

Kennedy's Disease: Caspase Cleavage of the Androgen Receptor Is a Crucial Event in Cytotoxicity

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Abstract: X-linked spinal and bulbar muscular atrophy (SBMA), Kennedy's disease, is a degenerative disease of the motor neurons that is associated with an increase in the number of CAG repeats encoding a polyglutamine stretch within the androgen receptor (AR). Recent work has demonstrated that the gene products associated with open reading frame triplet repeat expansions may be substrates for the cysteine protease cell death executioners, the caspases. However, the role that caspase cleavage plays in the cytotoxicity associated with expression of the disease-associated alleles is unknown. Here, we report the first conclusive evidence that caspase cleavage is a critical step in cytotoxicity; the expression of the AR with an expanded polyglutamine stretch enhances its ability to induce apoptosis when compared with the normal AR. The AR is cleaved by a caspase-3 subfamily protease at Asp¹⁴⁶, and this cleavage is increased during apoptosis. Cleavage of the AR at Asp¹⁴⁶ is critical for the induction of apoptosis by AR, as mutation of the cleavage site blocks the ability of the AR to induce cell death. Further, mutation of the caspase cleavage site at Asp¹⁴⁶ blocks the ability of the SBMA AR to form perinuclear aggregates. These studies define a fundamental role for caspase cleavage in the induction of neural cell death by proteins displaying expanded polyglutamine tracts, and therefore suggest a strategy that may be useful to treat neurodegenerative diseases associated with polyglutamine repeat expansions. **Key Words:** Triplet repeat disease—Caspase—Kennedy's disease—Androgen receptor—Aggregates.

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that was originally described to underlie developmental cell death (Lockshin and Williams, 1964; Kerr et al., 1972). For example, presenilin-2 (PS2) appears to play a fundamental role in apoptosis regulation (Wolozin et al., 1996), and mutations of PS2 associated with familial Alzheimer's disease increase apoptosis. Furthermore, both presenilin-1 (PS1) and PS2 protein products are substrates for the caspases (Kim et al., 1997), which are cysteine proteases required for apoptosis (Salvesen and Dixit, 1997). Mutations in the β -amyloid precursor protein at residue 717 that are associated with Alzheimer's disease are also proapoptotic (Yamatsuji et al., 1996). Spinal muscular atrophy is caused by mutations in the neuronal IAP gene (NIAP), an IAP (inhibitor of apoptosis)-related protein (Roy et al., 1995). XIAP has been shown to be a direct inhibitor of some caspases (Deveraux et al., 1997). How and at what point in the progression of these neurodegenerative diseases the apoptotic process is invoked, however, remain to be clarified.

Perhaps the most convincing evidence for involvement of the apoptotic pathway in neurodegeneration comes from studies of the CAG trinucleotide expansion diseases. Huntington's disease (HD) and other polyglutamine expansion diseases have an interesting relationship to the apoptotic process, as Huntingtin, as well as at least four other polyglutamine disease proteins, is a sub-

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Abbreviations used: AR, androgen receptor; DRPLA, dentatorubropallidolusian atrophy; HD, Huntington's disease; MJD, Machado-Joseph disease; NIAP, neuronal inhibitor of apoptosis; PBS, phosphate-buffered saline; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PS, presenilin; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia.

The basic mechanisms that underlie neurodegenerative diseases are unknown. However, over the last few years more evidence has accumulated, suggesting that the neuronal cell death in at least some neurodegenerative diseases may involve an inappropriate activation of the programmed cell death pathway (apoptotic pathway)

strate for the proapoptotic caspase-3 (Goldberg et al., 1996; Miyashita et al., 1997; Wellington et al., 1998; Sheldon et al., unpublished data).

Thus far, eight different dominantly inherited neurodegenerative diseases are associated with polyglutamine tract expansions in their respective proteins (Ross, 1995; Perutz, 1996; Nance, 1997). These include HD, spinal and bulbar muscular atrophy (SBMA; or Kennedy's disease), Machado-Joseph disease (MJD; or SCA-3), dentatorubropallidoluysian atrophy (DRPLA), and spinocerebellar ataxia types 1, 2, 6, and 7 (SCA-1, SCA-2, SCA-6, and SCA-7) (La Spada et al., 1991; Huntington's Disease Collaborative Research Group, 1993; Orr et al., 1993; Kawaguchi et al., 1994; Koide et al., 1994; Imbert et al., 1996; Sanpei et al., 1996; David et al., 1997; Zhuchenko et al., 1997). In all cases, there is selective death of neurons in different regions of the brain, and the clinical symptoms correlate with the affected regions. These neurodegenerative disorders show a strong correlation between polyglutamine tract length and age at onset of the disease (for review, see Nance, 1997). Anticipation, the phenomenon of progressively earlier onset of symptoms in succeeding generations, is a second common feature of these disorders.

As each of these disease-associated proteins shares a similar mutation, they may have a common pathological mechanism leading to neuronal cytotoxicity. This mechanism must involve the expansion of the polyglutamine repeat, as the causative proteins do not share other structural or functional similarities. Recent studies from several groups suggest that a critical step in the pathogenic mechanism shared among several of these triplet repeat proteins is the generation of a truncated protein containing the polyglutamine repeat, with the subsequent formation of intracellular aggregates. Perinuclear aggregates and intranuclear aggregates of truncated huntingtin are found in the brains of HD patients and HD transgenic mice expressing an N-terminal mutant fragment for HD (Davies et al., 1997; DiFiglia et al., 1997; Scherzinger et al., 1997; Martindale et al., 1998). Neuronal intranuclear inclusions are also found in the brains of MJD and DRPLA patients, and *in vitro* studies again have shown these inclusions to be present only with truncated forms of these proteins (Paulson et al., 1997; Igarashi et al., 1998).

We have recently reported that four of the polyglutamine-containing proteins, *i.e.*, huntingtin, the androgen receptor (AR), atrophin-1 (DRPLA), and ataxin-3 (MJD), are cleaved by caspases (Wellington et al., 1998), suggesting that a caspase-dependent apoptotic pathway may be a critical factor in the generation of truncated proteins in some of these polyglutamine repeat disease proteins.

Given our recent findings, we wished to determine if the caspase cleavage is required for the cytotoxicity exhibited by these proteins. Further, we wished to address whether the caspase cleavage site is required for the formation of the protein aggregates characteristic of these diseases. Here, we report the first conclusive evi-

dence that caspase cleavage is a critical step in the cytotoxicity of one polyglutamine disease protein, the AR. We show that expression of the AR with an expanded polyglutamine stretch enhances its cytotoxicity when compared with the normal AR. Protein aggregates are formed on apoptosis induction only for the expanded repeat protein, suggesting a gain-of-function for the proapoptotic fragment with an expanded polyglutamine stretch. Further, mutation of the caspase cleavage site at Asp¹⁴⁶ blocks the ability of the SBMA androgen receptor to form perinuclear aggregates, and substantially blocks its cellular cytotoxicity. Caspase cleavage of SBMA AR and other polyglutamine-containing disease proteins may thus be a required step leading to the progression of polyglutamine expansion disorders.

MATERIALS AND METHODS

Culture and transfection of cells

Cells from the human embryonic kidney cell line 293T were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with 1% penicillin/streptomycin. Transient transfection was performed with control plasmid pRc/CMV, full-length androgen constructs pRc/CMV-AR12, pRc/CMV-AR50, pRc/CMV-AR12D146N, pRc/CMV-AR50D146N, pRc/CMV-ARΔQ₅₈₋₈₀, and control plasmid pRc/CMV-*LacZ* according to Jordan et al. (1996). Optimal transfection efficiency was determined to occur with the following procedure: DNA (5 μl, 1 μg/μl) was mixed with 2 M CaCl₂ (6.2 μl) and tissue culture water (38.8 μl) in polystyrene tubes and incubated at room temperature for 10 min. During the 10-min incubation, the medium in the six-well plates was replaced with 1 ml of fresh medium. Then, 50 μl of 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM sodium phosphate, 12 mM dextrose, 50 mM HEPES, pH 7.05) was added to the polystyrene tube. The DNA mixture was incubated for 2 min at 37°C and then carefully added to the cells. After incubation overnight, the cells were washed twice with fresh medium. Using pRc/CMV-*LacZ*, transfection efficiency was determined by staining for the expression of β-galactosidase. Cell death was measured by trypan blue exclusion, acridine orange/ethidium bromide, and *LacZ* reporter gene cotransfection, either in the presence of testosterone (1 μM) or in its absence. To assess cell viability, 1.5 ml of medium was gently removed from the six-well dish, leaving 0.5 ml behind. Then, 0.5 ml of trypan blue dye solution (0.1%) was added to the adherent cells. Cells were incubated briefly at room temperature to allow for dye uptake. Cells were scored for dye uptake in three separate fields with a minimum of 300 cells counted. Values for percentages of viable cells were corrected for transfection efficiency, which was determined by *LacZ* staining. Testosterone was added to some cultures 12 h after transfection, and cell death was measured 36–50 h after transfection. Cellular death in confluent cells was induced with tamoxifen citrate at a concentration of 35 μM 36–48 h after transfection (Ellerby et al., 1997). Data were collected for three to five experiments and then compared by Student's *t* test for statistical significance.

Death was established as apoptotic based on acridine orange/ethidium bromide staining and activation of caspase-3. For adherent cells undergoing early apoptosis, morphological changes associated with apoptotic cell death were monitored using an acridine orange/ethidium bromide solution by a procedure based on the method of McGahon et al. (1995) with

modifications. At certain time points, cell culture medium was aspirated from adherent cells growing in 24-well plates, and the cells were washed gently once with room temperature phosphate-buffered saline (PBS). Then 1–2 ml of a 20-fold dilution of the dye mixture (composed of 100 mg/ml acridine orange and 100 mg/ml ethidium bromide), in PBS with formalin, was gently pipetted on the cells and viewed on an inverted fluorescence microscope.

Acridine orange passes freely through the plasma membrane, so it intercalates into the DNA, giving it, and therefore the nucleus, a green appearance. This dye also binds the RNA, but because it cannot intercalate, the RNA, and therefore the cytoplasm, appears red. Early apoptotic cells whose membranes are still intact, but which have started to fragment their DNA, still have green nuclei, because ethidium bromide still cannot enter the cell, but chromatin condensation will become visible as bright green patches in the nuclei. As the cell progresses through apoptosis, membrane blebbing occurs, so that in late apoptosis, ethidium bromide enters the cell and stains the nuclei so that they fluoresce bright orange. Nuclei were scored as apoptotic if they exhibited margination and condensation of the chromatin, and/or nuclear fragmentation similar to that observed in normal apoptotic cells (Kerr et al., 1972). A minimum of 200 cells were scored for each time point. Necrotic cells can be identified by ethidium bromide staining in the absence of the apoptotic features described above. Necrotic cells are typically stained bright orange with uniform color.

Western blot analysis

Western blot analysis was performed as described previously by Ellerby et al. (1997), using N-terminal and C-terminal AR antibody from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.).

In vitro translation reactions

Plasmids pRc/CMV, pRc/CMV-AR12, pRc/CMV-AR50, pRc/CMV-AR12D146N, pRc/CMV-AR50D146N, and pRc/CMV-ARΔQ_{58–80} were transcribed by using T7 polymerase, then translated by using the TNT system (Promega) in the presence of [³⁵S]methionine. Translations were incubated with the indicated caspase for 2 h, at identical specific activities, in the following buffer: 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10% sucrose, 10 mM dithiothreitol, and 0.1 mM EDTA, pH 7.2, at 37°C.

Purification of caspases and preparation of cell-free extracts

His-tagged caspases were purified by nickel-affinity chromatography as previously described (Orth et al., 1996; Cardone et al., 1997; Zhou et al., 1997). Translated AR constructs were treated with control extract, tamoxifen-primed neuronal cell-free extract, or cytochrome *c*/dATP extract as previously described (Ellerby et al., 1997).

Site-directed mutagenesis and plasmid construction

Human AR12D146N and AR50D146N were created using the QuikChange site-directed mutagenesis system from Stratagene. pRc/CMV-AR constructs were used as templates with the following synthetic primers according to manufacturer instructions: 5'-CCGGACGAGGATAACTCAGCTGCCCATCC-3'; 5'-GGATGGGGCAGCTGAGTTATCTCGTCCGG-3'; and 5'-GCTCTAGACCAATGCTTACTGGGTGTGG-3'. Site-directed mutagenesis was used to prepare

N-terminal fragments representing caspase cleavage products. AR12 and AR50 N-terminal constructs designated pLME443-12 and pLME443-50, encoding amino acids 1–146, were constructed by introducing termination codons with the following mutagenic primers: 5'-CCGGACGAGGATGACTGAGCTGCCCATCCAC-3' and 5'-GTGGATGGGGCAGCTCAGTCATCCTCGTCCGG-3'. C-terminal construct pLME2316, encoding amino acids 147–919 of AR was constructed by PCR amplification, using the following oligonucleotides containing *Xba*I and *Not*I restriction sites, respectively: 5'-GCTCTAGACCAATGCTTACTGGGTGTGG-3' and 5'-ATAAGAATGCGGCCGCGCACGATGGACTCAGCTGCCCATCCAC-3'.

pRc/CMV-ARΔQ_{58–80} was constructed, using overlap PCR. Using four oligonucleotides, two fragments of the AR gene were created via PCR. Fragment A was created, using the following oligonucleotides: 5'-CCGCTCGAGGCGGC-CGCTAGCTGCAGCGACTAC-3' and 5'-CTCACCCAGCAGCAGCAAAGTGGCGCCG-3' as the reverse primer. The former primes a region within the 5' untranslated region of the AR and contains an *Nhe*I restriction site. The latter primes up to the 5' end of the CAG region of AR and includes six nucleotides 3' to the AR. Fragment B was created with the following oligonucleotides: 5'-CTGCTGGGTGAGGATGGT-TCTCCCCAAAG-3' as the forward primer and 5'-CAGCTGCTTAAGCCGGGAAAGTG-3' as the reverse primer. The former primes six nucleotides 5' upstream of the CAG region and 22 nucleotides downstream from the 3' end of the region. The latter primes a region within the open reading frame of AR and contains an *Afl*III site. The PCR products, fragment A and fragment B, were then purified and used in a third PCR reaction. Using the forward primer used to make fragment A and the reverse primer for fragment B, a third PCR product was created using fragments A and B, which overlap by 12 nucleotides, as the template. This fragment (AB) was purified, digested with *Nhe*I and *Afl*III, and subsequently ligated into pRc/CMV-AR digested by the same enzymes.

Mapping of caspase cleavage sites by radiosequencing

Radiosequencing was performed as previously described (Salvesen and Enghild, 1990; Cardone et al., 1997). Plasmid pRc/CMV-AR12 was transcribed and translated with T7 polymerase, using the TNT system (Promega) with either [³⁵S]methionine or [³H]leucine. The translation was treated with caspase-3, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene difluoride membrane. After autoradiography, the position of the [³⁵S]methionine-labeled AR fragments was used to cut out the [³H]leucine AR bands from the polyvinylidene difluoride membrane. The samples were subjected to automated sequencing, using an Applied Biosystems 476A sequencer, and the anilinothiazolinone derivatives in each cycle were counted in a scintillation counter. Comparison of the known positions of leucines relative to the caspase cleavage site aspartate allowed identification of one of the AR cleavage sites.

Immunofluorescence microscopy

293T cells were grown on glass coverslips and transiently transfected with the indicated AR construct as described above. At 36 h after transfection, the cells were treated with 35 μM tamoxifen for 1 h. After fixation in 3% paraformaldehyde/PBS [or PHEM (60 mM PIPES, 25 mM HEPES, 5 mM glycine, 10 mM EGTA, 2 mM MgSO₄, pH 6.9)] solution for 20 min, the cells were permeabilized in 0.5% Triton/PBS (or PHEM) for

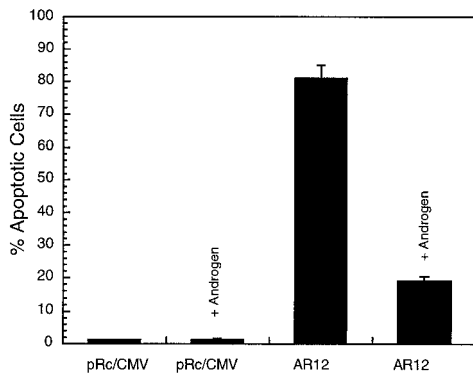


FIG. 1. Expression of the wild-type AR (AR12) is proapoptotic, with testosterone inhibiting the proapoptotic effect. 293T cells were transiently transfected at low confluency (10%) with pRc/CMV-AR12, and cellular death was assessed using trypan blue exclusion in the presence and absence of testosterone (1 μ M). Testosterone was added 12 h after transfection, and death was measured 48 h after transfection. Differences between control and pRc/CMV-AR12 and pRc/CMV-AR12 with and without testosterone were statistically significant ($p < 0.01$). Very similar results were obtained with the motor neuronal cell line NSC-19; transient transfection of AR12 resulted in apoptotic cell death and addition of testosterone blocked cell death.

5 min. The cells were incubated at room temperature with anti-AR antibody from Santa Cruz Biotech (1:200) for 1 h, washed with PBS (3 \times or PHEM), and then incubated in Texas Red-conjugated anti-rabbit antibody (1:1,000) for 30 min. PBS- (or PHEM-) washed cells on coverslips were then mounted onto slides with 4',6'-diamidino-2-phenylindole (DAPI; Sigma) as a nuclear counterstain. Either PHEM or PBS solutions were used for these studies. Immunofluorescence was observed by using a Zeiss confocal microscope. Control experiments were performed, including incubation with secondary antibody only, and immunofluorescence of cells transfected with control plasmids.

RESULTS

Increased cytotoxicity of SBMA mutant AR

To investigate the cytotoxicity of the AR and SBMA mutant AR, we transiently transfected 293T cells as well as motor neuronal cell line NSC-19 (Cashman et al., 1992) with expression constructs encoding the full-length human AR gene (Jordan et al., 1996). Untransfected 293T cells do not express the AR and are not dependent on androgen. The expression of wild-type AR in 293T cells enhanced apoptosis in the absence of androgen, whereas apoptosis was inhibited by the addition of testosterone (Fig. 1). The viability of the cells was measured by trypan blue exclusion and the mode of death was shown to be apoptotic, based on acridine orange/ethidium bromide staining. Expression of β -galactosidase was used as a control and used to determine transfection efficiency.

As the syndrome of complete androgen insensitivity is not associated with a motor neuron syndrome similar to that displayed by SBMA patients (Fischbeck, 1997), Kennedy's disease is more likely to be caused by a

gain-of-function rather than a loss-of-function mutation. Gain-of-function can entail enhancement of a normal function and/or establishment of a novel function. We hypothesized that the AR mutations associated with Kennedy's disease may enhance the proapoptotic effect of the wild-type AR. Therefore, we compared the cytotoxic effect of the wild-type AR (AR12) to that of a mutant form of AR with an expanded polyglutamine tract (AR50) in our tissue culture model. As shown in Fig. 2, cell death was increased by expansion of the polyglutamine repeat (AR50), and decreased by deletion of the polyglutamine tract in 293T cells. Vector alone or *LacZ*-transfected 293T cells served as controls.

Furthermore, stable transfections of AR12 and AR50 into the motor neuronal cell line NSC-19 (Cashman et al., 1992) confirmed that the cytotoxicity of the AR expression increases with expansion of the polyglutamine tract. In this case, transfection of the motor neuronal cell line NSC-19 with pRc/CMV-AR12 gave 21% death in the controls (pRc/CMV), 36% cell death with AR12 expression, and 54% death with AR50 expression. It is noteworthy that stable transfections of AR24 and AR65 into motor neurons, which are androgen responsive, have demonstrated poor expression of AR65 in comparison with AR24, which may have obscured a potential proapoptotic effect of AR65 (Brooks et al., 1997).

Cleavage of the AR by caspases

Because the AR had been shown previously to be processed proteolytically in the intracellular compartment (Kempainen et al., 1992), we evaluated its cleavage in a cell-free system of neuronal apoptosis (Ellerby et al., 1997). We showed previously that extracts prepared from neurons and neuronal cell lines committed to

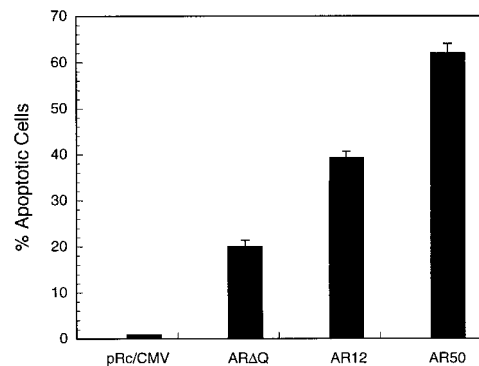


FIG. 2. Expansion of the polyglutamine repeat within the AR, from 12 to 50 glutamines, enhances the proapoptotic effect of AR expression. 293T cells were transiently transfected with pRc/CMV-AR Δ Q, pRc/CMV-AR12, or pRc/CMV-AR50 at 25% confluency. Death was induced 48 h after transfection with tamoxifen (35 μ M). Cellular death was assessed by using trypan blue exclusion in the presence and absence of testosterone (1 μ M) 5 h after tamoxifen treatment at 80% confluency. The difference between AR Δ Q, AR12, and AR50 was statistically significant ($p < 0.01$). Expression of the AR lacking the polyglutamine tract is less cytotoxic than the expression of wild-type or expanded AR. See Materials and Methods.

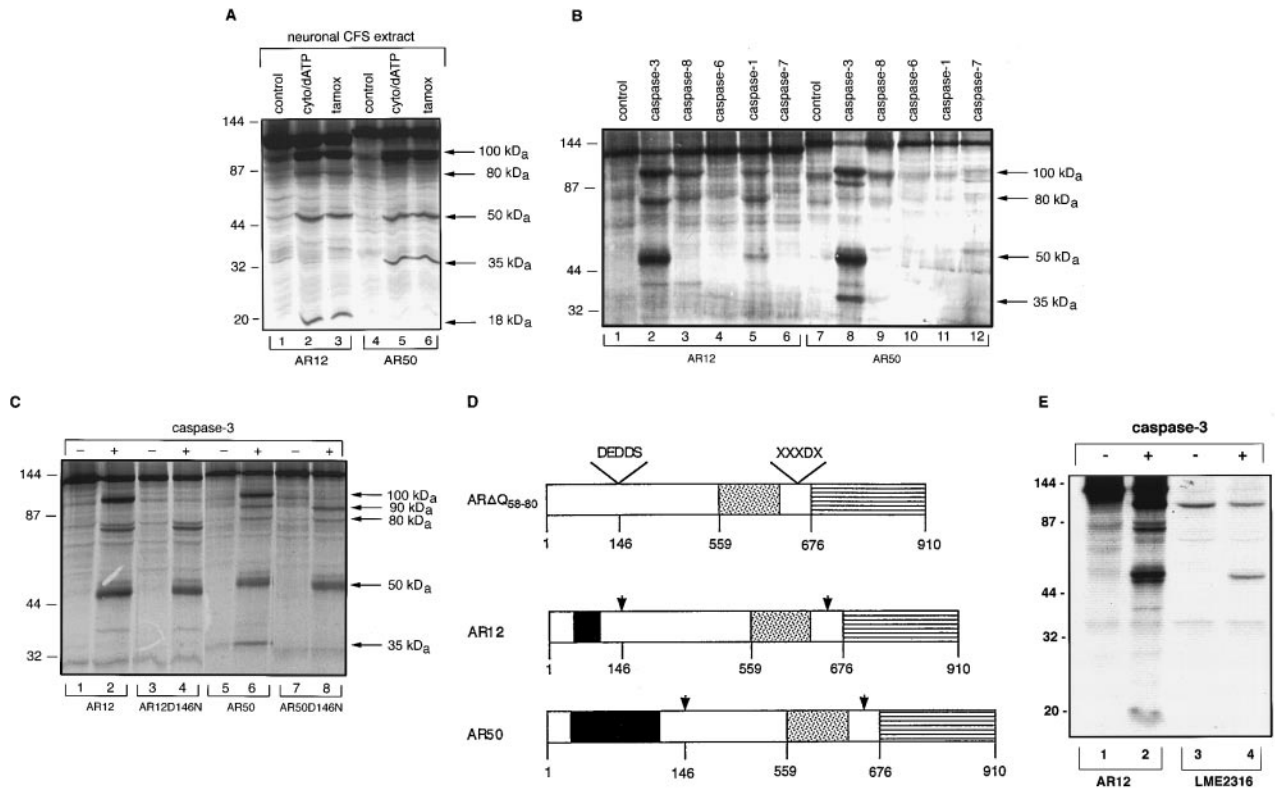


FIG. 3. **A:** In vitro translated wild-type AR12 (lanes 1, 2, and 3) and AR50 (lanes 4, 5, and 6) were treated with neuronal cell-free extracts. Control and activated extracts are noted (see Ellerby et al., 1997). cyto/dATP, cytochrome c/dATP; tamox, tamoxifen. **B:** In vitro translated AR12 (lanes 1–6) and AR50 (lanes 7–12) were treated with the indicated purified caspases (20 nM). **C:** In vitro translated AR12 (lanes 1 and 2), AR12D146N (lanes 3 and 4), AR50 (lanes 5 and 6), and AR50D146N (lanes 7 and 8) were treated with caspase-3. **D:** Diagrams of the primary structure of the normal and mutant (SBMA-derived) human AR protein are shown: ■, polyglutamine tract; □, DNA binding domain; ▨, hormone binding domain. Caspases cleave the AR in the N-terminal and C-terminal regions of the protein. Radiosequencing of in vitro translated AR with [³H]leucine was used to map the N-terminal cleavage site. The cleavage site in the N-terminal region and the approximate location of the C-terminal cleavage site are indicated (↓). AR functional domains are noted. The predicted molecular masses of the fragments resulting from cleavage near the N terminus of the AR12 are 82 kDa (for both AR12 and AR50) and 16 kDa (21 kDa for AR50). **E:** In vitro translated AR12 (lanes 1 and 2) and C-terminal AR construct pLME2316 (lanes 3 and 4) were treated with caspase-3.

apoptosis cleave several cellular death substrates (e.g., poly-ADP-ribose polymerase, the δ isoform of protein kinase C, and lamin-B) specifically, and that this cleavage is caspase dependent (Ellerby et al., 1997). Therefore, we tested whether in vitro translated AR is cleaved by activated neuronal extracts. As shown in Fig. 3A, both AR12 (lane 1) and AR50 (lane 4) remained intact when incubated with nonapoptotic extracts. In contrast, incubation of AR12 (Fig. 3A, lanes 2 and 3) or AR50 (Fig. 3A, lanes 5 and 6) with neuronal extracts committed to undergoing apoptosis yielded four AR fragments. These fragments had apparent sizes of 100 kDa (AR12 and AR50), 80 kDa (90 kDa for AR50), 50 kDa (AR12 and AR50), and 18 kDa (35 kDa for AR50). As shown in Fig. 3A, the predominant fragments generated in neuronal cell-free extracts are the 100 kDa (AR12 and AR50), 50 kDa (AR12 and AR50), and the 18 kDa (35 kDa for AR50). The fragment generated at 80 kDa (90 kDa for AR50) is cleaved considerably less efficiently in our neuronal cell-free extracts. Therefore, characterization of

the former (i.e., major) cleavage products was pursued further.

The finding that the AR is cleaved by apoptotic extracts but not by nonapoptotic extracts suggested, but did not prove, that the cleavage was mediated by caspases. This is because both caspases and noncaspase proteases may be activated during apoptosis (Zhivotovsky et al., 1996, 1997). However, previous work by Goldberg et al. (1996) had shown that huntingtin is cleaved in vitro by caspase-3, and the AR, like huntingtin, contains consensus caspase-3 cleavage sites. Therefore, we determined whether AR12 and AR50 are caspase substrates, by incubating in vitro translated AR products with purified caspases. As shown in Fig. 3B, we found that caspase-3 (CPP32/YAMA/apopain), caspase-1 (ICE), and caspase-8 (FLICE/Mach-1), but not caspase-6 (Mch2) or caspase-7 (Mch3), are capable of cleaving the AR (AR12 or AR50) in vitro. Caspase-3 cleavage of AR was more efficient than that catalyzed by either caspase-6 or caspase-8, based on both protein concentra-

tion and specific activity of these enzymes. We found no evidence that the expanded polyglutamine tract of AR influenced the susceptibility of AR to cleavage by purified caspases, and the results of these studies have been reported elsewhere (Wellington et al., 1998).

We next sought to determine whether this potential cleavage event plays a role in the *in vivo* proapoptotic effect of the AR. To determine this, we first used radio-sequence analysis to map the site of cleavage of the AR by caspase-3 to Asp¹⁴⁶ (see Fig. 3D). We localized this site to the N-terminal transcription modulatory domain of AR, ~70 residues C-terminal to the polyglutamine region of the protein. Cleavage at this site gave two cleavage products, with apparent sizes of 100 kDa (C-terminal product) and 18 kDa (N-terminal product) for AR12 (see Fig. 3A, lane 2). For AR50, a 35-kDa product containing the glutamine repeat was generated, as well as the same 100-kDa product (see Fig. 3A, lane 5). In addition, a second site was identified between the receptor steroid binding domain and DNA binding domain (see Fig. 3D). This second site yields a large N-terminal 80-kDa fragment with polyglutamine repeat for AR12 (90 kDa for AR50).

As shown in Fig. 3C, mutation of Asp¹⁴⁶ to Asn (D146N) blocked the *in vitro* cleavage by caspase-3 at this site. The cleavage products at 100 and 18 kDa (35 kDa for AR50) for AR12 are no longer present in the *in vitro* translated mutant proteins (see Fig. 3C). Mutation to Asn was chosen rather than to Glu because of the structural similarity of Asn to Asp and the previous finding that Glu in the P1 position may in some cases still allow caspase cleavage, although with lower efficiency than with Asp in the P1 position (Howard et al., 1991; Kayalar et al., 1996). Substitution of Asn for Asp at position 146 also blocked the AR cleavage in apoptotic neuronal extracts, providing confirmation that AR proteolytic processing in these extracts is mediated by caspases, and that Asp¹⁴⁶ is a caspase cleavage site (data not shown).

To test further whether the cleavage products of AR at Asp¹⁴⁶ were consistent with the observed cleavage products generated in neuronal apoptotic extracts and in cell culture, a series of truncated cDNAs representing the caspase cleavage products of normal and SBMA AR were prepared. *In vitro* translation of plasmids representing N-terminal cleavage product for AR12 (18 kDa) and AR50 (35 kDa) resulted in translated products identical to those generated from caspase cleavage of full-length AR (data not shown). As shown in Fig. 3E, *in vitro* translation of a plasmid coding for the C-terminal fragment (amino acids 147–919) produced a 100-kDa product identical to that generated during caspase cleavage of the full-length AR (Fig. 3E, lane 2 and 3). It is interesting that incubation of the C-terminal fragment with purified caspase-3 generates two 50-kDa fragments (Fig. 3E, lane 4). Thus, AR cleavage at Asp¹⁴⁶ generates a small N-terminal product and a large C-terminal product. The C-terminal product is further cleaved to generate the two 50-kDa products. As shown in Fig. 4A and B, western

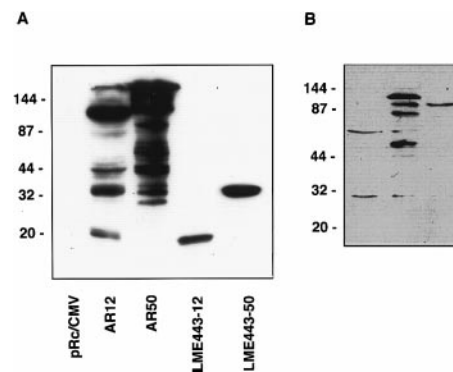


FIG. 4. Western analysis of 293T cells transfected with truncated or full-length AR constructs. **A:** Western blot of tamoxifen (35 μ M)-treated 293T cells transfected with pRc/CMV (lane 1), AR12 (lane 2), AR50 (lane 3), pLME443-12 (lane 4), and pLME443-50 (lane 5). Truncated N-terminal AR cDNAs were prepared as described in Materials and Methods and N-terminal AR antibody was used to detect expression of these constructs. **B:** Western blot of pRc/CMV (lane 1), AR12 (lane 2), and pLME2316 (lane 3) transfected into 293T cells treated with tamoxifen (35 μ M). C-terminal AR antibody was used to detect expression of AR and the C-terminal fragment.

analysis of the various truncated AR cDNAs and full-length AR cDNA introduced into 293T cells confirmed that these fragments are generated during apoptotic death. Similar cleavage products were observed in numerous cell lines undergoing apoptosis, e.g., NT2, NSC-19, and COS-7 (data not shown).

The proapoptotic effect of the AR requires caspase cleavage

Having determined that a caspase cleavage site is located just carboxy-terminal to the polyglutamine tract in the AR, and that mutation of this site blocks caspase cleavage, we assessed the effect of blocking caspase cleavage of AR12 and AR50 on the proapoptotic effects of these proteins in culture. As shown in Fig. 5A, transient transfection of AR12D146N and AR50D146N resulted in a two- to threefold reduction in apoptotic cell death when compared with AR12 and AR50. The expression level of all four proteins was similar in the transient expression assay (see Fig. 5B), suggesting that the reduction in proapoptotic effect was not simply due to a decrease in the expression of the transfected mutant genes. Further, western blot analysis using an antibody directed against the N-terminal domain of AR12 confirmed the generation of caspase cleavage product of 18 kDa (35 kDa for AR50), and substitution of Asn for Asp at position 146 in AR blocked the generation of these cleavage products in 293T cells.

Increased cytotoxicity of SBMA AR correlates with formation of large perinuclear aggregates

As our cytotoxicity studies indicated that the caspase cleavage site is crucial to the proapoptotic effect of AR, we investigated whether apoptosis induction with tamoxifen modulated the formation of

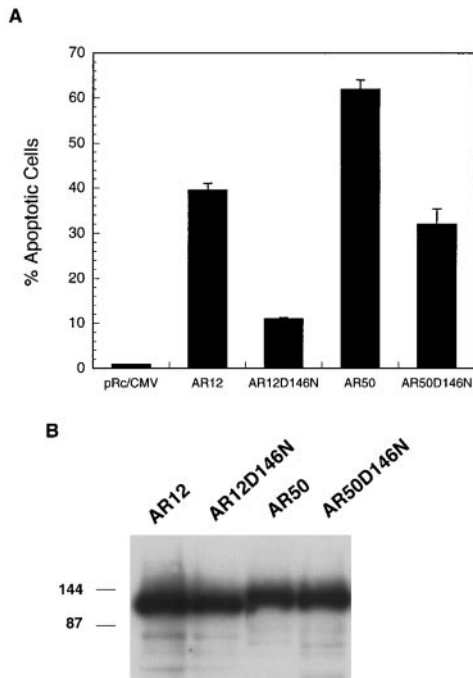


FIG. 5. Inhibition of the cytotoxic effect of both wild-type and expanded ARs by mutation of the N-terminal caspase cleavage site. **A:** 293T cells were transiently transfected with pRc/CMV-AR12, pRc/CMV-AR50, pRc/CMV-AR12D146N, and pRc/CMV-AR50D146N. Death was induced 48 h after transfection with tamoxifen (35 μ M). Cellular death was assessed by using trypan blue exclusion 5 h after tamoxifen treatment at 80% confluency. Differences between AR12 and AR12D146N as well as AR50 and AR50D146N were statistically significant ($p < 0.01$). **B:** Western analysis of transiently transfected 293T cells with pRc/CMV-AR12, pRc/CMV-AR50, pRc/CMV-AR12D146N, and pRc/CMV-AR50D146N demonstrates a similar level of expression of androgen receptor.

aggregates. Expression of the AR50, but not AR12, led to formation of large perinuclear aggregates in the cytoplasm, as determined by confocal microscopy (Fig. 6C and D) on apoptosis induction. Modulation of aggregate formation did not occur in the absence of apoptosis induction (Fig. 6A and C) for AR50 or AR12. The perinuclear aggregates described in these studies are observed in several cell culture models of triplet repeat expansion disease proteins and in patients with HD (DiFiglia et al., 1997; Paulson et al., 1997; Martindale et al., 1998). Transfection of 293T cells with truncated MJD resulted in perinuclear and intranuclear clusters (Paulson et al., 1997). In a similar manner, we have recently reported that cells transfected with amino-terminal huntingtin constructs contain both perinuclear and intranuclear aggregates, depending on the size of the fragment (Martindale et al., 1998). The fact that initial studies on patients with Kennedy's disease have shown aggregates in the nucleus rather than perinuclear may reflect further truncation or specific neuronal protein interactions with AR fragments that localize the inclusions to the nucleus.

Mutation of caspase cleavage site in SBMA AR blocks formation of aggregates

To test whether the caspase cleavage site is required for the formation of aggregates in cell culture, we evaluated whether mutation of the caspase cleavage site in the AR affects the formation of aggregates. Cells transfected with pRc/CMV-AR12 expressed AR protein diffusely in the cytoplasm with a homogeneous pattern during apoptotic cell death with tamoxifen (Fig. 6C). Cells transfected with pRc/CMV-AR50 showed large aggregates in perinuclear locations (Fig. 6D) during apoptotic cell death. In sharp contrast, cells transfected with pRc/CMV-AR50D146N did not form these large aggregates, suggesting that, at least in this system, caspase cleavage of SBMA AR is required for aggregation (Fig. 6F). Cells transfected with pRc/CMV-AR12D146N also showed a homogeneous cytoplasmic distribution of AR (Fig. 6E).

To determine whether the formation of aggregates correlated with the cytotoxicity of SBMA AR and caspase cleavage, we quantified the frequency of aggregate bodies in 293T cells transfected with each of the AR constructs. During apoptotic stimulation with tamoxifen, aggregate formation was observed at high frequency for SBMA AR (38–43%) when compared with normal AR (5–10%) (Fig. 7). Mutation of the caspase cleavage site Asp¹⁴⁶ of the expanded AR substantially blocked aggregate formation (12–18%) (Fig. 7).

Androgen blocks cleavage of AR and prevents formation of aggregates in SBMA AR

The AR functions as a transcription factor after nuclear translocation that is initiated by binding to androgen. We have shown that the wild-type AR is proapoptotic (Fig. 1), and that this cytotoxicity is completely blocked by the addition of androgen (Fig. 1). In separate experiments, we have shown that the cytotoxicity of the AR is blocked by mutation of the caspase cleavage site Asp¹⁴⁶, suggesting that the generation of a proapoptotic fragment is responsible for this effect. Together these data suggest that testosterone may block the cleavage of the AR. As shown in Fig. 8A and B, addition of testosterone to 293T cells transfected with AR results in translocation of AR into the nucleus. Western analysis reveals that testosterone addition blocks cleavage of AR during apoptotic stimulation (Fig. 8C).

Given our findings, we assessed whether androgen was a critical factor in the formation of aggregates in cells transfected with SBMA AR. As shown in Fig. 9, aggregate formation was observed at high frequency for SBMA AR (38–43%) when compared with normal AR (5–10%). Addition of testosterone blocked aggregate formation (13–18%) in cells transfected with SBMA AR.

DISCUSSION

In this study, we show that cells transfected with expression constructs encoding the AR undergo enhanced apoptotic cell death that is mediated by a pro-

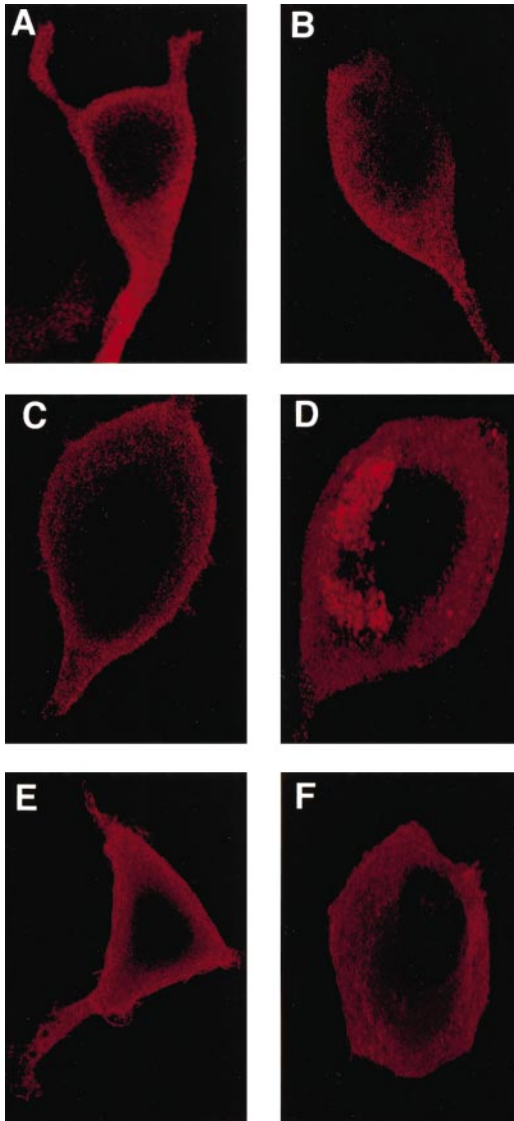


FIG. 6. Immunofluorescence of AR with expanded polyglutamine repeat shows the formation of protein aggregates in 293T cells. **A:** Untreated 293T cells transfected with AR (AR12). **B:** Tamoxifen treated (1 h, 35 μ M) 293T cells transfected with normal AR (AR12). Immunofluorescence of AR in 293T cells transfected with wild-type AR before tamoxifen treatment was identical to tamoxifen-treated cells. **C:** Untreated 293T cells transfected with disease-associated AR (AR50). **D:** Tamoxifen-treated (1 h, 35 μ M) 293T cells transfected with disease-associated AR (AR50). Immunofluorescence studies show that mutation of the N-terminal caspase cleavage site in SBMA AR blocks formation of perinuclear aggregates. **E:** Tamoxifen-treated (1 h, 35 μ M) 293T cells transfected with AR12D146N. **F:** Tamoxifen-treated (1 h, 35 μ M) 293T cells transfected with AR50D146N.

apoptotic caspase cleavage product. In vitro mutagenesis of the N-terminal caspase site blocks production of this proapoptotic fragment and dramatically reduces cellular toxicity. Further, we show that caspase cleavage of SBMA AR plays a central role in the formation of perinuclear aggregates in cell culture. Our results suggest

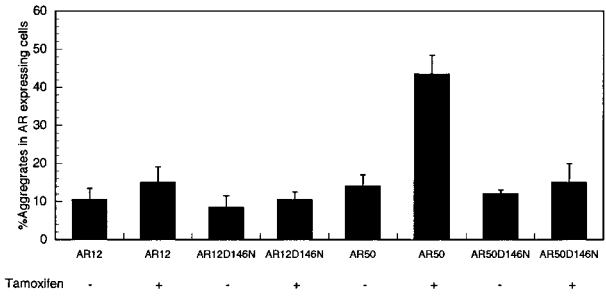


FIG. 7. Mutation of the caspase cleavage site abolishes formation of perinuclear aggregates in SBMA AR. 293T cells were transfected with the indicated AR constructs. AR was detected by immunofluorescence. The total number of cells containing aggregates relative to the total number of cells expressing AR were counted. The percentage aggregates in AR-expressing cells are presented for each construct before and after tamoxifen addition. Differences between AR50 and the other AR constructs were statistically significant ($p < 0.01$).

that the enhanced cytotoxicity of SBMA AR observed in our tissue culture paradigm may relate to the cause of neuronal cell death in patients with Kennedy's disease. Our initial characterization of the SBMA AR in 293T cells demonstrated a marked reduction in cytotoxicity after mutation of the caspase cleavage site. However, this reduction was not a proportionally greater reduction in cytotoxicity when compared with the wild-type protein. The reason for this is likely to be related to the following three factors: (1) the cytotoxicity of normal AR and expanded form are modulated by androgen levels; (2) we

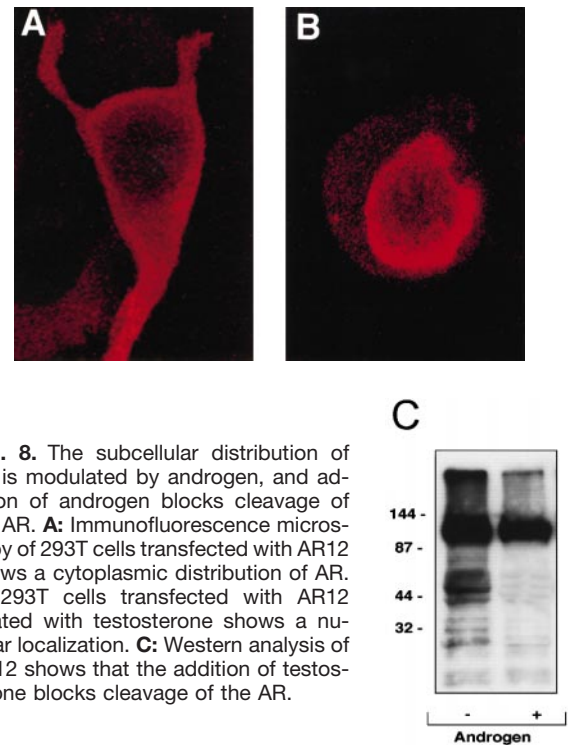


FIG. 8. The subcellular distribution of AR is modulated by androgen, and addition of androgen blocks cleavage of the AR. **A:** Immunofluorescence microscopy of 293T cells transfected with AR12 shows a cytoplasmic distribution of AR. **B:** 293T cells transfected with AR12 treated with testosterone shows a nuclear localization. **C:** Western analysis of AR12 shows that the addition of testosterone blocks cleavage of the AR.

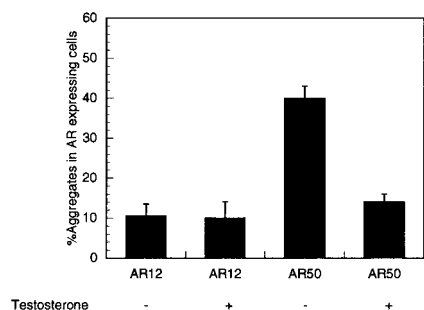


FIG. 9. Addition of testosterone modulates aggregate formation in SBMA AR. 293T cells were transfected with the indicated AR constructs. AR was detected by immunofluorescence. The total number of cells containing aggregates relative to the total number of cells expressing AR were counted. The percentages of aggregates in AR-expressing cells treated with tamoxifen are presented for each construct without and with testosterone (100 nM) addition. Differences between AR50 with and without androgen were statistically significant ($p < 0.01$).

have mutated only one of the two caspase cleavage sites contained within the AR; (3) the SBMA AR is transcriptionally less active than the wild-type form.

One intriguing finding is the effect of androgen addition on the formation of aggregates by SBMA (i.e., expanded) AR. We found that addition of testosterone reduces cleavage of AR, and this correlates with the ability of androgen to reduce the frequency of aggregates found in cells transfected with SBMA AR. Androgen treatment results in translocation of AR into the nucleus and high levels of transcriptional activity. SBMA AR is similarly translocated into the nucleus, with a slightly reduced transactivation activity (50–80%) (Kazemi-Esfarjani et al., 1995). One possible explanation for our results is that the transcriptionally active form of AR may bind to proteins and/or DNA in a fashion that blocks cleavage by caspases. An alternative possibility is that transcriptional activation of AR by androgen may result in the expression of antiapoptotic proteins such as BCL2 or IAPs.

The results reported here, coupled with our recent reports (Martindale et al., 1998; Wellington et al., 1998), suggest that one potential similarity between the eight different proteins for which expanded polyglutamine regions are associated with neurodegenerative disease states is that they are cleaved by caspases to produce proapoptotic fragments; i.e., they may function as caspase amplifiers. In this model, initial cleavage would produce a toxic fragment with a gain of toxic function, e.g., aggregation. This initial generation of toxic fragment would lead to amplification of caspases through a feedback loop. This amplification loop would be highly dependent on cellular context such as caspase/inhibitor distribution within the cell, protein–protein, and/or protein–ligand interaction with each type of polyglutamine repeat protein. Thus, generation of a proapoptotic fragment may be specifically blocked by binding to the appropriate substrates (such as androgen for the AR).

The physiological role of caspase cleavage of these proteins is unknown. However, the reported results demonstrate that caspase cleavage is crucial to the cytotoxic effect of the expanded repeat AR, as well as to its aggregation. In contrast, expression of the wild-type AR did not lead to aggregation, whether or not caspase activation was induced. These results do not, however, exclude the possibility that noncaspase proteases may also play a role in processing of the AR or other polyglutamine tract proteins.

The results also do not offer an explanation for the specific pattern of neuronal loss in Kennedy's disease or other CAG repeat diseases. It is possible that alterations in caspase expression, androgen concentration, AR expression, or downstream targets may determine the selective vulnerability of motor neurons in Kennedy's disease. The effect of testosterone to block both AR cleavage and the proapoptotic effect suggests that physiological inhibitors and enhancers of caspase cleavage of polyglutamine-containing proteins may explain the selective vulnerability of specific neuronal populations in this group of diseases.

Finally, as blocking the cleavage of the AR inhibits its proapoptotic effect, such a strategy may prove useful for the treatment of neurodegenerative diseases associated with polyglutamine repeat expansions. It is noteworthy that general caspase inhibitors have been developed, but not substrate-specific caspase inhibitors. The latter may prove to be effective in halting the continued progression of neurodegenerative diseases associated with polyglutamine tract expansions.

Note added in proof: Recent studies by Butler et al. (1998) and Abdullah et al. (1998) have demonstrated a 74-kDa C-terminally truncated fragment of the AR, which is increased in cells expressing polyglutamine-expanded AR. It is possible that this corresponds to the fragment generated by cleavage at the C-terminal caspase site, between the DNA binding domain and the hormone binding domain (referred to as an 80-kDa fragment in the current article).

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