Polyglutamine aggregates stimulate ER stress signals and caspase-12 activation

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Accumulation of unfolded and malfolded proteins causes endoplasmic reticulum (ER) stress, stimulating unfolded protein response (UPR) and c-Jun N-terminal kinase (JNK) activation and activating caspase-12 located on the ER. Little is known about the relationship between the ER stress and polyglutamine [poly(Q)] aggregates. Poly(Q)72 repeats [poly(Q)72] induced the stimulation of ER stress signals such as JNK activation, upregulation of Grp78/Bip and caspase-12 activation in C2C5 cells. We prepared antiserum against the cleavage site of mouse caspase-12 at D318 (anti-m12D318), and showed that poly(Q)72 with perinuclear aggregates, cytoplasmic inclusions and nuclear inclusions stimulated JNK activation and anti-m12D318 immunoreactivity, but poly(Q)72 with dispersed aggregates and small nuclear aggregates showed a significantly less effect. Poly(Q)72 and poly(Q)11 dispersed in cytoplasm did not. Anti-m12D318-positive cells showed apoptotic features. Unlike anti-m8D387 immunoreactivity, the anti-m12D318 immunoreactivity was not coaggregated with poly(Q). Ac-IETD-fmk (caspase-8 inhibitor) and Ac-DEVD-CHO (caspase-3 inhibitor) did not prevent the anti-m12D318 immunoreactivity induced by poly(Q)72 aggregates. Anti-m12D318 immunoreactivity was detected in caspase-81 and caspase-31 mouse embryonic fibroblasts expressing poly(Q)72 aggregates. Thus, caspase-12 was activated by poly(Q)72 aggregates via a pathway independent of caspase-8 and caspase-3 activation, and caspase-12 activation was closely associated with poly(Q) aggregate-mediated cell death. Stimulation of ER stress signals may be involved in the pathogenesis of neurodegenerative disorders with poly(Q) expansion.

INTRODUCTION

Autosomal dominant neurodegenerative disorders, including dentatorubral–pallidolysian atrophy (DRPLA), Huntington’s disease (HD) and spinocerebellar ataxia (SCA) types 1, 2, 3 (Machado–Joseph Disease; MJD), 6 and 7, and the X-linked recessive disease spinal and bulbar muscular atrophy (SBMA), have been found to be caused by expansion of CAG trinucleotide repeats coding polyglutamine [poly(Q)] (1). Recently, neuronal intranuclear inclusions have been demonstrated in mice transgenic for exon 1 of the human HD gene harboring expanded CAG repeats (2). Similar nuclear inclusions were demonstrated in neurons in the autopsied tissues of patients with HD, DRPLA, SCA1, 3 and 7, and SBMA (1,3–6).

In cultured cells, truncated poly(Q) forms intracellular aggregates in the cytoplasm and nuclei of cells (7), leading to cell toxicity (8) and cell death with apoptotic features (3,7–9). The chaperone proteins, Hsp70 and Hsp40, inhibit the poly(Q) aggregates and poly(Q)-mediated cell death (10). However, the involvement of poly(Q) inclusions in cell death remains unclear, because not all cells expressing poly(Q) aggregates show cell death.

Caspases are components of the apoptotic pathway in mammals (11). The relationship between the activation of caspases and cell death induced by poly(Q) aggregates has been studied only recently (8,9,12–15). Caspase-8 and its active form are coaggregated with poly(Q) in the cytoplasm and around and within the nucleus. As caspases are activated...
via sequential processing by members of the caspase family (16), it has been speculated that the poly(Q)-mediated apoptotic pathway is triggered by activated caspase-8 coaggregated with poly(Q), i.e. that the caspase-8 apoptotic pathway is involved in poly(Q)-mediated cell death. However, while cells expressing nuclear coaggregates of poly(Q) and caspase-8 show apoptotic features, cells expressing cytoplasmic coaggregates of poly(Q) and caspase-8 exhibit cell death with low frequency. Thus, caspase-8 activation in the cytoplasmic poly(Q) aggregates may not be directly associated with poly(Q)-mediated cell death.

Unfolded or malfolded proteins in the endoplasmic reticulum (ER) activate stress signals via ER stress sensor proteins called IREs (17) leading to the induction of ER stress. For instance, IRE1-α mediates ER stress signals induced by agents such as tunicamycin, which blocks N-linked protein glycosylation in the ER. Upon activation, IRE1-α can recruit TNF receptor-associated factor 2 (TRAF2), which in turn recruits and activates the proximal components of the c-Jun N-terminal kinase (JNK) pathway (18). The ER stress also leads to the activation of genes possessing a unfolded protein response (UPR), which either improves local protein folding or results in cell death (17). The UPR controls the levels of molecular chaperones and enzymes involved in protein folding in the ER. ATF6 located at the ER membrane is one of the candidates for the UPR-specific transcription factor (19). Grp78/Bip (Bip) protein is the chaperone that increases protein folding in the ER lumen (20). Upon induction of ER stress, ATF6 is processed and the processed cytoplasmic region translocates into the nucleus and activates transcription of the endogenous Bip gene (19). When these stress signals are unable to rescue cells, the apoptotic pathway is activated. Caspase-12, which is located at the outer layer of the ER, is one of the apoptotic pathways of ER stress-mediated cell death (21). Tunicamycin induces the processing of caspase-12 at D318 (21,22).

Little is known about the relationship between ER stress signals and poly(Q) aggregates. Recently, it has been shown that nuclear poly(Q) aggregates stimulate stress signals such as SAPK/ERK kinase 1 (SEK1)/JNK and apoptosis signal-regulating kinase 1 (ASK1) that are closely linked to cell death (23). In the present study, we examined the relationship between ER stress signals and cytoplasmic and nuclear poly(Q) aggregates. Some types of cytoplasmic and nuclear poly(Q) aggregates stimulated ER stress signals and induced caspase-12 activation via a pathway independent of caspase-8 activation.

RESULTS

Poly(Q) aggregates and ER stress

C2C5 cells are the differentiated P19 EC cells constitutively expressing c-Jun (24). In contrast with P19 EC cells (15), C2C5 cells preferentially showed cytoplasmic poly(Q)72 aggregates rather than nuclear poly(Q)72 aggregates when pEGFP–72CAG was transfected (Fig. 1). EGFP–poly(Q)72 was dispersed in the cytoplasm of C2C5 cells in the initial time after transfection of pEGFP–72CAG [poly(Q)72/DC in Fig. 1A], concentrated in irregular and small cytoplasmic aggregates distributed ubiquitously in the cell body [poly(Q)72/DA in Fig. 1C]. They then formed a complex of interconnected aggregates in the perinuclear region [poly(Q)72/perinuclear aggregates: poly(Q)72/PA in Fig. 1E] or were heavily concentrated as cytoplasmic inclusions [poly(Q)72/CI in Fig. 1G], while small poly(Q) aggregates also initially appeared in nuclei [poly(Q)72/NA in Fig. 1I] and then formed nuclear inclusions [poly(Q)72/NI in Fig. 1K]. The percentage of cells expressing poly(Q)72/DC decreased 24–48 hours after transfection, while the percentage of cells expressing poly(Q)72/CI

![Figure 1](https://academic.oup.com/hmg/article-abstract/11/13/1505/744915)
increased (Table 1). The percentages of cells showing poly(Q)_{72}/DC, DA, PA, CI, NA and NI were 34.1%±6.4%, 9.2%±4.6%, 8.2%±5.2%, 38.9%±7.5%, 4.3%±0.8% and 5.3%±0.6%, respectively at 48 hours after transfection. Thus EGFP–poly(Q)_{72} preferentially formed cytoplasmic aggregates such as poly(Q)_{72}/DA, PA and CI. EGFP–poly(Q)_{11} did not show the aggregate in C2C5 cells (Fig. 1M).

ER stress stimulates JNK activation (18) and upregulates the level of Bip, which increases protein folding in the ER lumen (20). We examined the relationship between the poly(Q) aggregates and stimulation of ER stress signals, JNK activation and upregulation of Bip in C2C5 cells (Fig. 2). When pEGFP–72CAG was transfected into C2C5 cells, the EGFP–poly(Q)_{72} band appeared as a soluble protein at 24 hours, and its level was decreased in a time-dependent manner, probably owing to the insoluble aggregation of EGFP–poly(Q)_{72}. Upregulation of Bip and phosphorylation of serine at 63 of c-Jun (JNK activation) were faintly detected at 24 hours after transfection. EGFP–poly(Q)_{72} induced upregulation of Bip and stimulated JNK activation 36–48 hours after transfection (Fig. 2A). In contrast to EGFP–poly(Q)_{72}, poly(Q)_{11} did not upregulate Bip and did not stimulate JNK activation (Fig. 2B,C).

We examined the relationship between the distribution of poly(Q)_{72} aggregates and activation of JNK (Fig. 3). Most cells expressing poly(Q)_{72}/PA, CI and NI showed anti-phosphorylation of serine at 63 of c-Jun (anti-c-Jun-p) immunoreactivity (Fig. 3E,G,K), but much less was observed in cells expressing poly(Q)_{72}/DA and NA (Fig. 3C,I); i.e. the percentages of anti-c-Jun-p-positive cells were 3.9%±0.8%, 85.1%±6.8%, 88.0%±7.2%, 8.3%±2.4% and 75.0%±7.0% of cells showing poly(Q)_{72}/DA, PA, CI, NA and NI, respectively (Fig. 3). The percentage of anti-c-Jun-p-positive cells in cells expressing poly(Q)_{72}/DC and poly(Q)_{11} was the same as that in unstimulated cells (Fig. 3A,M). In the cells expressing poly(Q)_{72}/NI, anti-c-Jun-p immunoreactivity co-localized with nuclear poly(Q)_{72} aggregates (Fig. 3K), as shown previously (23). While anti-c-Jun-p immunoreactivity did not co-localize with poly(Q) aggregates in the cells expressing poly(Q)_{72}/PA and CI, its immunoreactivity was detected in nuclei (Fig. 3E,G). Thus the cytoplasmic aggregates of poly(Q)_{72}/PA and CI stimulated stress-signal JNK activation, resulting in the phosphorylation of serine at 63 of c-Jun in nuclei, as did nuclear aggregates of poly(Q)_{72}/NI.

**Processing of caspase-12 in cells expressing poly(Q) aggregates**

Excess ER stress induces caspase-12 activation leading to apoptotic pathways (21). To examine the relationship between

**Table 1. Time-dependent changes of poly(Q)_{72} configurations (percentages)**

<table>
<thead>
<tr>
<th></th>
<th>DC</th>
<th>DA</th>
<th>PA</th>
<th>CI</th>
<th>NA</th>
<th>NI</th>
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<tr>
<td>24 h</td>
<td>66.0±5.3</td>
<td>8.9±3.5</td>
<td>10.6±3.0</td>
<td>8.3±1.8</td>
<td>2.8±2.1</td>
<td>3.4±1.3</td>
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<td>36 h</td>
<td>53.9±7.8</td>
<td>4.6±1.9</td>
<td>7.1±5.3</td>
<td>24.4±5.6</td>
<td>3.0±5.3</td>
<td>7.0±4.0</td>
</tr>
<tr>
<td>48 h</td>
<td>34.1±6.4</td>
<td>9.2±4.6</td>
<td>8.2±5.2</td>
<td>38.9±7.5</td>
<td>4.3±0.8</td>
<td>5.3±0.6</td>
</tr>
</tbody>
</table>

DC, dispersed in cytoplasm; DA, dispersed aggregates; PA, perinuclear aggregates; CI, cytoplasmic inclusions; NA, nuclear aggregates; NI, nuclear inclusions.

**Figure 2.** ER stress signals induced by EGFP–poly(Q)_{72}. (A) The relationship between the appearance of the EGFP–poly(Q)_{72} in the soluble fraction and the upregulation of Bip and JNK activation. After pEGFP–72CAG was transfected into C2C5 cells, the levels of EGFP–poly(Q)_{72}, Bip, c-Jun and c-Jun-p in the soluble fraction were examined in time-dependent manner by immunoblot analysis. (B) Upregulation of Bip induced by poly(Q)_{72} aggregates. The level of Bip was examined 48 hours after transfection of pEGFP–72CAG and –11CAG. Lane 1, unstimulated C2C5 cells; lane 2, cells expressing poly(Q)_{11}; lane 3, cells expressing poly(Q)_{72}. The level of Bip was upregulated by poly(Q)_{72} but not by poly(Q)_{11}. Expression of tubulin and EGFP–poly(Q) in the soluble fraction was examined by immunoblot analysis using antitubulin and anti-GFP, respectively. (C) Activation of JNK induced by poly(Q)_{72}. JNK activation was detected by immunoblot analysis using anti-c-Jun-p 48 hours after transfection. Lane 1, cells expressing poly(Q)_{11}; lane 2, cells expressing poly(Q)_{72}; c-Jun-p was detected only in cells expressing poly(Q)_{72}. Expression of c-Jun was examined by immunoblot analysis using anti-c-Jun.
ER stress-mediated cell death and the activation of caspase-12, we prepared anti-m12D318, antiserum against the putative cleavage site of mouse caspase-12 (mcaspase-12) at D318. When pFLAG–mcaspase-12 was transfected into COS cells, it was processed into a 43 kDa fragment corresponding to FLAG–mcaspase-12D318. Anti-m12D318 reacted with FLAG–mcaspase-12D318 and the 43 kDa processing fragment, but not with FLAG–mcaspase-12 (61 kDa) (Fig. 4A).

When pEGFP–mcaspase-12 and pEGFP–mcaspase-12D318 were transfected into COS cells, anti-m12D318 immunoreactivity was detected in cells expressing EGFP–mcaspase-12D318 (Fig. 4Bb), but not in cells expressing EGFP–caspase-12 at the initial time (at 12 hours) after transfection (Fig. 4Bd). At 30 hours after transfection, some of the cells overexpressing EGFP–mcaspase-12 showed anti-m12D318 immunoreactivity (Fig. 4Bf).

Anti-m12D318 reacted with a 37 kDa band 18 hours after tunicamycin treatment (Fig. 4C). An increased level of the 37 kDa band was detected at 24 hours. The molecular size of the processing fragment suggests that unlike autoprocessing of caspase-12 induced by overexpression, caspase-12 is processed at the N-terminal region by tunicamycin treatment in addition to being processed at D318 as described previously (21,22). Consistent with the immunoblot analysis, anti-m12D318 immunoreactivity was detected in the tunicamycin-treated cells (Fig. 4Dc), but not in the untreated cells (Fig. 4Da).

We examined whether poly(Q)72 aggregates stimulate the processing of caspase-12 (Fig. 5A). Transfection of pEGFP–72CAG induced the processing of caspase-12 into a 37 kDa band, which reacted with anti-m12D318. The 37 kDa band was detected 24 hours after transfection with pEGFP–72CAG. An increased level of the 37 kDa band was detected at 48 hours. EGFP–poly(Q)11 was detected in the soluble fraction and did not induce the processing of caspase-12 (Fig. 5B).

**Activation of caspase-12 in cells expressing poly(Q) aggregates**

We have shown that active forms of caspase-8 and -3 are coaggregated with poly(Q) (15); i.e. immunoreactivities of anti-m8D387 and anti-m3D175, antisera against the cleavage sites of caspase-8 and -3, respectively (25,26), were restricted to poly(Q)72 aggregates (Fig. 6K,M). In contrast to anti-m8D387 and anti-m3D175 immunoreactivities, anti-m12D318 immunoreactivity was detected in the cytoplasm of cells expressing poly(Q)72/PA and CI (Fig. 6G) at some frequency, but not in the cells expressing poly(Q)72/DC (Fig. 6C), DA (Fig. 6E) and NA: anti-m12D318-positive cells were 30.2% ± 3.9% and 22.1% ± 1.0% of cells expressing poly(Q)72/PA and CI, respectively, and 78.6% ± 4.0% of cells expressing poly(Q)72/NI (Table 2). Unlike poly(Q)72, poly(Q)11 did not induce the appearance of the anti-m12D318-immunoreactive cells (Fig. 6A). As well as cells expressing poly(Q)72/NI, more than 90% of anti-m12D318-positive cells expressing poly(Q)72/PA and CI showed apoptotic features (Fig. 6G,I, Table 2), but anti-m8D387- and anti-m3D175-positive cells showed such features only slightly (Fig. 6K,M).

In contrast with the close relationship between anti-m12D318 immunoreactivity and apoptotic features, the correlation between anti-c-Jun-p immunoreactivity and apoptotic features was different in the cells expressing poly(Q)72/NI and the cells expressing poly(Q)72/PA and CI; 84.3% and about 30% of anti-c-Jun-p-positive cells expressing poly(Q)72/PA and CI, respectively, showed apoptotic features (Table 2).

**Independent activation of caspase-12 from caspase-8**

We examined the relationship between the activation of caspase-8 in poly(Q) aggregates and poly(Q) aggregate-induced caspase-12 activation (Fig. 7). The anti-m12D318
Figure 4. Antiserum against the putative cleavage site of mcaspase-12 at D\textsuperscript{318} (anti-m12D318). (A) Immunoblot analysis of the specificity of the anti-m12D318. pFLAG–mcaspase-12 and pFLAG–mcaspase-12D318 were transfected into COS cells and anti-m12D318 immunoreactivity was examined by immunoblot analysis. Lane 1, untransfected COS cells; lane 2, cells expressing FLAG–mcaspase-12; lane 3, cells expressing FLAG–mcaspase-12D318. (B) Anti-m12D318 immunoreactivity against the processing fragment of mcaspase-12 at D\textsuperscript{318}. pEGFP–mcaspase-12D318 (a,b) and pEGFP–mcaspase-12 (c–f) were transfected into COS cells. The processing of mcaspase-12 was examined by double staining of EGFP-labeling (green) and anti-m12D318 immunostaining (red) at 12 hours (c,d) and at 30 hours (e,f) after transfection of pEGFP–mcaspase-12. (a,c,e) shows EGFP labeling; (b,d,f) shows anti-m12D318 immunostaining. (C) Anti-m12D318 immunoreactivity against the processing fragment of caspase-12 induced by tunicamycin on C2C5 cells. The processing of caspase-12 at D\textsuperscript{318} was examined in a time-dependent manner by immunoblot analysis using anti-m12D318. (D) Anti-m12D318 immunoreactivity in C2C5 cells treated with tunicamycin: (a,b) untreated cells; (c,d) tunicamycin-treated cells. (a,c) shows anti-m12D318 immunoreactivity; (b,d) are phase-contrast images. Arrowheads indicate anti-m12D318-positive cells. Scale bars: 25 μm.
immunoreactivity was not inhibited by Ac-IETD-fmk, an inhibitor of caspase-8 (Fig. 7Ab,e), or by Ac-DEVD-CHO, an inhibitor of caspase-3 (Fig. 7Ac,f). Ac-IETD-fmk inhibited anti-m3D175 immunoreactivity in poly(Q)72 aggregates (Fig. 7Ah,k). Furthermore, anti-m12D318 immunoreactivity was also detected in caspase-3/7/7 mouse embryonic fibroblasts (MEF) (Fig. 7Be,f) and caspase-8/7/7 MEF cells (Fig. 7Bh,i) expressing poly(Q)72 aggregates. Thus, caspase-12 was independently processed from caspase-8 and/or caspase-3 in cells expressing poly(Q)72 aggregates.

DISCUSSION

ER stress induced by poly(Q) aggregates

Nuclear poly(Q) aggregates activate stress signals, including SEK1/JNK and ASK1 (23). Lymphoblasts derived from HD patients shows increased stress-induced apoptotic cell death via an increase in mitochondrial depolarization (27). However, the relationship between the ER stress and poly(Q) aggregates has been unclear. Bip increases protein folding in the ER lumen. ER stress initially leads to activation of the Bip gene via the UPR element in the promoter region (20). Upon ER stress, IRE1-α can recruit TRAF2, which in turn recruits and activates the proximal components of the JNK pathway (17,18). Caspase-12 is processed by ER stress and is closely associated with ER stress-mediated cell death (21). At present the molecular mechanism by which poly(Q)72/PA and CI and poly(Q)72/NI cause ER stress is not clear. On the basis of the timing of the appearance of EGFP–poly(Q)72 aggregates and the activation of ER stress signals (Figs 2A, 3, 5 and 6 and Tables 1 and 2), it is likely that poly(Q)72/DC, the major poly(Q)72 probably composed of a soluble monomeric (or oligomeric) form, and even small aggregates such as poly(Q)72/DA and NA, are not sufficient to induce ER stress.
Poly(Q)_{72} aggregates probably composed of insoluble poly(Q)_{72}, are closely associated with ER stress, causing Bip up-regulation and JNK activation, suggesting that ER stress is not caused by accumulation of unfolded monomeric or oligomeric poly(Q)_{72} but by large poly(Q) aggregates and inclusions themselves.

It seems unlikely that these poly(Q) aggregates directly cause ER stress via IREs, because poly(Q)_{72}/PA and CI partly interact with the ER membrane but are not located in the ER (data not shown). One of the possible explanations is that poly(Q)_{72}/PA and CI may cause dysfunction of cellular organelles. Mitochondrial dysfunction is reported in brain from patients with HD (28). In the caudate nucleus of the HD brain, severe defects of mitochondrial respiratory chain function (complex II, III and IV activities) were demonstrated. Poly(Q)_{72}/PA and CI may cause dysfunction of the ER, reducing the functions of molecular chaperones and/or enzymes involved in protein folding and in turn causing the accumulation of the unfolded proteins in the ER and leading to ER stress.

However, poly(Q)_{72}/PA and CI are not always associated with cell death (Table 2). Most poly(Q)_{72}/NI (80%) induced cell death, but only about 20–30% of poly(Q)_{72}/PA and CI did so.
Poly(Q)72/NI may also cause nuclear dysfunction inducing ER potentially induce cell death than cytoplasmic aggregates (29). Previous reports that nuclear poly(Q) aggregates more preferentially at its N terminus, and has been shown to play a role in ER stress and then cause the accumulation of the unfolded proteins in the ER, leading to ER stress.

Table 2. Relations of cell populations with certain types of poly(Q) configurations, incidence of anti-c-Jun-p and anti-12D318 reactivity and apoptosis

<table>
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<th>Positive cells (%)</th>
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<tr>
<td>DC</td>
<td>Apoptotic feature</td>
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<tr>
<td>c-Jun-p</td>
<td>2.1 ± 0.7</td>
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<tr>
<td>Apoptosis/c-Jun-p</td>
<td>ND</td>
</tr>
<tr>
<td>m12D318</td>
<td>1.6 ± 1.4</td>
</tr>
<tr>
<td>m12D318/apoptosis</td>
<td>ND</td>
</tr>
<tr>
<td>DA</td>
<td>Apoptotic feature</td>
</tr>
<tr>
<td>c-Jun-p</td>
<td>2.8 ± 4.4</td>
</tr>
<tr>
<td>Apoptosis/c-Jun-p</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>m12D318</td>
<td>2.6 ± 4.3</td>
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<tr>
<td>m12D318/apoptosis</td>
<td>ND</td>
</tr>
<tr>
<td>PA</td>
<td>Apoptotic feature</td>
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<tr>
<td>c-Jun-p</td>
<td>33.1 ± 0.3</td>
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<tr>
<td>Apoptosis/c-Jun-p</td>
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<td>m12D318</td>
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<td>m12D318/apoptosis</td>
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<tr>
<td>CI</td>
<td>Apoptotic feature</td>
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<tr>
<td>c-Jun-p</td>
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<td>Apoptosis/c-Jun-p</td>
<td>88.0 ± 7.2</td>
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<tr>
<td>m12D318</td>
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<td>c-Jun-p</td>
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<td>Apoptosis/c-Jun-p</td>
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<td>c-Jun-p</td>
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<td>m12D318</td>
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</tr>
<tr>
<td>m12D318/NI</td>
<td>98.2 ± 0.8</td>
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</table>

The percentages are positive cells, in the cells expressing certain types of poly(Q) configuration. DC, dispersed in cytoplasm; DA, dispersed aggregates; PA, perinuclear aggregates; CI, cytoplasmic inclusions; NA, nuclear aggregates; NI, nuclear inclusions. ‘Apoptosis/c-Jun-p’ is the percentage of cells showing apoptotic features among anti-c-Jun-p-positive cells, and ‘m12D318/apoptosis’ is the percentage of anti-m12D318-positive cells among the showing cells in apoptotic features. ND, not determined because of the low number of cells.

suggesting that poly(Q)72/NI induces more excess ER stress than poly(Q)72/PA and CI. This result is consistent with previous reports that nuclear poly(Q) aggregates more preferentially induce cell death than cytoplasmic aggregates (29). Poly(Q)72/NI may also cause nuclear dysfunction inducing ER stress. Nuclear dysfunction may inhibit the transcription of molecular chaperones and/or enzymes, increasing protein folding in the ER or inducing the suppression of ER stress.

It has been shown that poly(Q) interacts with TATA-binding protein (TBP)-associated factor (TAF1)130 and interferes with cAMP-responsive element-binding protein (CREB)-dependent transcription (30). We do not exclude the other possibility that poly(Q)72/NI may also directly inhibit the transcription of specific chaperone molecules and enzyme involved in protein folding and then cause the accumulation of the unfolded proteins in the ER, leading to ER stress.

Caspase-12 activation by poly(Q) aggregates

Caspase-12 has a caspase-associated recruit domain (CARD) at its N terminus, and has been shown to play a role in ER stress-mediated cell death (21). Several possible molecular mechanisms have been postulated for ER stress-mediated caspase-12 activation. Upon excess ER stress, caspase-12 is processed at its N-terminal region by a calcium-dependent neutral protease, calpain, activated by calcium released from the ER and then autoprocessed at D318 (22). Recently, it has been shown that TRAF2 forms a stable complex with procaspase-12 in unstressed cells. Stimuli that induce ER stress lead to the dissociation of procaspase-12 from TRAF2 and simultaneously promote oligomerization of procaspase-12, inducing the autoprocessing of caspase-12, probably via the CARD domain (31).

Poly(Q) aggregates induced the processing of caspase-12 at D318 (Fig. 5) and anti-m12D318-positive cells showed apoptotic features (Fig. 6), suggesting that poly(Q) aggregates induce ER stress, leading to cell death via activation of caspase-12. However, it is possible that, like caspase-8, caspase-12 may be activated independently of the ER stress. One possibility is that caspase-12 is autoprocessed by co-aggregation with poly(Q) via the CARD domain. Caspase-8 and caspase-9 are autoprocessed by oligomerization (16) and caspase-8 is activated during poly(Q) aggregation (12,13,15). However, unlike anti-m8D387 immunoreactivity, anti-m12D318 immunoreactivity was not detected in the poly(Q) aggregates but was detected in the cytoplasm in cells expressing poly(Q)72/PA and CI (Fig. 6G) and even in cells expressing poly(Q)72/NI (Fig. 6I), suggesting that caspase-12 is not activated during co-aggregation with poly(Q).

The other possibility is that caspase-12 is processed by activated caspase-8 in poly(Q) aggregates. Caspases are activated via sequential processing by caspase family members (16). However, Ac-IETD-fmk prevented caspase-3 activation in poly(Q)72 aggregates, but did not affect caspase-12 activation induced by poly(Q)72 aggregates (Fig. 7A). Furthermore, caspase-8 or caspase-3 deficiency did not affect caspase-12 activation (Fig. 7B). These observations suggest that caspase-12 is not downstream of caspase-8 in the caspase activation induced by poly(Q)72 aggregates. Thus, poly(Q)72 aggregates stimulate the activation of caspase-8 during aggregation and also activate caspase-12 independently of the caspase-8/caspase-3 pathway.

More than 90% of anti-m12D318-positive cells showed apoptotic features (Fig. 6, Table 2), suggesting that caspase-12 activation is closely associated with poly(Q)-aggregates-mediated cell death. In contrast with anti-m12D318-positive cells, only 30–40% of anti-c-Jun-p-positive cells expressing poly(Q)72/PA and CI showed apoptotic features, suggesting that poly(Q)72/PA- and CI-induced JNK activation is not sufficient to induce cell death. Some of the poly(Q)72/PA and CI and most poly(Q)72/NI, which induce excess ER stress via ER and nuclear dysfunction, respectively, may induce caspase-12 activation and cell death. Further analysis of the molecular mechanism of the activation of caspase-12 induced by poly(Q)72/PA and /CI or poly(Q)72/NI is underway in our laboratory.

In conclusion, poly(Q)72/PA and CI and poly(Q)72/NI induced stress signals related to ER stress, leading to activation of caspase-12 and cell death. Activation of caspase-12 may be one of the apoptotic pathways induced by poly(Q)72 aggregates. ER stress may be involved in the pathogenesis of neurodegenerative disorders with poly(Q) expansion.
Figure 7. Relation between the activation of caspase-8/caspase-3 and the processing of caspase-12 induced by poly(Q)72 aggregates. (A) Effect of Ac-IETD-fmk and Ac-DEVD-CHO on the anti-m12D318 or anti-m3D175 immunoreactivity induced by EGFP–poly(Q)72 aggregates. C2C5 cells were transfected with pEGFP–72CAG and incubated in the presence or absence of Ac-IETD-fmk (b,e,h,k) or Ac-DEVD-CHO (c,f,i,l), and then anti-m12D318 (a–f) and anti-m3D175 (g–l) immunoreactivities (red) were examined by the immunostaining. Anti-m12D318 immunoreactivity (red) was detected in the cytoplasm (a–f), while anti-m3D175 immunoreactivity (red) co-localized with EGFP–poly(Q)72 aggregates (green) (g, i) and was detected as yellow. (B) Effect of deficiency of caspase-8 and -3 on the anti-m12D318 immunoreactivity induced by EGFP–poly(Q)72 aggregates. pEGFP–72CAG was transfected into wild-type MEF cells (a–c), caspase-3−/− MEF cells (d–f) and caspase-8−/− MEF cells (g–i), and anti-m12D318 immunoreactivity (red) was examined by immunostaining. (a,d,g) EGFP–poly(Q)72 aggregates (green); (b,e,h) anti-m12D318 immunoreactivity; (c,f,i) superimposition of EGFP labeling and anti-m12D318 immunoreactivity. Arrows indicate EGFP–poly(Q)72 aggregates and arrowheads indicate anti-m12D318 or anti-m3D175 immunoreactivity. Scale bars: 25 µm.
MATERIALS AND METHODS

JNK activity

JNK activity was examined using the PhosphoPlus c-Jun (Ser63) II Antibody Kit (Cell signaling Technology, Beverly, MA) according to the manufacter’s manual. Briefly, JNK activity was detected by immunoblot and immunostaining analysis using antiserum against phosphorylation of serine at 63 of c-Jun (anti-c-Jun-p) (32).

Preparation of EGFP- and FLAG-fused mcaspase-12 and its processing fragment

The cDNA fragments encoding mcaspase-12 and its putative processing fragment at D$^{318}$ (mcaspase-12D318) were amplified from RNA of C2C12 cells by RT–PCR using the following primers: forward primer for mcaspase-12: 5'-ATGCCGCAC-3' and reverse primer for mcaspase-12: 5'-CTACTTCCGAGAAAGGTAG-3' or reverse primer for mcaspase-12D318: 5'-TCAATCGACATGGC-3'. The cDNA fragments were amplified as follows: one cycle at 95°C for 2 min, 25 cycles at 95°C for 1 min and 60°C for 2 min, and one cycle at 60°C for 7 min. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and then subcloned inframe into the EcoRI site of the pEGFP vector (Clontech Laboratories, Inc., Palo Alto, CA) and the pCMV-FLAG vector (Kodak, New Haven, CT). The nucleotide sequence was confirmed by dyeoxy sequencing with a fully automated DNA sequencer ALFII (Pharmacia, Milwaukee, WI).

Preparation of antiserum against cleavage site of mcaspase-12

Antiserum against a cleavage site of mcaspase-12 was prepared basically as described previously (25,26,33). Briefly, a peptide corresponding to a putative C-terminal processing site (D$^{318}$) of mcaspase-12 and cysteine, CIATAD (34), was synthesized (Sawady Technology, Tokyo). Antiserum against CIATAD (anti-m12D318) was generated by injecting CIATAD conjugated to keyhole limpet hemocyanin (KLH) into rabbit. Anti-m12D318 antibody was purified by corresponding peptide affinity column chromatography. Anti-m12D318 did not react with the processing fragments of other caspases, including caspase-2, -3, -7, -8 and -9. Anti-m12D318 was highly specific for the processing fragment of mcaspase-12 at D$^{318}$. Anti-m8D387 and anti-m3D175 antibodies were prepared as described previously (25,26).

Immunoblot analysis

pEGFP–72CAG and pEGFP–11CAG were prepared as described previously (15). pEGFP–72CAG and pEGFP–11CAG (5 μg) were transfected into C2C5 cells, and pFLAG–mcaspase-12 and pFLAG–mcaspase-12D318 were transfected into COS cells using the calcium phosphate method (35). Cells were washed two times with fresh medium 5 hours after transfection and incubated for the indicated period, then lysed with RIPA buffer [phosphate-buffered saline (PBS) containing 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)] or PBS containing 0.2% Triton X-100. After centrifugation at 10 000 g for 10 min, the cell extracts (50 μg protein) were subjected to SDS–polyacrylamide gel (12%) electrophoresis and immunoblot analysis. Proteins of the gels were electrophoretically transferred to nitrocellulose filters. After the filters were incubated with anti-m12D318, antitubulin (Sigma, St Louis, MO), anti-c-Jun (Cell Signaling Technology), anti-c-Jun-p, anti-Bip (StressGen Biotechnology Corp., Victoria, Canada), anti-FLAG (Sigma) and anti-GFP antibodies (Boehringer Mannheim, Mannheim, Germany), the reactivities on the filters were detected by alkaline phosphatase-conjugated, goat anti-rabbit or anti-mouse immunoglobulin (Promega), respectively, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate.

Immunostaining

C2C5 cells were transfected with pEGFP–72CAG and pEGFP–11CAG, and COS cells were transfected with pEGFP–mcaspase-12D318 and pEGFP–mcaspase-12, using the calcium phosphate method, and were cultured in the presence or absence of a protease inhibitor: Ac-IETD-fmk (20 μM) or Ac-DEVD-CHO (20 μM). pEGFP–72CAG was also transfected into wild-type MEF cells, caspase-3$^{-/-}$ MEF cells (36), and caspase-8$^{-/-}$ MEF cells (37). After the cells were fixed at the indicated time with 4% parafomaldehyde in PBS at room temperature for 20 min, they were incubated with anti-m12D318, anti-m3D175 or anti-m8D387 for 24 hours at 4°C. They were then incubated with rhodamine-labeled goat anti-rabbit or anti-mouse immunoglobulin (Leinco Technologies Inc., St Louis, MO) for 1 hour at 37°C, and cell nuclei were labeled with Hoechst 33342 (Molecular Probes, Eugene, OR) and viewed with a confocal laser scanning microscope (CSU-10, Yokokawa, Tokyo).

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