BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 145–150 (1998)
ARTICLE NO. RC989624

Caspase-3 Cleaves the Expanded Androgen Receptor Protein of Spinal and Bulbar Muscular Atrophy in a Polyglutamine Repeat Length-Dependent Manner

Yasushi Kobayashi,* Shigeru Miwa,* Diane E. Merry,† Akito Kume,*
Li Mei,* Manabu Doyu,* and Gen Sobue*,[1]

**Department of Neurology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan; and †Department of Neurology, University of Pennsylvania School of Medicine, 415 Curie Boulevard, Clinical Research Building, Philadelphia, Pennsylvania 19104*

Received October 5, 1998

Spinal and bulbar muscular atrophy (SBMA) is one of a group of human inherited neurodegenerative diseases caused by polyglutamine expansion. There is increasing evidence that generation of truncated proteins containing an expanded polyglutamine tract may be an important step in the pathogenesis of these disorders. We have previously demonstrated that the SBMA gene product, the androgen receptor (AR) protein, is toxic when truncated. We now report that *in vitro* translated full-length AR proteins containing different sized polyglutamine repeats (24, 65 and 97 repeats, respectively) are specifically cleaved by recombinant caspase-3, liberating a polyglutamine containing fragment, and that the susceptibility to cleavage is polyglutamine repeat length-dependent. These findings suggest that AR protein is one of the "death substrates" cleaved by caspase-3 and that caspase-3 might be involved in the pathogenesis of SBMA. **© 1998 Academic Press**

Spinal and bulbar muscular atrophy (SBMA) is an X-linked neurodegenerative disease caused by the expansion of a CAG repeat in the first exon of the androgen receptor (AR) gene (1). In SBMA patients, a normally polymorphic CAG repeat (10-36 CAGs) expands to 37-66 CAGs. The number of CAGs is inversely correlated with the age of onset of the disease (2, 3, 4). To date, seven other CAG-repeat diseases have been identified, including Huntington's disease (HD) (5), dentatorubralpallidoluysian atrophy (DRPLA) (6, 7), and five spinocerebellar ataxias (SCAs 1, 2, 3, 6, 7) (8-14). These disorders likely share a common pathogenesis caused by the gain of a toxic function associated with the expanded polyglutamine tract.

Truncated, expanded polyglutamine tracts cause neurodegeneration in transgenic mice as well as *Drosophila,* and cell death in transfected cells (15-20). In addition to cellular toxicity, truncated and expanded polyglutamine tracts have been shown to form aggregates through hydrogen bonding or transglutaminase activity (21, 22, 23). Furthermore, recent evidence indicates that neuronal intranuclear inclusions (NII) formed by the disease protein are a common pathological feature of these diseases (24-30). Processing of the polyglutamine containing disease proteins may be a key step in the pathogenesis of these neurodegenerative diseases. Previous reports indicated that polyglutamine containing disease proteins are good substrates for caspase-3, a member of the cysteine protease family (31, 32, 33). Polyglutamine length-dependent processing of disease proteins would be an important process for the polyglutamine length-dependent phenotypic expression including formation of NII and neuronal cell loss. Although cleavage of huntingtin by caspase-3 was originally described to be modulated by the polyglutamine length (31), subsequent reports indicated that the polyglutamine length does not modulate the susceptibility to cleavage of disease proteins including AR (32, 33). To determine whether the polyglutamine length modulates the susceptibility to cleavage of AR by caspase-3, we created recombinant full length AR with three different sized polyglutamine repeats (24, 65 and 97 repeats). The 97 polyglutamine repeat AR protein exceeds even the longest reported polyglutamine repeat in SBMA. We have assessed the polyglutamine length-modulation of susceptibility to caspase-3 cleavage in an *in vitro* cleavage assay system using these proteins. Here we show that AR protein is

[1] To whom correspondence should be addressed. Fax: +81-52-744-2384. E-mail: sobueg@med.nagoya-u.ac.jp.

Abbreviations used: Ab, antibody; AR, androgen receptor; DRPLA, dentatorubralpallidoluysian atrophy; HD, Huntington's disease; NII, neuronal nuclear inclusions; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia.

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specifically cleaved by recombinant caspase-3 and that longer polyglutamine tracts render AR protein more susceptible to caspase-3 cleavage.

MATERIALS AND METHODS

Antibodies. Antibodies used were PG-21 (anti-N-terminal of AR, Affinity BioReagents, Inc., Golden, CO), AR(N-20) (anti-N-terminal of AR, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and AR(C-19) (anti-C-terminal of AR, Santa Cruz Biotechnology, Inc.)

Construction of plasmids. For construction of the full length ARs, AR inserts derived from pCMV-AR24, pCMV-AR65 and AR97ΔHA (20, 34) were cloned into pSP64 Poly(A) (Promega Corporation, Madison, WI). Resulting clones were designated pSP64-AR24 (24 polyglutamine repeats), pSP64-AR65 (65 polyglutamine repeats) and pSP64-AR97 (97 polyglutamine repeats), respectively. All constructs were confirmed by sequencing.

Cleavage of in vitro translated AR protein. In vitro translated AR proteins were prepared using the TNT coupled reticulocyte lysate systems (Promega Corporation) according to manufacture's protocol. The *in vitro* translation mixture was incubated in cleavage buffer (50 mM HEPES/KOH (pH 7.2), 10 % sucrose, 0.1 % (w/v) CHAPS, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol) together with recombinant caspase-3 (Pharmingen, San Diego, CA) at different concentrations. The mixture was incubated at 37 °C for 0-8 h, terminated with equal amount of 2x SDS Laemmli loading buffer and subjected to denaturation by heating to 95 °C for 2 min. For some of the reactions, we added Ac-DEVD-CHO (100 nM) (Pharmingen), a specific inhibitor of caspase-3 protease.

Western blot analysis. An aliquot of each sample was fractionated on 5-20 % SDS-PAGE, and transferred to a PVDF membrane at 100 V for 1.5 h. The blots were blocked in 5 % dry milk/TBS-T (Tris buffered saline pH7.6, 0.1% Tween-20) overnight at 4 °C and then incubated with the primary antibody at room temperature for 1 h. The blots were subsequently washed TBS-T, and then incubated with secondary antibody, donkey anti-rabbit IgG coupled to HRP at room temperature for 1 h. After washing in TBS-T, detection was performed using the ECL system (Amersham, UK). Finally the blots were exposed to Hyperfilm-ECL (Amersham). The signal intensity was quantified by densitometry.

Statistical analysis. Results were analyzed using a one-way analysis of variance (ANOVA) and unpaired t-test if appropriate from the Statview statistical program.

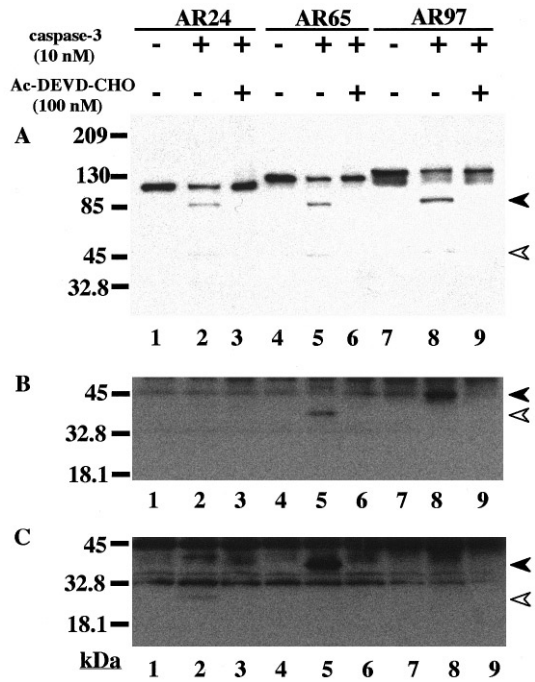

FIG. 1. AR protein-cleavage by recombinant caspase-3. *In vitro* translated full-length AR protein (1 μl) was incubated in cleavage buffer along with recombinant caspase-3 (10 nM) (10 μl final volume). The mixture was incubated at 37 °C for 8 h, and terminated with equal amounts of 2x SDS Laemmli loading buffer and heat denaturation. For some of the reactions, we added Ac-DEVD-CHO (100 nM). Half of the mixture (10 μl) was subjected to Western blotting. (A) Detection with anti-C-terminal of AR Ab (AR(C-19)). *Closed* and *open arrowhead*s indicate 85 kDa and 45 kDa C-terminal cleaved fragments, respectively (*lane 2, 5, 8*). (B) Detection with anti-N-terminal of AR Ab (PG-21) (shorter exposure). *Closed* and *open arrowhead*s indicate 45 kDa (truncated AR97, *lane 8*) and 35 kDa (truncated AR65, *lane 5*) N-terminal cleaved fragments, respectively. (C) Detection with anti-N-terminal of AR Ab (PG-21) (longer exposure). *Closed* and *open arrowhead*s indicate 35 kDa (truncated AR65, *lane 5*) and 25 kDa (truncated AR24, *lane 2*) N-terminal cleaved fragments, respectively.

RESULTS

AR protein-cleavage by recombinant caspase-3. Western blot analysis revealed different molecular weights of *in vitro* translated full-length AR proteins (AR24; 110 kDa, AR65; 120 kDa, AR97; 130 kDa, respectively) (Fig. 1A; *lane 1, 4, 7*). We first confirmed that caspase-3 specifically cleaved AR protein. Incubation of *in vitro* translated AR proteins with caspase-3 yielded 85 kDa and 45 kDa fragments that were detected with anti-C-terminal of AR Ab (AR(C-19)) (Fig. 1A; *lane 2, 5, 8*). In addition, anti-N-terminal of AR Abs (PG-21, AR(N-20)) detected the different sizes of N-terminal cleaved fragments of AR protein (AR24; 25 kDa, AR65; 35 kDa, AR97; 45 kDa, respectively) (Fig. 1B and C). Ac-DEVD-CHO, a specific inhibitor of caspase-3 protease completely blocked the cleavage of AR protein by caspase-3, indicating that caspase-3 specifically cleaved the AR proteins (Fig. 1A; *lane 3, 6, 9*). While anti-C-terminal of AR Ab revealed the same sized cleaved AR fragments despite the polymorphic polyglutamine repeats, anti-N-terminal of AR Ab showed a progressive increase in the size of the cleaved fragments with increasing repeat length. This suggests that the cleavage sites are located downstream from the polyglutamine tract in AR and demonstrates that AR protein-cleavage by caspase-3 generates N-terminal polyglutamine containing fragments.

The blots showed a tendency such that the longer the repeat, the stronger the signal intensity of the fragment cleaved by recombinant caspase-3 (Fig. 1A, B and C). In the literature there is a controversy over polyglutamine length-modulation of cleavage by caspase-3 (30, 31, 32). It was important , therefore, to determine whether polyglutamine length modulates AR protein cleavage by caspase-3.

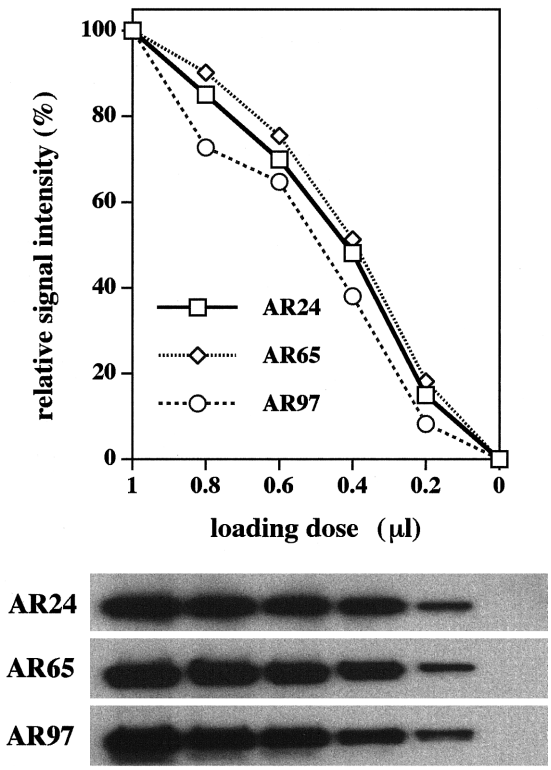

FIG. 2. Quantification of AR protein on Western blots. Varying amounts of *in vitro* translated full-length AR protein were subjected to Western blotting using anti-C-terminal of AR Ab. The signal intensity was quantified by densitometry. The relative signal intensity was expressed as a percentage of the signal intensity with 1 μl of loading dose.

Quantification of AR protein on Western blots. To quantify the cleavage of AR on Western blots, we plotted standard curves of AR protein. The standard curves demonstrated a linear relationship between loading dose of AR protein and signal intensity (Fig. 2). Validating the quantitative Western blot analysis for further study.

Polyglutamine tract modulates AR protein-cleavage by recombinant caspase-3. In an effort to assess the influence of polyglutamine length on the rate of AR-protein cleavage by caspase-3, two controlled experiments were performed. For the first, equivalent amounts of *in vitro* translated full-length AR protein containing 24, 65 or 97 polyglutamine repeats was incubated with varying concentrations of recombinant caspase-3 for 2 h. The extent of the cleavage of AR protein was significantly increased according to polyglutamine lengths ($p<0.05$ at 1 and 10 nM; $p<0.01$ at 100 nM by ANOVA: $p<0.05$ in AR24 vs. AR65 and AR65 vs. AR97 by unpaired t-*test*) (Fig. 3A). For the second experiment, equivalent amounts of *in vitro* translated full-length AR protein containing 24, 65 or 97 polyglutamine repeats were incubated with 10 nM of recombinant caspase-3. Reactions were terminated in parallel at 2 h intervals up to 8 h. The time course analysis of AR protein-cleavage by caspase-3 also revealed significant differences of rate of cleavage between different sized polyglutamine repeats ($p<0.05$ at 6 h; $p<0.01$ at 8 h by ANOVA: $p<0.05$ in AR24 vs. AR65; $p<0.01$ in AR65 vs. AR97 at 6 h; $p<0.01$ in AR24 vs. AR65 and AR65 vs. AR97 at 8 h by unpaired t-*test*) (Fig. 3B). These results showed that polyglutamine length modulates AR protein-cleavage by recombinant caspase-3.

DISCUSSION

In this study, we show that AR protein is specifically cleaved by recombinant caspase-3, liberating polyglutamine containing fragments and that longer polyglutamine tracts render AR protein more susceptible to caspase-3 cleavage. This finding suggests that AR protein-cleavage by caspase-3 may be an important mechanism for generating truncated AR protein fragments containing the expanded polyglutamine tract.

We previously reported that a truncated fragment of AR protein with expanded polyglutamine is toxic to COS-7 and MN-1 cell (20). In other CAG repeat diseases, truncated and expanded polyglutamine fragments of disease proteins have been shown to have toxicity in transfected cells, transgenic mice and flies (16-19). Generation of truncated, expanded polyglutamine fragments of the disease proteins, therefore, may be a key step in the pathogenesis of the CAG repeat diseases. The disease proteins of HD, DRPLA, MJD and SBMA have been reported to be good substrates for caspase-3 (31, 32, 33). While huntingtin-cleavage by caspase-3 has been shown to generate polyglutamine containing fragments (31), DRPLA protein-cleavage by caspase-3 has not (32). In SBMA, we demonstrated that AR protein-cleavage by caspase-3 liberated polyglutamine containing fragments of a length similar to these we found to have toxicity in living cells. Although polyglutamine length-dependent modulation has been controversied (31, 32, 33), the results reported here clearly demonstrated that polyglutamine length modulates AR protein-cleavage by caspase-3 in SBMA. We found a significantly different susceptibility to cleavage by caspase-3 between AR protein with 24 polyglutamine repeats and 65 polyglutamine repeats. Moreover the use of AR protein with exaggerated length of 97 repeats in this assay confirmed repeat-length dependent AR protein-cleavage susceptibility by caspase-3. One previous study (33) used smaller polyglutamine repeats in AR protein (12 and 50 repeats) than this report (24, 65 and 97 repeats), and consequently might not have demonstrated the significant difference we found in susceptibility to cleavage by caspase-3 between normal and expanded AR protein. Alternatively their use of a recombinant

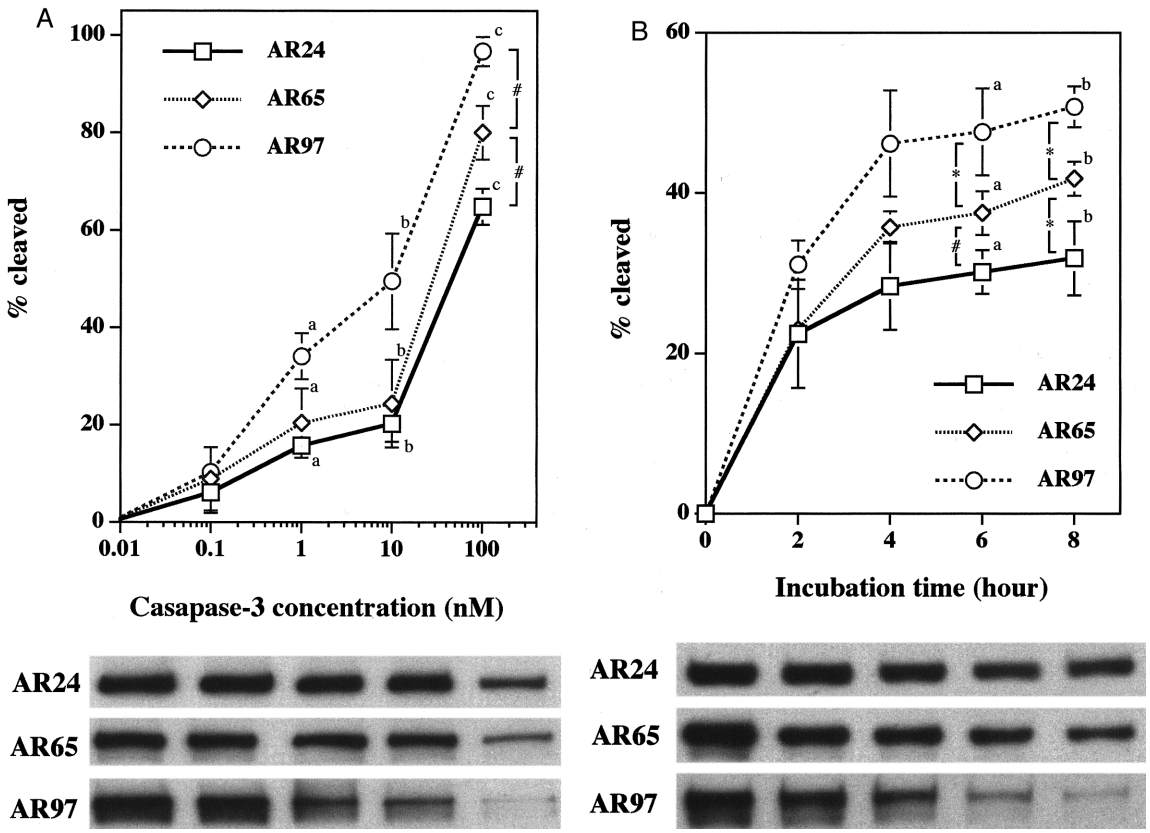

FIG. 3. Polyglutamine tract modulates AR protein-cleavage by recombinant caspase-3. (A) *In vitro* translated full-length AR protein (1 μl) was incubated in cleavage buffer along with recombinant caspase-3 at varying concentrations (10 μl final volume). The mixture was incubated at 37 °C for 2 h, and terminated with equal amounts of 2x SDS Laemmli loading buffer and heat denaturation. Three quarters of the mixture (15 μl) was subjected to Western blotting using anti-C-terminal of AR Ab. The signal intensity was quantified by densitometry and expressed as a percentage of the signal intensity with no added caspase-3. ^{a} $p<0.05$, $^{b}p<0.05$, $^{c}p<0.01$ by ANOVA. #$p<0.05$ by unpaired *t-test*. (B) *In vitro* translated full-length AR protein (1 μl) was incubated in cleavage buffer with recombinant caspase-3 at 10 nM (10 μl final volume). The mixture was incubated at 37°C, and terminated with equal amounts of 2x SDS Laemmli loading buffer at 2 h intervals up to 8 h and heat denaturation. Three quarters of the mixture (15 μl) was subjected to Western blotting using anti-C-terminal of AR Ab. The signal intensity was quantified by densitometry and expressed as a percentage of the signal intensity with no added caspase-3 at same intervals. $^{a}p<0.05$, $^{b}p<0.01$ by ANOVA. #$p<0.05$, *$p<0.01$ by unpaired *t-test*. Values are means ± SD. Both experiments were performed independently between 3 and 6 times.

csapase-3 of a different source from us might contribute to the opposite result.

Since the activated caspase-3 is expressed only in the execution phase of cell death (35, 36), there is still a question of how caspase-3 is responsible for pathogenesis of SBMA. Recently, the proenzyme form of caspase-3 has been found to have 60-fold lower catalytic activity than the activated form (33), hence the proenzyme form of caspase-3 could to some extent cleave the expanded AR proteins. However, caspase-3 has been shown to constitutively express in neurons throughout brain (37), and the expanded AR protein has been observed in the central nervous system of SBMA despite of affected or non-affected neurons (29). Taken together, expression of toxicity of expanded polyglutamine tract may not necessarily be related to caspase-3. Thus, caspase-3 might commit to additional deterioration rather than initiating disease process in SBMA.

Our findings indicate that the caspase-3 cleavage site is located downstream from the polyglutamine tract in the AR protein and liberates N-terminal polyglutamine containing fragments. To identify the cleavage site of the AR protein, we searched for a DXXD motif, which is the consensus cleavage site for caspase-3 (38). Two such motifs were found in the AR protein, both of them were located downstream from the polyglutamine tract (^{152}DEDD^{155} and ^{178}DLKD^{181}). The close proximity of these motif sites would enable them to be candidates for cleavage, generating a 85 kDa C-terminal cleaved fragment of AR protein . On the other hand, the cleavage site for the observed 45 kDa C-terminal cleaved fragment remains to be identified, because the DXXD motif was not found in the appropriate region of the AR protein.

In summary, caspase-3 specifically cleaves the expanded AR protein of SBMA in a polyglutamine repeat length-dependent manner, and liberates polyglutamine containing fragments. Because truncated and expanded polyglutamine has toxicity to living cells, caspase-3 might be involved in the disease progression of SBMA. Therapeutic approaches that inhibit caspase-3 activity in motor neurons might be helpful in the treatment of SBMA.

ACKNOWLEDGMENTS

We are grateful to Dr. Kenneth H. Fischbeck for discussion and for critically reading the manuscript. This study was supported by grants from the Ministry of Welfare and Health of Japan and by a COE grant from the Ministry of Science, Culture and Education of Japan.

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