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Adenovirus-mediated expression of mutant DRPLA proteins with expanded polyglutamine stretches in neuronally differentiated PC12 cells. Preferential intranuclear aggregate formation and apoptosis

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To investigate the molecular mechanisms of neurodegeneration caused by expanded CAG repeats in dentatorubral-pallidoluysian atrophy (DRPLA), an autosomal dominant neurodegenerative disorder caused by unstable expansion of a CAG trinucleotide repeat in the *DRPLA* gene on 12p13.31, we established an efficient expression system for truncated and full-length DRPLA proteins with normal or expanded polyglutamine stretches in neuronally differentiated PC12 cells and fibroblasts using an adenovirus expression system. Although aggregate body formation was observed both in neuronally differentiated PC12 cells and in fibroblasts expressing truncated DRPLA proteins with Q82, >97% (*n* = 3) of neuronally differentiated PC12 cells showed intranuclear inclusions, while only 31 ± 21% (*n* = 3) of fibroblasts had intranuclear inclusions at 3 days after infection. The percentage of apoptotic cells was significantly higher in neuronally differentiated PC12 cells expressing the truncated DRPLA protein with Q82 than in fibroblasts, suggesting the possibility that intranuclear aggregate bodies are formed preferentially in neuronally differentiated PC12 cells and that these cells are more vulnerable than fibroblasts to the toxic effects of expanded polyglutamine stretches in the DRPLA protein. When the full-length DRPLA protein with Q82 was expressed, aggregate bodies were found exclusively in the nuclei of the neuronally differentiated PC12 cells, while they were found in the cytoplasm of fibroblasts. Despite the presence of aggregate bodies, apoptosis was not induced by expression of the full-length DRPLA protein with Q82 in either neuronally differentiated PC12 cells or fibroblasts, suggesting that the presence of intranuclear aggregate bodies is in itself not necessarily toxic to cells.

INTRODUCTION

Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder caused by unstable expansion of a CAG trinucleotide repeat in exon 5 of the *DRPLA* gene on chromosome 12p13.31, which codes for a polyglutamine stretch (1–3). In addition to DRPLA, seven neurodegenerative diseases, spinal and bulbar muscular atrophy (SBMA) (4), Huntington's disease (HD) (5), spinocerebellar ataxia type 1 (SCA1) (6), SCA2 (7–9), Machado–Joseph disease (MJD) (10), SCA6 (11) and SCA7 (12), have also been found to be caused by expansion of CAG trinucleotide repeats coding for polyglutamine stretches.

Evidence suggesting that the polyglutamine stretches are cytotoxic to neuronal cells by a mechanism of 'gain of function' is increasing: (i) the mode of inheritance in all of the aforementioned diseases, except for SBMA, is autosomal dominant; (ii) the gene products of the mutant genes have been shown to be expressed at levels comparable with those of wild-type genes (13–17); (iii) CAG trinucleotide repeats are the only common domain shared among the genes for these diseases; and (iv) expanded polyglutamine stretches have been demonstrated to exhibit toxicity in cultured cells and transgenic mice (18–20).

Ikeda *et al*. (18) reported the induction of apoptosis in COS-7 cells expressing a truncated MJD1a protein containing expanded polyglutamine stretches, although expression of the full-length MJD1a protein with expanded polyglutamine stretches did not reveal such toxicity. We have constructed expression vectors carrying full-length mutant and wild-type *DRPLA* cDNAs as well as those with various lengths of deletions, and demonstrated that the expression of a truncated mutant DRPLA protein with expanded polyglutamine stretches in COS-7 cells results in intra- and perinuclear aggregate formation and apoptotic cell death. Similar cytotoxicity has also been demonstrated for fragments of mutant huntingtin (20–22), and MJD1a protein (23). All these data indicate that truncated proteins containing expanded polyglutamine stretches have a stronger potential to induce aggregate forma-

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tion and more potent cytotoxicity compared with full-length proteins.

Recently, neuronal intranuclear inclusions (NIIs) have been demonstrated in mice transgenic for exon 1 of the human *HD* gene harboring expanded CAG repeats (24). Subsequently, similar NIIs were demonstrated in neurons in the autopsied tissues of patients with HD (24), MJD (23), DRPLA (19,25), SCA7 (26), SCA1 (27) and SBMA (28), suggesting the possibility that NIIs play a key role in the mechanisms of neurodegeneration in CAG repeat diseases.

Expression studies of DRPLA, however, have so far been conducted using non-neuronal cell lines such as COS cells. One of the characteristic features of CAG repeat diseases is selective neuronal degeneration despite the ubiquitous expression of the genes (13,16,29,30), suggesting the possibility that neuronal cells are more vulnerable than non-neuronal cells to the toxic effects of expanded polyglutamine stretches. To investigate this possibility, expression systems which allow high expression levels in neuronal cells and comparison of aggregate formation and toxicity in these cells with those in non-neuronal cells are required.

With this background, we constructed adenovirus vectors harboring truncated and full-length *DRPLA* cDNAs with normal or expanded CAG repeats using the COS-TPC (cosmid cassettes and adenovirus DNA terminal protein complex) method introduced by Miyake *et al*. (31), which increased the efficiency of obtaining recombinant adenovirus clones by ~100-fold. Using this expression system, we analyzed the expression patterns of DRPLA proteins, either with wild-type or with expanded polyglutamine stretches and their cytotoxicities in neuronally differentiated PC12 cells, and compared the results with those in cultured fibroblasts.

RESULTS

Generation of adenovirus vectors

Adenovirus vectors expressing truncated or full-length DRPLA proteins with Q19 or Q82 were constructed by the COS-TPC method. To facilitate analysis of the expressed proteins, the FLAG tag epitope was inserted at their N-termini (Fig. 1). During the process of generation of adenovirus vectors containing a truncated cDNA with expanded CAG repeats (78 repeat units), the number of repeats was reduced to 30, 49 or 59 in three among the 10 adenovirus clones analyzed, while $(CAG)_{15}$ repeats were stable in all of the 10 clones analyzed. The number of repeats in the adenovirus vectors containing the full-length cDNAs was preserved in each of the 10 clones with $(CAG)_{15}$ or $(CAG)_{78}$.

Virus clones with preserved numbers of repeats (15 and 78 repeat units) were amplified. The purified adenovirus clones containing truncated cDNA with $(CAG)_{15}$ or $(CAG)_{78}$, which, together with their flanking sequences, coded for 19 and 82 glutamine residues, respectively, were named Ax1CAAQ19 and Ax1CAAQ82, respectively. The adenovirus clones containing the full-length cDNAs with $(CAG)_{15}$ and $(CAG)_{78}$ were named Ax1CAAFD19 and Ax1CAAFD82, respectively (Fig. 1). The titers of Ax1CAAQ19, Ax1CAAQ82, Ax1CAAFD19 and Ax1CAAFD82 were 4×10^{10}, 1×10^{10}, 1×10^{9} and 4×10^{8} p.f.u./ml, respectively.

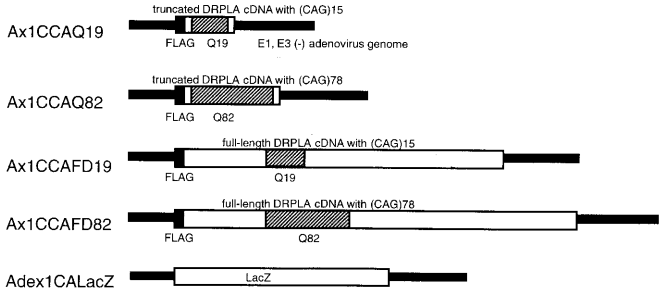

Figure 1. Construction of the adenovirus vectors. The full-length and truncated *DRPLA* cDNAs coding for Q19 or Q82 along with the FLAG tag at the N-terminus were inserted into the adenovirus vectors. The open boxes correspond to truncated (Ax1CCAQ19 and Ax1CCAQ82) or full-length (Ax1CCAFD19 and Ax1CCAFD82) *DRPLA* cDNA. The hatched boxes represent the CAG repeats. The adenovirus genome is devoid of E1 and E3 genes and contains the CAG promoter (cytomegarovirus enhancer and β-actin promoter) and the rabbit β-globin polyadenylation signal.

Optimal multiplicity of infection and culture time for neuronally differentiated PC12 cells and fibroblasts

To determine the optimal multiplicity of infection (m.o.i.) and culture time, preliminary experiments were performed using Adex1CALacZ harboring the β-galactosidase gene. Neuronally differentiated PC12 cells and fibroblasts (1×10^4 cells) were infected with 100 µl of Adex1CALacZ virus solution containing 1×10^5, 5×10^5, 1×10^6 or 5×10^6 p.f.u. (m.o.i. = 10, 50, 100 and 500, respectively), and cultured for 1, 2, 3, 6 or 9 days. In neuronally differentiated PC12 cells, 100% of the cells expressed β-galactosidase activity at 3 days after infection with Adex1CALacZ at an m.o.i. of 100 or 500. In cultured human fibroblasts, expression of β-galactosidase activity was observed in 60% of the cells at 3 days after infection and in 100% of the cells at 6 or 9 days after infection at an m.o.i. of 100. The percentage of β-galactosidase positivity in fibroblasts infected at an m.o.i. of >100 was essentially the same. Based on these results, an m.o.i. of 100 was used for infecting neuronally differentiated PC12 cells and fibroblasts. The number of fibroblasts infected with Adex1CALacZ at m.o.i. 100 increased to 1.6, 1.7 and 2.1 times at 3, 6 and 9 days after infection, respectively, while in the case of the neuronally differentiated PC12 cells, the number of cells did not increase throughout the period.

Preferential intranuclear aggregate formation in neuronally differentiated PC12 cells expressing truncated mutant DRPLA protein with expanded polyglutamine stretches

In neuronally differentiated PC12 cells infected with Ax1CAAQ19, truncated DRPLA protein containing 19 glutamine residues (Q19) was observed homogeneously throughout the cells not only in the cytoplasm but also in the nucleus, but no aggregate bodies were observed. In contrast, varying numbers (2–39) of intranuclear aggregate bodies were observed in most of the neuronally differentiated PC12 cells infected with the Ax1CAAQ82 coding for the truncated DRPLA protein with 82 glutamine residues (Q82), while aggregate bodies were scarcely observed in the cytoplasm

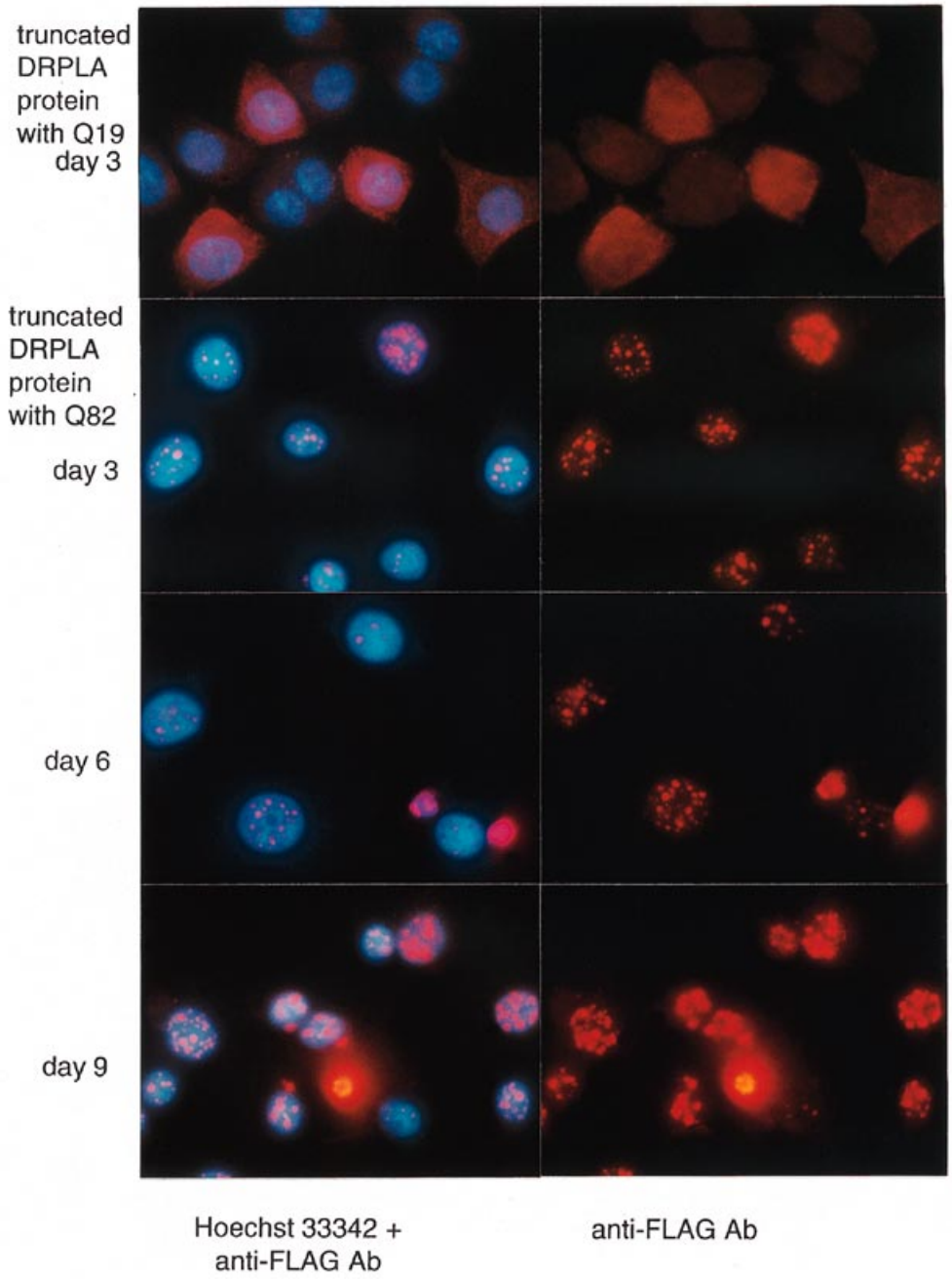

Figure 2. Expression of truncated DRPLA proteins in neuronally differentiated PC12 cells. Neuronally differentiated PC12 cells expressing truncated wild-type DRPLA protein (Q19) exhibited diffuse staining in both the cytoplasm and the nucleus with anti-FLAG M5 monoclonal antibody at 3 days after infection. In cells expressing mutant truncated DRPLA protein (Q82), multiple intranuclear inclusions were observed at 3, 6 and 9 days after infection.

(Fig. 2). The percentages of cells containing the intranuclear aggregate bodies in the cells expressing the FLAG epitope were 98 ± 0.6, 99 ± 1.0 and 100 ± 0.6% at 3, 6 and 9 days after infection with Ax1CAAQ82, respectively (n = 3) (Fig. 3). The mean size of the intranuclear inclusion bodies increased to a size comparable with that of the nucleolus during this culture period, and the intranuclear inclusions were strongly stained with the anti-FLAG M5 monoclonal antibody at 6 and 9 days after infection.

The percentages of TUNEL-positive PC12 cells in the FLAG-positive cells were 4.7 ± 3.1, 39 ± 16 and 64 ± 10% at 3, 6 and 9 days after infection with Ax1CAAQ82, respectively, while they were 5.7 ± 6.4, 14 ± 8.1 and 20 ± 8.6%, at 3, 6 and 9 days, respectively, after infection with Ax1CAAQ19 (n = 3), and 8.0 ± 5.0, 13 ± 3.5 and 13 ± 5.3% at 3, 6 and 9 days, respectively, after infection with Adex1CALacZ (n = 3) (Fig. 4).

In contrast to the PC12 cells, in the fibroblasts infected with Ax1CAAQ82 coding for the truncated DRPLA protein with

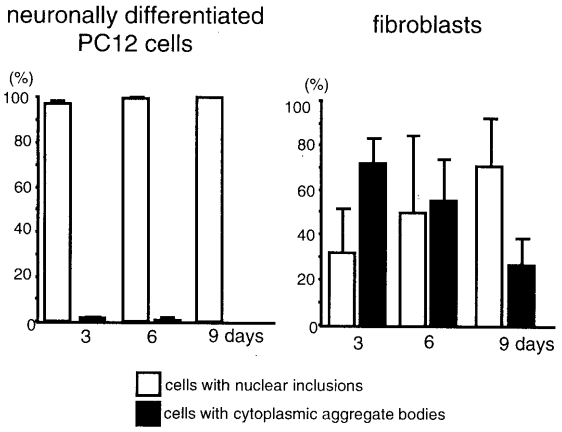

Figure 3. Percentages of cells with the intranuclear aggregate bodies in cells expressing the FLAG epitope and those of cells with the cytoplasmic aggregate bodies.

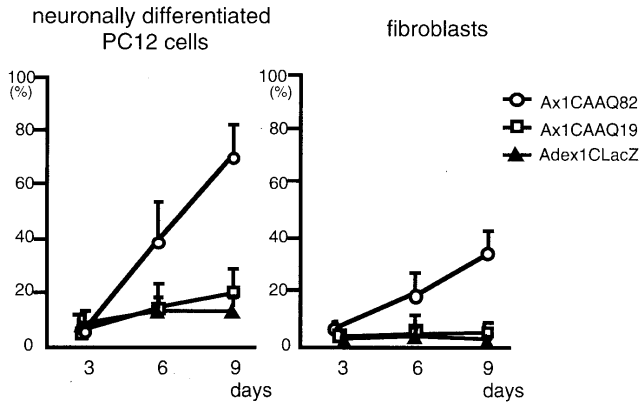

Figure 4. Percentages of TUNEL-positive cells in cells expressing the FLAG epitope.

Q82, irregularly shaped aggregate bodies of variable sizes were observed mainly in the cytoplasm or adjacent to the nuclei at 3 days after infection (Fig. 5). Intranuclear aggregate bodies were also observed in 31 ± 21% of the FLAG-positive cells at 3 days after infection, with the frequencies increasing to 52 ± 34 and 71 ± 23% at 6 and 9 days after infection, respectively (n = 3) (Fig. 3). In the fibroblasts infected with Ax1CAAQ19 coding for the truncated DRPLA protein with Q19, homogenous staining was observed in the cytoplasm, but not in the nucleus, throughout the culture period of 9 days (Fig. 5).

The percentages of TUNEL-positive fibroblasts in the FLAG-positive cells were 5.0 ± 1.7, 18 ± 6.2 and 37 ± 6.1% at 3, 6 and 9 days after infection with Ax1CAAQ82, respectively (n = 3), while they were 2.3 ± 3.0, 3.7 ± 5.2 and 4.7 ± 4.0% at 3, 6 and 9 days after infection, respectively, in those infected with Ax1CAAQ19 (n = 3), and 2.3 ± 3.0, 3.7 ± 5.2 and 4.7 ± 4.0% at 3, 6 and 9 days after infection, respectively, in those infected with Adex1CALacZ (Fig. 4).

Intranuclear aggregate bodies were formed in neuronally differentiated PC12 cells expressing full-length mutant DRPLA protein with expanded polyglutamine stretches

The nuclei of all of the neuronally differentiated PC12 cells infected with Ax1CAAFD19 coding for the full-length DRPLA protein with Q19 were stained strongly with the anti-FLAG M5 monoclonal antibody, while staining of the cytoplasm was much weaker compared with that in the nucleus, and no aggregate bodies were observed at any time during the culture period of 9 days after infection. Neuronally differentiated PC12 cells infected with Ax1CAAFD82 coding for full-length DRPLA protein with Q82, however, were stained diffusely in the nuclei, and less strongly in the cytoplasm. It is of particular interest that 1–3 spherical intranuclear inclusions were observed in 73 ± 31% (n = 3) of FLAG-positive cells at 3 days after infection. The mean size of the inclusions was larger than that in cells expressing the truncated DRPLA protein with Q82. While the percentages of cells with intranuclear inclusions increased to 92 ± 7.2 and 94 ± 5.9%, at 6 and 9 days after infection, respectively, the number of inclusions per cell was <5, and most of the cells carried only 1–2 inclusions (Fig. 6).

The percentages of TUNEL-positive cells in neuronally differentiated PC12 cells expressing full-length mutant DRPLA protein with Q82 and those expressing full-length wild-type DRPLA protein with Q19 were not significantly increased compared with those in cells expressing β-galactosidase.

While in the fibroblasts infected with Ax1CAAFD19 coding for the full-length wild-type DRPLA protein with Q19, the nuclei of the majority (90 ± 4.3%, n = 3) were stained strongly with the anti-FLAG M5 monoclonal antibody at 3 days after infection, in ~10% of the cells only the cytoplasm was stained weakly with the antibody without staining of the nucleus. In the fibroblasts infected with Ax1CAAFD82 coding for the full-length mutant DRPLA protein with Q82, multiple aggregate bodies were observed in the cytoplasm, associated with diffuse staining of the cytoplasm in ~80% of FLAG-positive cells or diffuse staining of the nuclei in a minor proportion of the cells. In contrast to the intranuclear inclusions observed in the neuronally differentiated PC12 cells expressing full-length mutant protein with Q82, the fibroblasts exhibited aggregate bodies in the cytoplasm throughout the culture period of 9 days (Fig. 6).

The percentages of TUNEL-positive fibroblasts in the cells expressing full-length mutant DRPLA protein with Q82 or those expressing full-length wild-type DRPLA protein with Q19 were not significantly increased compared with those in fibroblasts expressing β-galactosidase.

Western blotting analysis of mutant DRPLA protein

Since expression levels of the mutant DRPLA proteins are expected to affect the kinetics of aggregate formation, the expression levels of truncated and full-length mutant DRPLA proteins were analyzed by western blot analysis (Fig. 7). Western blot analysis of proteins from neuronally differentiated PC12 cells or fibroblasts infected with Ax1CAAQ82 coding for truncated mutant DRPLA protein with Q82 revealed only bands at the top of the stacking gel accompanied by smearing, and bands corresponding to the expected size of

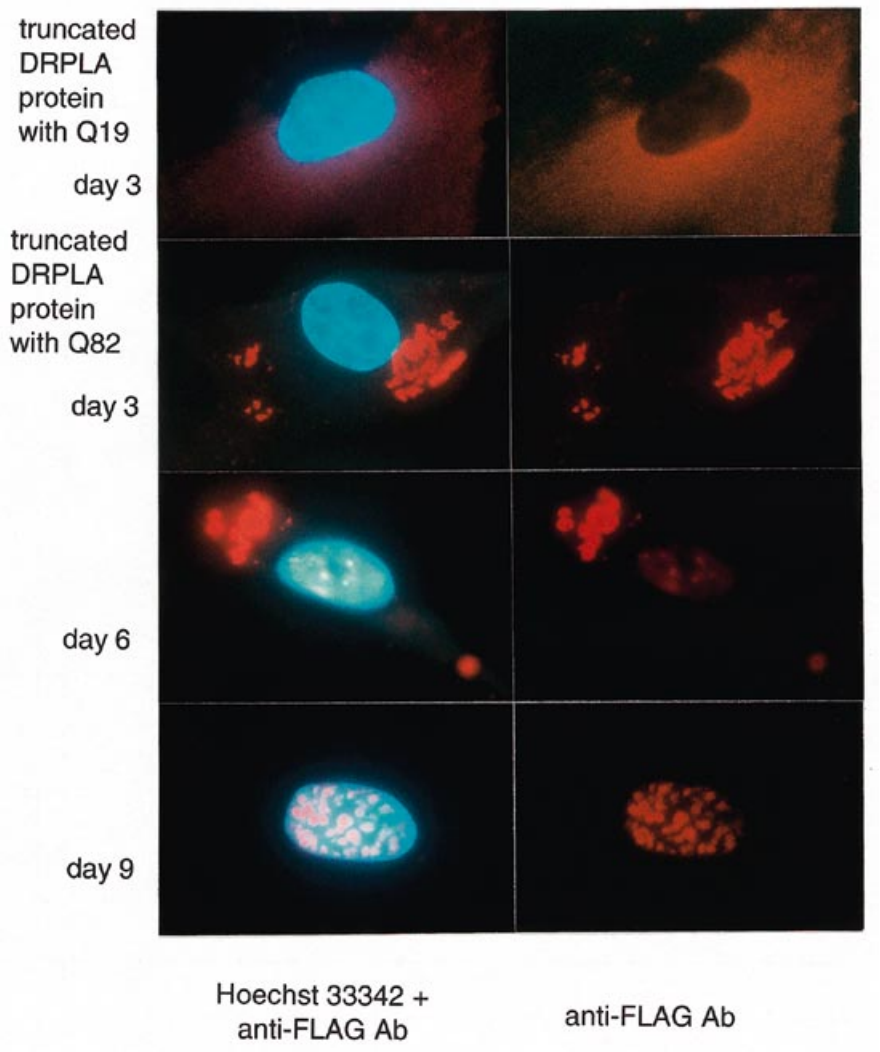

Figure 5. Expression of truncated DRPLA protein in fibroblasts. The cytoplasm of the fibroblasts expressing the truncated DRPLA protein with Q19 was diffusely stained. In contrast, in the cytoplasm of fibroblasts expressing the truncated DRPLA protein with Q82, irregular aggregate bodies were noted. These aggregate bodies tended to be localized in the cytoplasm at 3 and 6 days, but were later localized in the nucleus at 6 and 9 days after infection.

11.7 kDa were not detected, suggesting that the truncated mutant DRPLA protein (Q82) formed aggregates, restricting its progress even through the stacking gel. Analysis of proteins from the neuronally differentiated PC12 cells or the fibroblasts infected with Ax1CAAFD82 coding for full-length mutant DRPLA protein with Q82 revealed a band at 195 kDa, consistent with the mobility of the full-length mutant DRPLA protein (13). In addition, bands at the top of the stacking gel were also observed, similar to those for the truncated mutant DRPLA protein.

DISCUSSION

In this study, we successfully developed adenovirus vectors which allow the expression of truncated and full-length DRPLA proteins containing normal or expanded polyglutamine stretches in neuronally differentiated PC12 cells. During the process of constructing these adenovirus vectors, instability of the expanded CAG repeats of the truncated *DRPLA* cDNA was observed in three of the 10 adenovirus clones, while no such instability was observed in adenovirus vectors containing CAG repeats of normal size. Thus, expanded CAG repeats may lead to instability in the replication of the adenovirus genome.

Using the adenovirus vectors, we were able to express truncated and full-length wild-type or mutant DRPLA proteins in neuronally differentiated PC12 cells and fibroblasts. Formation of aggregate bodies was observed in the neuronally differentiated PC12 cells as well as in fibroblasts expressing the truncated DRPLA protein with Q82, which is consistent with our previous observations using transient expression of the truncated mutant DRPLA protein in COS-7 cells (19). Of particular interest is the higher frequency of intranuclear inclusions in neuronally differentiated PC12 cells and, furthermore, the much higher percentage of TUNEL-positive cells in neuronally differentiated PC12 cells compared with fibroblasts (Figs 3 and 4, Table 1). These results raise the possibilities that neuronally differentiated PC12 cells have the property of promoting intranuclear aggregation of proteins with expanded polyglutamine stretches, and that they are more vulnerable

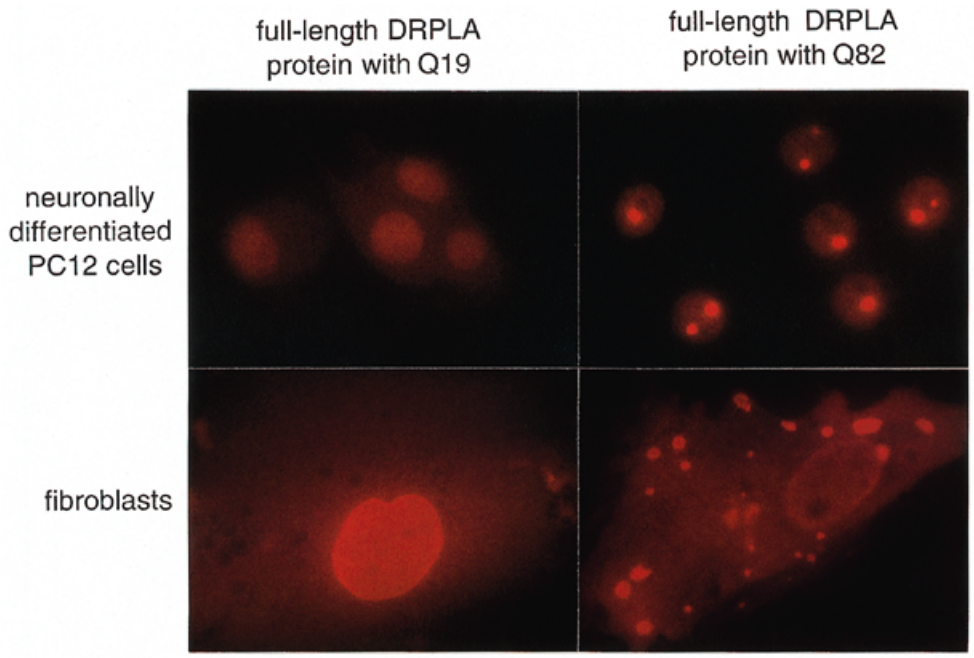

Figure 6. Expression of full-length DRPLA proteins in neuronally differentiated PC12 cells and fibroblasts. Neuronally differentiated PC12 cells expressing the full-length DRPLA protein with Q19 revealed diffuse staining of the nucleus and less intense staining of the cytoplasm. Neuronally differentiated PC12 cells expressing the full-length DRPLA protein with Q82 showed 1–4 intranuclear inclusions. In fibroblasts expressing the full-length DRPLA protein with Q19, diffuse staining of the nucleus was observed in the majority of cells. Fibroblasts expressing full-length DRPLA protein with Q82 showed only cytoplasmic aggregate bodies and no intranuclear inclusions.

than fibroblasts to the toxic effects of the truncated mutant DRPLA protein with Q82. Since formation of aggregate bodies is assumed to be a concentration-dependent process, the following parameters would affect the kinetics of aggregate formation: (i) differences in infection rate between the neuronally differentiated PC12 cells and fibroblasts; (ii) differences in cell division rate; (iii) differences in the expression level or processing of the proteins; and (iv) differences in the subcellular distribution of the proteins. In the present study, there are differences in the aforementioned parameters between the PC12 cells and the fibroblasts, in particular in the cell division rate and distribution of the expressed proteins. Nevertheless, the preferential localization of the truncated wild-type DRPLA protein (Q19) and full-length wild-type protein within the nucleus of the PC12 cells compared with the fibroblasts is interesting. The difference in the subcellular distribution of the truncated as well as the full-length DRPLA proteins between the neuronally differentiated PC12 cells and fibroblasts may in part account for the selective neuronal involvement in DRPLA despite the ubiquitous expression of the causative proteins (13,16,29,30). To investigate further the mechanisms of selective neuronal degeneration, detailed studies concerning the above-mentioned parameters using various types of neuronal cell lines will be required.

Interestingly, 1–2 intranuclear aggregates were also observed in 73 ± 31% (n = 3) of PC12 cells expressing full-length DRPLA proteins with Q82 at 3 days after infection, while apoptotic cell death did not seem to be induced at any point during the culture period of 9 days after infection. The smaller number of intranuclear aggregate bodies in cells infected with Ax1CAAFD82 coding for full-length mutant DRPLA protein with Q82 than in those in cells infected with Ax1CAAQ82 coding for truncated

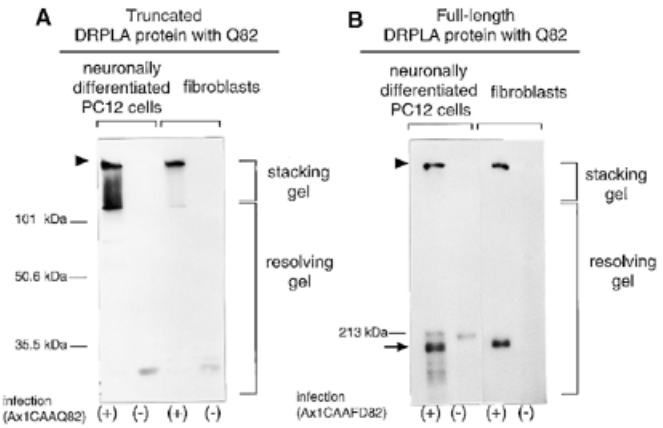

Figure 7. Western blot analysis of DRPLA proteins in neuronally differentiated PC12 cells and fibroblasts. (**A**) Neuronally differentiated PC12 cells or fibroblasts infected with Ax1CAAQ82 coding for truncated mutant DRPLA protein with Q82 were harvested at 3 days after infection and subjected to western blot analysis using an anti-FLAG M5 monoclonal antibody. Immunoreactive bands were detected (arrowhead) at the top of the stacking gel (6% SDS–polyacrylamide gel) accompanied by a smearing pattern, while bands corresponding to the expected size (11.7 kDa) were not detected. (**B**) Neuronally differentiated PC12 cells or fibroblasts infected with Ax1CAAFD82 coding for the full-length mutant DRPLA protein with Q82 were harvested at 3 days after infection and subjected to western blot analysis using an anti-FLAG M5 monoclonal antibody. An immunoreactive band corresponding to a size of 195 kDa (arrow), consistent with the mobility of full-length mutant DRPLA protein (13), was detected in both the PC12 cells and fibroblasts. In addition, bands at the top of the stacking gel (arrowhead) were also observed, similar to those for the truncated mutant DRPLA protein (A).

DRPLAP with Q82 may reflect the different kinetics of aggregate formation of full-length and truncated DRPLA proteins. In the present study, we could not determine whether the aggregates

Table 1. Expression patterns, aggregate body formation and apoptosis in neuronal PC12 cells and fibroblasts expressing truncated or full-length DRPLA proteins

	Truncated DRPLA protein			Full-length DRPLA protein		
	Expression pattern	Aggregates	TUNEL reaction	Expression pattern	Aggregates	TUNEL reaction
Neuronally differentiated PC12 cells						
Q19	Nuclear and cytoplasmic	–	–	Nuclear	–	–
Q82	Nuclear	+ many/nucleus (nuclear)	++	Nuclear	+ 1–2/nucleus (nuclear)	–
Fibroblasts						
Q19	Cytoplasmic	–	–	Predominantly nuclear	–	–
Q82	Cytoplasmic and nuclear	+ (cytoplasmic–nuclear)	+ to ++	Cytoplasmic and nuclear	+ (cytoplasmic)	–

contain the intact full-length DRPLA protein as the major component or processed DRPLA protein fragments, due to unavailability of antibodies recognizing the various epitopes on the DRPLA protein, including the C-terminus. Nonetheless, the results indicate that the presence of intranuclear aggregate bodies in cells expressing full-length DRPLA protein is in itself not sufficient for expression of toxic effects on the cells. These findings are in accordance with recent reports demonstrating that aggregate formation is not a prerequisite for neurodegeneration of Purkinje cells in mice transgenic for SCA1 (32), and that the intranuclear inclusions may reflect a cellular mechanism to protect cultured striatal neurons expressing mutant huntingtin (33).

In terms of the size and number of the aggregate bodies, intranuclear aggregate bodies observed in cells expressing full-length mutant DRPLA protein seem to share more similarities with the NIIs observed in the autopsied brains of DRPLA patients compared with those observed in cells expressing the truncated mutant DRPLA protein (19,25). The results suggest the possibility that the expression of the full-length DRPLA protein with expanded polyglutamine stretches replicates more closely the events occurring in the brain of DRPLA patients. Thus, the expression system using adenovirus vectors containing full-length mutant *DRPLA* cDNA seems to offer a better model system for investigating the mechanisms of aggregate formation and neurodegeneration, compared with systems based on the transient expression of truncated cDNAs.

Another interesting finding in the present study is the localization of DRPLA proteins in PC12 cells (Table 1). The observation that truncated DRPLA protein with Q19 was expressed diffusely in the cytoplasm and the nucleus of PC12 cells suggests that the truncated DRPLA protein presumably gets into the nucleus by a diffusion mechanism, since the expected molecular weight of the truncated DRPLA protein (4 kDa) is small enough to allow it to cross the nuclear pores (34). In contrast to the truncated DRPLA protein with Q19, the truncated DRPLA protein with Q82 (11.7 kDa) was localized as aggregate bodies exclusively in the nucleus of PC12 cells. In fibroblasts, truncated DRPLA protein with Q19 was localized in the cytoplasm, while that with Q82 was localized both in the cytoplasm and the nucleus at 3 days after infection, and almost exclusively in the nucleus at 9 days after infection. Preferential localization of the truncated DRPLA with Q82 but not of that with Q19 in the nucleus seems to be a phenomenon dependent on the length of the polyglutamine stretches. Since there are no nuclear localization signals (NLSs) in the truncated DRPLA proteins, the existence of nuclear transport systems that recognize expanded polyglutamine stretches would be expected. Alternatively, the difference in the speed of aggregate formation between the nucleus and the cytoplasm might account for the preferential intranuclear aggregate formation. Preferential localization of the aggregate bodies in the nuclei of neuronally differentiated PC12 cells compared with fibroblasts suggests that the nuclear transport systems or intranuclear aggregate formation is more active in neuronally differentiated PC12 cells than in fibroblasts. Although the mechanisms underlying the preferential localization of aggregate bodies in the nuclei of neuronally differentiated PC12 cells remain to be elucidated, it could be speculated that proteins with NLSs which bind to expanded polyglutamine stretches might be involved in the nuclear transport of proteins with expanded polyglutamine stretches.

In fibroblasts expressing the truncated DRPLA protein with Q82, aggregate bodies were localized both in the cytoplasm and the nucleus at 3 days after infection, and almost exclusively in the nucleus at 9 days after infection. Although the mechanism of disappearance of the cytoplasmic aggregate bodies during the culture period would be interesting to study, the present study does not provide an unequivocal answer to whether the aggregate bodies are transferred to the nucleus from the cytoplasm, or the cytoplasmic aggregate bodies disappear as a result of cell death or of processing of the aggregates in the cytoplasm.

In contrast to the diffuse localization of the truncated DRPLA protein with Q19 in the cytoplasm and nucleus of the PC12 cells, the full-length wild-type DRPLA protein with Q19 was localized more abundantly in the nucleus of neuronally differentiated PC12 cells than in their cytoplasm. Since the expected molecular weight of the full-length DRPLA protein is 125 kDa, it cannot be expected to travel through the nuclear pores by simple diffusion. The nuclear localization could be ascribed to the putative NLSs located in the N-terminus of the DRPLA protein [amino acids 16–32 (35) and 87–93]. When full-length DRPLA protein with Q82 was expressed in these cells, the formation of aggregate bodies was observed in the

nuclei of the PC12 cells, while in fibroblasts the aggregate bodies were localized in the cytoplasm. These results also suggest that the nuclear transport systems for expanded polyglutamine stretches are more active in PC12 cells than in fibroblasts.

The expression system described in the present study is an excellent expression system which allows high expression levels of proteins with expanded polyglutamine stretches in cultured neuronal cells, and the formation of intranuclear inclusions of not only truncated mutant DRPLA protein but also of full-length mutant DRPLA protein. Our results indicate that neuronally differentiated PC12 cells have the property of preferentially forming intranuclear aggregate bodies consisting of proteins with expanded polyglutamine stretches, and the high vulnerability of neuronal cells to the toxic effects of the expanded polyglutamine stretches. The results also suggest the possibility that aggregate formation by itself may not necessarily lead to toxicity as expected. The expression system described in the present study is expected to be extremely useful for elucidation of the molecular mechanisms of neurodegeneration induced by expanded polyglutamine stretches and the formation of NIIs. Long-term expression of full-length mutant DRPLA proteins in cultures or *in vivo* using the adenovirus vectors described herein will be of particular interest for investigating the mechanisms underlying neuronal degeneration.

MATERIALS AND METHODS

Construction of adenovirus vectors

The DNA segments coding for a FLAG tag consisting of eight amino acids, seven amino acids upstream of the polyglutamine stretch, the polyglutamine stretches (19 or 82 glutamine residues) and seven amino acids downstream of the polyglutamine stretch were obtained by PCR using full-length *DRPLA* cDNAs (CAG = 15 of 78) as templates and the primer pair 5'-GGA TAT CAT GGA CTA CAA AGA CGA TGA CGA CAA GTC AAC ACA TCA CCA TC ACC AC-3' and 5'-GGA TAT CGG CCC AGA GTT TCC GTG ATG-3'. According to the COS-TPC method (31), the resultant PCR products were inserted into the *Swa*I site of the cassette cosmid, pAdex1CAwt, and the sequences were ascertained by direct nucleotide sequence analysis. Full-length *DRPLA* cDNAs containing $(CAG)_{15}$ or $(CAG)_{78}$ with the FLAG tag at the 5' ends derived from pEF-BOS-AFN or pEF-BOS-AFE (19) were inserted similarly into pAdex1CAwt. The adenovirus vectors carrying the *DRPLA* cDNAs were generated by homologous recombination of pAdex1CAwt with the adenovirus DNA terminal protein complex, kindly provided by Miyake *et al.* (31).

Virus clones were isolated by limiting dilution analysis in 96-well microtiter plates. The culture medium and 293 cells in a well showing cytopathic effects (CPEs) were harvested. The cells were disrupted by alternate freezing and thawing six times, followed by centrifugation (2300 *g*, 5 min) at 4°C. The supernatants were used as the first seeds.

To ascertain the number of CAG repeats, 293 cells in 24-well microtiter plates were infected with 10 µl of the first seed of each clone, and the cells and the medium from the well showing CPEs were harvested at 3 days after infection. To determine the number of CAG repeat units, the genomic DNAs extracted from the cell pellets were subjected to PCR using the primer pair 5'-GAG AAG TCA ACA CAT CAC CAT CAC C-3' and 5'-GGC CCA GAG TTT CCG TGA TG-3' for the truncated cDNA, and the primer pair 5'-CAC CAG TCT CAA CAC ATC ACC ATC-3' and 5'-CCT CCA GTG GGT GGG GAA ATG CTC-3' for the full-length cDNA. Virus clones with the correct repeat numbers were amplified to obtain the second and third seeds for the virus solution and purified by centrifugation using CsCl step gradients as described by Kanegae *et al.* (36).

Titration of the adenovirus vectors

Titers of the virus solutions were determined by an end-point CPE assay (37) with modifications (36). A 50 µl aliquot of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) was dispensed into each well of a 96-well tissue culture plate, and then eight rows of 3-fold serial dilutions of the virus, starting from a 10^{-4} dilution, were prepared. Then, 293 cells (3×10^5) in 50 µl of DMEM supplemented with 5% FCS were added to each well and incubated at 37°C; 12 days later, the end-point of the CPE was determined by microscopy, and the 50% tissue culture infectious dose (TCID) was calculated by Karber's method (38). One $TCID_{50}$ per milliliter corresponds approximately to 1 p.f.u./ml (36).

Infection of cultured cells with adenovirus vectors

Neuronally differentiated PC12 cells were prepared as described by Greene *et al.* (39). Undifferentiated PC12 cells were cultured in DMEM with 10% horse serum and 5% FCS. For the differentiation to neuronally differentiated PC12 cells, PC12 cells were cultured in DMEM containing 1% horse serum and 50 ng/ml of 2.5S nerve growth factor (NGF) (Takara, Ootsu, Japan). To maintain the PC12 cells in a differentiated state, the medium was replaced every 3 days.

For the expression experiments using the adenovirus vectors, the cells were seeded at a density of $1 \times 10^4/cm^2$ on 8-well chamber slides (Nunc, Roskilde, Denmark) coated with type I collagen (CELLGEN; Koken, Tokyo, Japan) and incubated at 37°C in 5% CO_2. Cultured human skin fibroblasts at passages 4–9 were seeded on the chamber slides at a density of $1 \times 10^4/cm^2$, and cultured in DMEM containing 10% FCS. Neuronally differentiated PC12 cells or fibroblasts were infected with the adenovirus vectors, Ax1CAAQ19, Ax1CAAQ82, Ax1CAAFD19 or Ax1CAAFD82. The adenovirus vector containing the *lacZ* gene (Adex1CALacZ; kindly provided by Dr Izumu Saito, University of Tokyo, Japan) was used as the control virus. After removal of the culture medium, 100 µl of the adenovirus solution was overlaid on the cells at an m.o.i. of 10, 50, 100 or 500 p.f.u./cell. After incubation for 1 h at 37°C, 100 µl of the culture medium was added and the cells were cultured for 1, 2, 3, 6 or 9 days.

Immunohistochemistry

The cultured cells were fixed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde for 1 h, treated with PBS containing 0.02% Triton X-100 and 10% goat serum at room temperature for 30 min to permeabilize the cells and block Fc receptors, and incubated for 2 h at room temperature in PBS containing 0.02% Triton X-100, 10% goat serum and anti-FLAG M5 monoclonal antibody (1:1000 dilution;

Eastman Kodak, Rochester, NY), followed by incubation with a rhodamine-conjugated anti-mouse IgG (1:200 dilution; Dako, Glostrup, Denmark) for 1 h. The cell nuclei were visualized using 2 mg/ml of Hoechst 33342 (Calbiochem-Novabiochem, San Diego, CA).

To visualize the expression of β-galactosidase, the cells were fixed with 4% paraformaldehyde for 1 h, followed by incubation in *N*,*N*-dimethylformamide containing 1 mg/ml halogenated indolyl-β-D-galactoside (Bluo-Gal; Gibco BRL, Rockville, MD), 5 mM K-ferricyanide, 5 mM K-ferrocyanide and 2 mM $MgCl_2$.

Evaluation of apoptotic cell death by TUNEL assay

Following immunolabeling, the cells were assayed for the presence of fragmented DNA by TUNEL assay using an In Situ Cell Death Detection kit (Boehringer Mannheim, Mannheim, Germany). Two hundred cells expressing the FLAG epitope were analyzed and quantified for determination of the percentage of apoptotic cells.

Western blot analysis

Neuronally differentiated PC12 cells (1×10^6 cells) or fibroblasts (2×10^5 cells) infected at an m.o.i. of 100 were harvested using cell scrapers and homogenized in 10 mM Tris–HCl (pH 7.4), containing 1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride. Protein samples (10 μg) were electrophoresed under reducing conditions (dithiothreitol) through a 7.5% (0.034% bis methylene acrylamide) or 15% (0.4% bis methylene acrylamide) SDS–polyacrylamide gel for full-length and truncated DRPLA proteins, respectively, followed by blotting onto Immobilon-P (Millipore, Bedford, MA). Immunostaining was performed by pre-incubation with 3% non-fat dry milk in Tris-buffered saline (pH 7.6), containing 0.05% Tween-20 overnight at room temperature, and incubation with anti-FLAG biotinylated M5 monoclonal antibody (1:2000 dilution; Eastman Kodak) for 4 h at room temperature, followed by incubation with a streptavidin-biotinylated horseradish peroxidase complex (1:5000 dilution; Amersham Pharmacia Biotech, Amersham, UK) for 1 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence reagent (ECL; Amersham).

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