cell death within specific regions of the central nervous system. However, the precise mechanism of their pathogenesis is yet to be elucidated. There is accumulating evidence which shows that the apoptotic process is involved in the neuronal cell death occurring not only in triplet repeat disorders but also in other neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis (13,14,15). Among them, several gene products responsible for neurodegenerative disorders have been identified by us and others as substrates for caspase-3-like proteases (5,16,17).

Therefore, it makes sense to assume that the caspase family has some role in the development of the neuronal cell loss found in these diseases.

We previously reported that the DRPLA protein was mainly detected in the cytoplasm using frozen tissue sections. The difference of the intracellular distribution could be explained by following possibilities. 1) DRPLA is phosphorylated in some circumstances (K. Nakamura, personal communication) and our previous antibody may only react with the phosphorylated protein. 2) The DRPLA protein in the post-mortem tissue may undergo rapid proteolysis and such a protein localized in the cytoplasm was detected. 3) We observed a prominent band at 100 kDa using a previous antibody and this protein, either the processed DRPLA protein or cross-reactive material could affect the subcellular distribution in immunostaining. In any case, since its location was recently reported to be cytoplasmic, using FLAG-tagged DRPLA protein (18), in order to resolve the contradictory findings, further study should obviously be needed.

Since the physiological roles of the DRPLA protein are still unknown, the significance of the protein redistribution after cleavage is elusive. As previously described, there is a bipartite nuclear localization signal (NLS) at the N terminus of the DRPLA protein ($^{16}$RK KEAPGPREEL RSRGR$^{32}$) (19). Therefore, the DRPLA protein, once the NLS sequence is cleaved off by caspase-3, should be localized not only in the nucleus but also in the cytoplasm.

Recently, neuronal intranuclear inclusion containing polyQ has been reported to be closely related to most of the disorders associated with polyQ such as HD, MJD, SCA1 and DRPLA (18,20,21,22). Although in our experimental system the DRPLA protein with a long polyQ aggregated mainly in the cytoplasm, it is possible that the aggregation may eventually lead to the formation of intranuclear inclusion in long-lived neurons. In this case, the mechanism of nuclear transport of the aggregates should apparently be elucidated. Two groups have reported that the DRPLA protein tends to form aggregates using a relatively short peptide or various deletion mutants with expanded polyQ fused to GFP (18,23). Here we confirmed their results and also demonstrated that a much longer protein also has the potential to form aggregates when it contains the extended polyQ. Moreover, we firstly demonstrated the possible link between proteolysis by a member of caspase and the aggregation of polyQ in the DRPLA protein. In this regard, it has recently been reported that proteolytic cleavage of mutant huntingtin, the product of the HD gene, also leads to the development of aggregates (24). Thus far, eight disorders have been reported to be caused by the elongated polyQ. Interestingly, other than HD and DRPLA, four out of six proteins encoded by the genes responsible for SBMA, SCA-6, SCA-2 and MJD (androgen receptor, $\alpha_{1A}$ voltage-dependent $Ca^{2+}$ channel, ataxin-2 and MJD-1, respectively) have one to three putative caspase-3 cleavage sites (25-29), DXXDs (30). Therefore, cleavage by caspase-3 like proteases possibly has some roles in the formation of aggregates or inclusions not only in DRPLA but also in other diseases.

Although a long-term challenge will be needed, further understanding of the role of caspase in these disorders could lead to therapeutic intervention where disruption of the caspase cascade is a fascinating target.

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