Brief Report

Cytoplasmic Localization and the Choice of Ligand Determine Aggregate Formation by Androgen Receptor with Amplified Polyglutamine Stretch

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Abstract. Polyglutamine tract expansion in androgen receptor is a recognized cause of spinal and bulbar muscular atrophy (SBMA), an X-linked motor neuronopathy. Similar mutations have been identified in proteins associated with other neurodegenerative diseases. Recent studies have shown that amplified polyglutamine repeat stretches form cellular aggregates that may be markers for these neurodegenerative diseases. Here we describe conditions that lead to aggregate formation by androgen receptor with polyglutamine stretch amplification. In transfection experiments, the mutant, compared with the wild-type receptor, was delayed in its cytoplasmic–nuclear translocation and formed large cytoplasmic aggregates in the presence of androgen. The cytoplasmic environment appears crucial for this aggregation, since retention of both the wild-type and mutant receptors in this cellular compartment by the deletion of their nuclear localization signals resulted in massive aggregation. Conversely, rapid nuclear transport of both receptors brought about by deletion of their ligand binding domains did not result in aggregate formation. However, androgen antagonists that altered the conformation of the ligand binding domain and promoted varying rates of cytoplasmic–nuclear translocation all inhibited aggregate formation. This demonstrates that in addition to the cytoplasmic localization, a distinct contribution of the ligand binding domain of the receptor is necessary for the aggregation. The finding that antiandrogens inhibit aggregate formation may provide the basis for in vivo determination of the role of these structures in SBMA.

Key words: cytoplasmic inclusions • spinal and bulbar muscular atrophy • antiandrogens • ligand binding domain • neurodegenerative disorder

Introduction

Expansion of CAG trinucleotide repeats encoding polyglutamine stretches has been identified as a common pathogenic mutation for eight neurodegenerative diseases, including spinal and bulbar muscular atrophy (SBMA),[1] Huntington disease, dentatorubral-pallidoluysian atrophy (DRPLA), and five dominantly inherited ataxias-spinocerebellar ataxia types 1–3, 6, and 7 (for reviews see Lin et al., 1999; Perutz, 1999).

Aggregation of the polyglutamine stretches has been recognized as a common feature of all these CAG repeat–associated disorders and may act as a marker of the disease process (Sisodia, 1998). However, a direct link of the aggregates to the disease process is still elusive. In some studies, the aggregates were reported as cytotoxic (Ross, 1997). As a result, suppression of aggregation by transglutaminase inhibitors prevented apoptosis induced by dentatorubral-pallidoluysian atrophy proteins containing polyglutamine stretch amplifications (Igarashi et al., 1998). On the other hand, the aggregates have been described as originating from protein misfolding. The mutant protein supposedly adopts an altered configuration, leading to ubiquitination, aggregation, and resistance to proteosomal degradation. Inhibition of ubiquitination in one study prevented aggregation and accelerated cell death, suggesting that the aggregates may rather be beneficial and may se-

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[1]*Abbreviations used in this paper:* AR, androgen receptor; DIC, differential interference contrast; DHT, dihydrotestosterone; LBD, ligand binding domain; NLS, nuclear localization signal(s); SBMA, spinal and bulbar muscular atrophy.

quester toxic components of the disorder (Saudou et al., 1998).

Although differences in the alleged function of the aggregates persist, their reported sites of accumulation in the cell are also not too clear. In Huntington, the aggregates are present as neuronal intranuclear inclusions (Davies et al., 1997; Li et al., 1999) or outside the nucleus in the form of dystrophic neurites and neuropil aggregates (DiFiglia et al., 1997; Gutekunst et al., 1999). In SCA2 and 6, the mutant proteins accumulated in the cytoplasm, not the nucleus (Huynh et al., 1999; Ishikawa et al., 1999), whereas aggregates formed by the androgen receptor (AR) in SBMA are reported both in the cytoplasm and in the nucleus (Stenoien et al., 1999). These different reports have prompted us to reassess the cellular localization of the AR isolated from SMBA patients and to define conditions that lead to the formation of aggregates.

SBMA is a rare motor neuron disorder characterized by the adult onset of proximal muscle weakness, atrophy, and fasciculations caused by the degeneration of motor neurons. This disease is slowly progressive and is complicated by the involvement of bulbar muscles, which may lead to dysphagia and repeated aspiration pneumonia. Affected males show signs of androgen insensitivity, including gynecomastia, impaired spermatogenesis, reduced fertility, and testicular atrophy (for reviews see Merry and Fischbeck, 1998).

The disease gene was mapped to the proximal long arm of the X-chromosome specifically to the region where the AR is localized. The link between SBMA and the AR was clarified when it was shown that the CAG repeat region in the AR gene is amplified in SBMA patients (Merry and Fischbeck, 1998). Beyond the identification of the CAG trinucleotide repeat, little is known about the molecular pathogenesis of SBMA, and no effective treatment is available.

Efforts to create mouse models of SBMA for the study of the mechanism and treatment of this disorder have fallen short of reproducing the disease phenotype because of low levels of expression of the protein (Bingham et al., 1995). Similarly, cell culture systems in which the transcriptional activity of the mutant receptor was analyzed did not consistently produce results that could explain the molecular mechanisms leading to the disease (for review see Merry and Fischbeck, 1998). Reports that the mutant AR formed inclusions in cells as other proteins with amplified polyglutamine stretches (Li et al., 1998; Stenoien et al., 1999) established aggregation as a common link among the neurodegenerative diseases caused by polyglutamine tract amplifications. We have therefore undertaken these studies to examine the conditions that lead to the formation of aggregates by the mutant AR.

Materials and Methods

Cell Culture

Simian kidney COS-7 cells were cultured in DME supplemented with 10% FCS at 37°C in an atmosphere of 5% CO_2. 24 h before transfection, the cells were plated onto coverslips in 35-mm tissue culture dishes at a concentration of 5×10^4 cells/dish. Transient transfection was carried out by the Fugene transfection procedure (Boehringer Mannheim) with 1.5 μg DNA. 24 h later, the cells were cultured in phenol red–free DME (GIBCO BRL Life Technology) supplemented with 3% charcoal-treated FCS for an additional 24 h. Unless otherwise stated, the transfected cells were treated without hormone (0.1% ethanol as vehicle), with hormone (vehicle containing 100 nM DHT), or with antihormones for different periods of time. For the determination of the different stages of cellular localization of the receptor, the transfected cells were precooled for 5 min on ice and treated with hormone on ice for 1 h before the final incubation at 37°C for different periods of time. The cells were then fixed with prechilled methanol for 15 min followed by two washing steps with PBS. The coverslips were treated at room temperature with PBS containing 0.2% gelatin, 0.1% Triton X-100 for 45 min and thereafter incubated with polyclonal AR44 antibody directed against amino acids 201–222 of the AR (1:200) for 1 h. The primary antibody was detected with a TRITC-conjugated goat anti–rabbit antibody (1:100) (Dianova GmbH).

Plasmid Constructs

ARQ1, ARQ22, and ARQ77 were obtained by subcloning the corresponding AR sequence (Chamberlain et al., 1994) into the expression vector pSG5. ARdNLSQ1, ARdNLSQ22, and ARdNLSQ77 were obtained by PCR-mediated deletion of sequences corresponding to amino acids 614–634 in the ARQ1, ARQ22, and ARQ77 constructs. ARdHBDQ22 and ARdHBDQ77 were generated by the introduction of a stop codon at amino acid 682 by PCR-mediated mutagenesis.

Fluorescence Microscopy

Fluorescence microscopy and differential interference contrast (DIC) images were performed with a Zeiss Axiovert 135 microscope. All image files were digitally processed for presentation using Adobe Photoshop and printed using a Tektronix 450 printer.

Western Blot

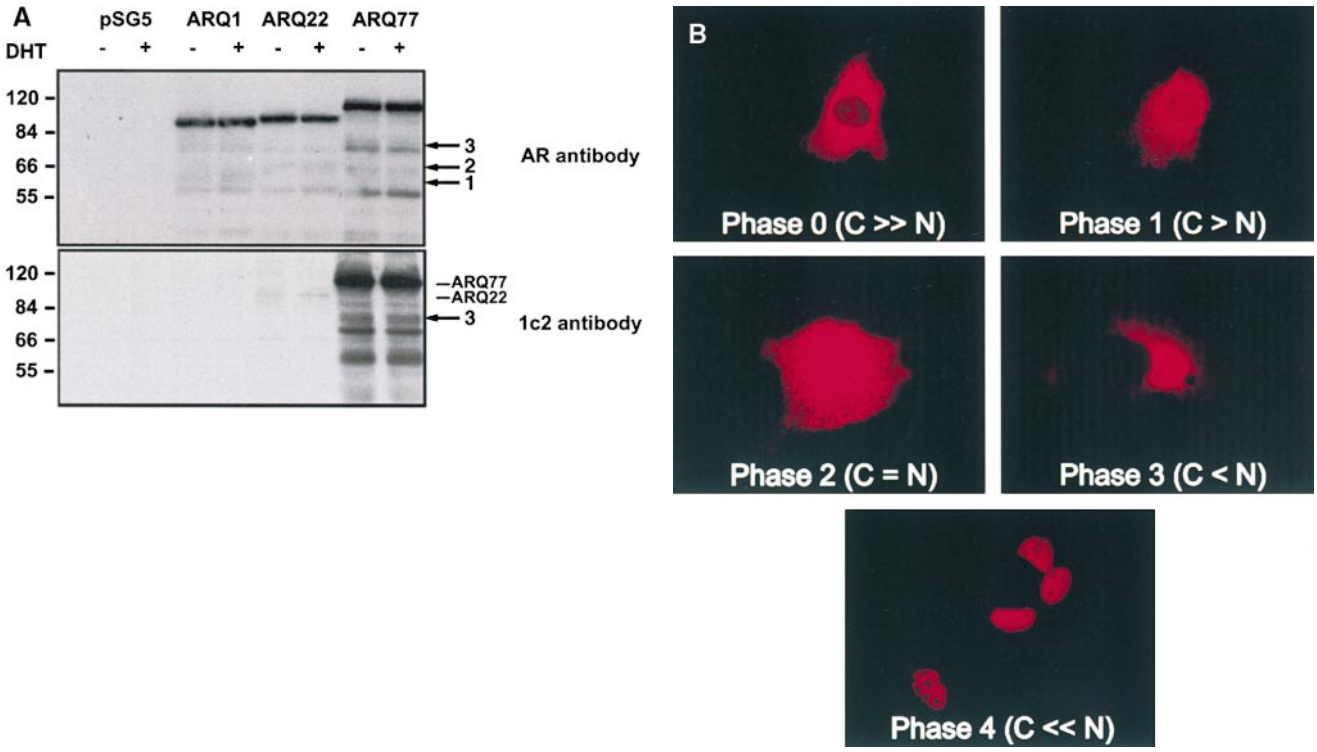
Western blot analyses to detect the AR or the polyglutamine stretch were performed as reported previously (Peterziel et al., 1999).

Results and Discussion

Nuclear Transport

To determine how amplification of the polyglutamine stretch in the AR affects subcellular localization of this receptor, the wild-type AR with 22 glutamine residues (ARQ22) and an AR with glutamine repeat amplification of 77 residues (ARQ77) were expressed in receptor-negative COS-7 cells in the absence and presence of the androgen dihydrotestosterone (DHT) (Fig. 1 A). As control, a receptor construct with deletion of its glutamine repeat sequence leaving only one residue (ARQ1) was also analyzed. Almost equal amounts of the receptors were detected in the transfected cells. In addition, low molecular weight species containing NH_2-terminal forms of the receptors were detected by the use of an antibody that recognized NH_2-terminal epitope of the AR (Fig. 1 A, arrows 1, 2, and 3 in upper panel). With the use of an antibody that detects amplified glutamine repeat stretches (Trottier et al., 1995), we identified in the lane with ARQ77 several receptor fragments containing the epitope for this antibody, including a characteristic 74-kD receptor fragment initially reported by Butler et al. (1998) (Fig. 1 A, see arrow 3 in lower panel). This fragment is supposed to be generated from a deletion of the hormone binding domain of ARQ77, and has been proposed as contributing to the toxicity of the mutant receptor by initiating the expression of specific genes in the absence of hormone (Butler et al., 1998).

The subcellular localization of the ARQ1, ARQ22, and



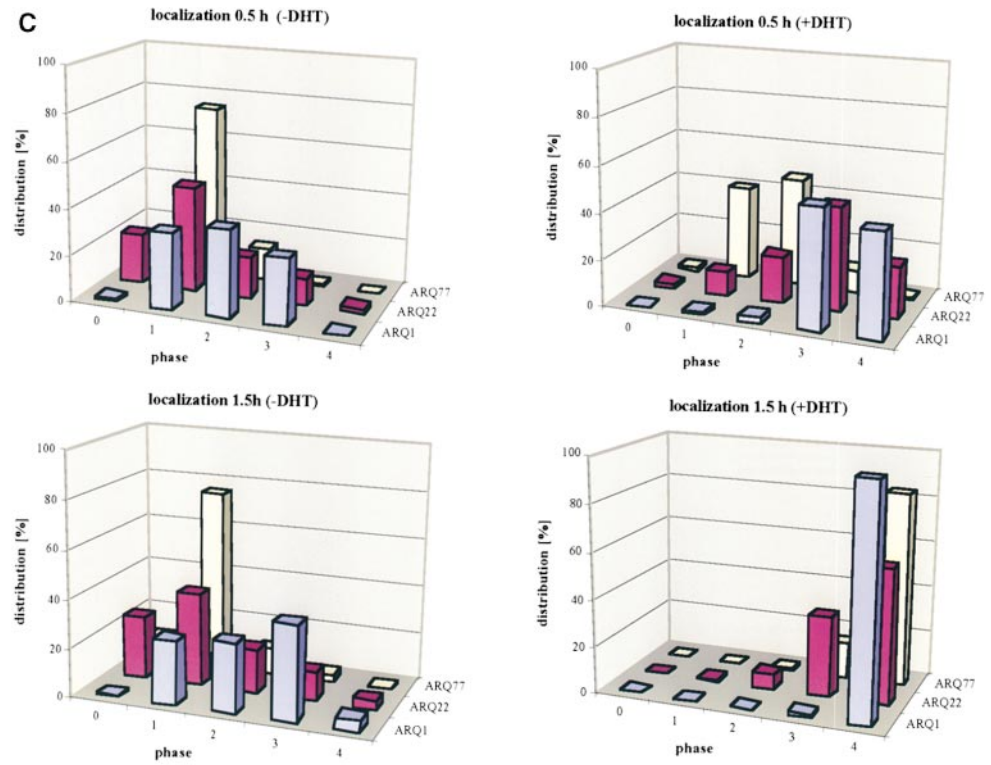


Figure 1. Subcellular localization of ARs with polyglutamine stretch of different lengths. COS-7 cells were transfected with an empty expression vector pSG5 and AR expression vectors ARQ1, ARQ22, and ARQ77, and treated with or without DHT (10^{-7} M) for 0.5 or 1.5 h. (A) Western blot analysis was performed with equal amounts of cellular proteins using the anti-AR antibody AR44 and an anti-polyglutamine repeat antibody Ic2. The bands marked 1, 2, and 3 in the upper panel are NH_2-terminal AR fragments as they are recognized by the AR antibody that detects an NH_2-terminal epitope. In the lower panel, bands corresponding to ARQ22 and ARQ77 have been indicated. Note that the Ic2 antibody recognizes the Q77 stretch more effectively than the Q22 stretch. Also recognized by the Ic2 antibody and indicated as band 3 is a 74-kD NH_2-terminal fragment of the AR containing an amplified glutamine stretch. (B) Five different phases of cellular localization of the AR defined numerically as 0–4. C, cytoplasm; N, nucleus. (C) Shown as bar diagrams are the number of cells with the transfected receptor at a particular stage of cellular localization in the presence or absence of DHT as determined by immunofluorescence microscopy.

ARQ77 was analyzed by fluorescence microscopy in the absence or after 30 or 90 min treatment with DHT. Five different phases depicting the cellular localization of the AR were scored and numerically classified as 0–4. Phase 0 was strictly cytoplasmic, whereas phase 4 showed complete nuclear localization. The phases in between 0 and 4 represented different stages of cytoplasmic–nuclear localizations (Fig. 1 B). In three independent experiments in

which a total of >11,000 cells were analyzed, it was in only a limited number of cells that nuclear localization of the AR was determined in the absence of hormone. The majority of cells containing ARQ22 and ARQ77 was scored between phases 0 and 2 (Fig. 1 C, −DHT, 0.5 and 1.5 h). Cells containing ARQ1 had a tendency to migrate into the nucleus even in the absence of hormone, and were scored between phases 1 and 3 (Fig. 1 C, −DHT, 0.5 and 1.5 h). In the presence of hormone, differences were observed in the rate of nuclear transport of the receptors. Half an hour after hormone treatment, ARQ1 was nearly nuclear (phases 3 and 4) (Fig. 1 C, +DHT, 0.5 h), whereas ARQ22 and ARQ77 were still between phases 1 and 4 (Fig. 1 C, +DHT, 0.5 h). At 1.5 h, ARQ1 was completely nuclear (phase 4), whereas the other receptors were distributed between phases 3 and 4, albeit with the majority at phase 4 (Fig. 1 C, +DHT, 1.5 h). Thus, the AR with the extended glutamine stretch less readily entered the nucleus, a trend that was more pronounced at 0.5 h than at 1.5 h of DHT treatment. At 1.5 h, when nuclear localization was nearly complete for ARQ1 and ARQ77, ARQ22 still showed some cytoplasmic localization. The reason for this is not quite clear at the moment.

Cytoplasmic Aggregates

In studies on the nuclear transport of the AR, we noticed that the receptors appeared in some of the cells as cytoplasmic aggregates. For example, 0.5 h after DHT treatment, 5% of the cells transfected with ARQ1 had several tiny inclusions in the cytoplasm (Fig. 2 A), whereas twice as many cells (11–13%) containing ARQ22 and ARQ77

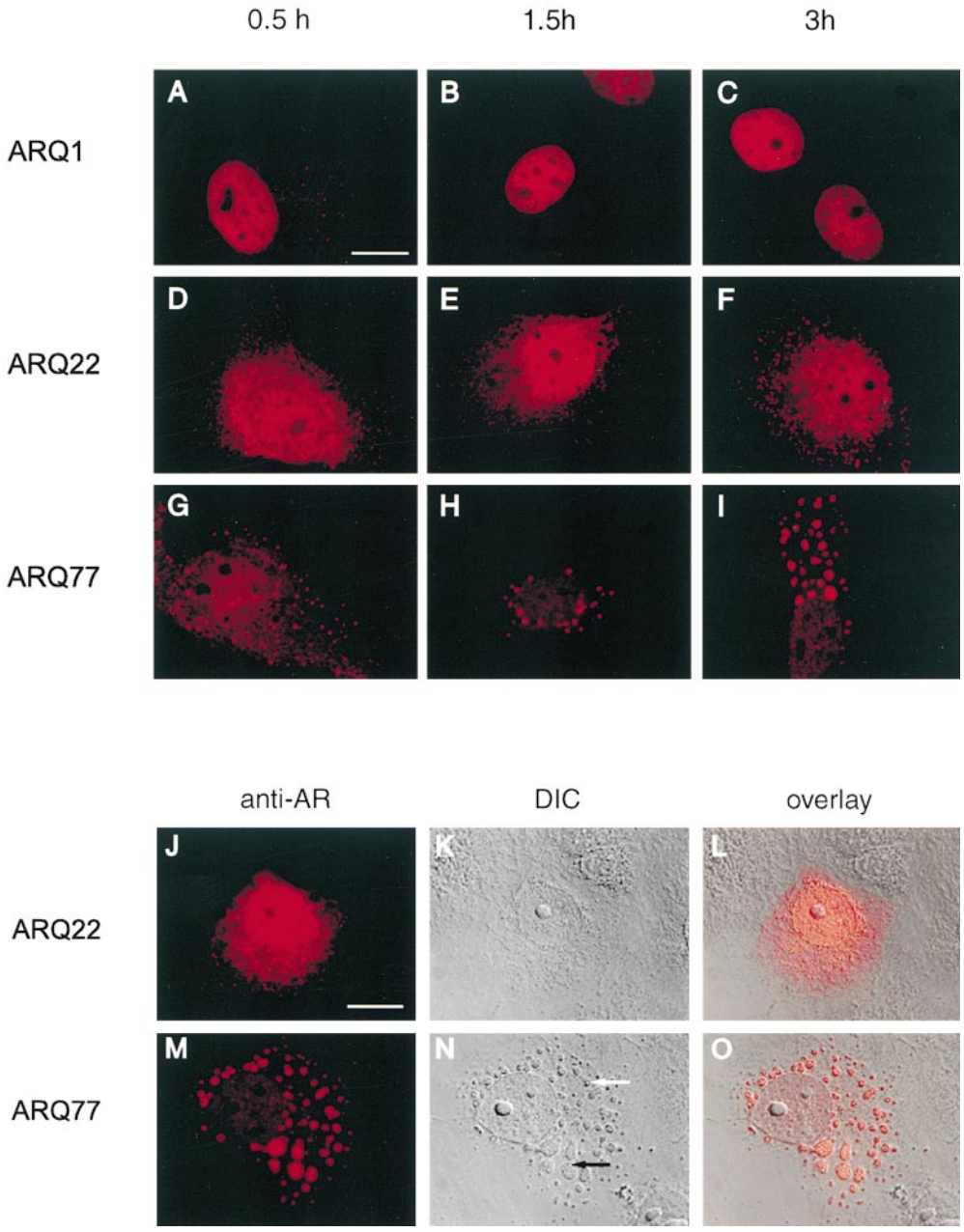


Figure 2. Detection of cytoplasmic aggregates formed by the AR constructs ARQ1, ARQ22, and ARQ77 in COS-7 cells. COS-7 cells were transfected with ARQ1, ARQ22, and ARQ77, treated with hormones and the cellular localization of the receptors was determined by fluorescence microscopy. (A–I) Shown are some of the aggregates formed by these receptors after 0.5, 1.5, and 3 h. (J–O) A comparison of the aggregates formed by ARQ22 and ARQ77 after 3 h of DHT treatment with the use of immunoflourescence experiments (J and M), DIC (K and N), and an overlay of the two images (L and O). The white arrow points to a single small inclusion and the black arrow shows aggregates in the process of being fused. Bars, 15 μm.

exhibited cytoplasmic inclusions (Fig. 2, D and G). At 1.5 and 3 h of hormone treatment, aggregates were no longer observed in the ARQ1-positive cells (Fig. 2, B and C), but 10% of the cells containing ARQ22 or ARQ77 still possessed aggregates (Fig. 2, E, F, H, and I). After 24 h of hormone treatment, no aggregates were detected in any of the cells (data not shown), possibly due to processing by the molecular chaperones of the cells.

Differences in the structure of the aggregates were evident. Those formed by ARQ1 were very small and rounded, whereas those formed by ARQ22 were slightly larger and quantitatively more than the ARQ1 aggregates (Fig. 2, A and B). In contrast, cytoplasmic aggregates formed by ARQ77 were larger and more clustered (Fig. 2, G–I). The differences in the morphology of the aggregates formed by ARQ22 and ARQ77 are more clearly demonstrated in Fig. 2, J–O, along with DIC images. It appeared that the aggregates formed by ARQ77 fused in time to form larger aggregates (Fig. 2 N, white and black arrows). The delayed nuclear transport of ARQ22 and ARQ77 compared with ARQ1 and the fact that those two receptors formed aggregates more efficiently than ARQ1 suggested that the cytoplasmic environment might contribute to aggregate formation. This notion was not shared by Perez et al. (1998), who identified the nuclear environment as the site of aggregation for a SCA3 protein fragment that contained a polyglutamine stretch amplification. The difference between that report and our study possibly arose from the use of two different gene products, the AR and SCA3.

We confirmed that the cytoplasmic environment is the site for the formation of aggregates by the AR in experiments in which we used ARQ1, ARQ22, and ARQ77 constructs lacking their nuclear localization signals (NLS)

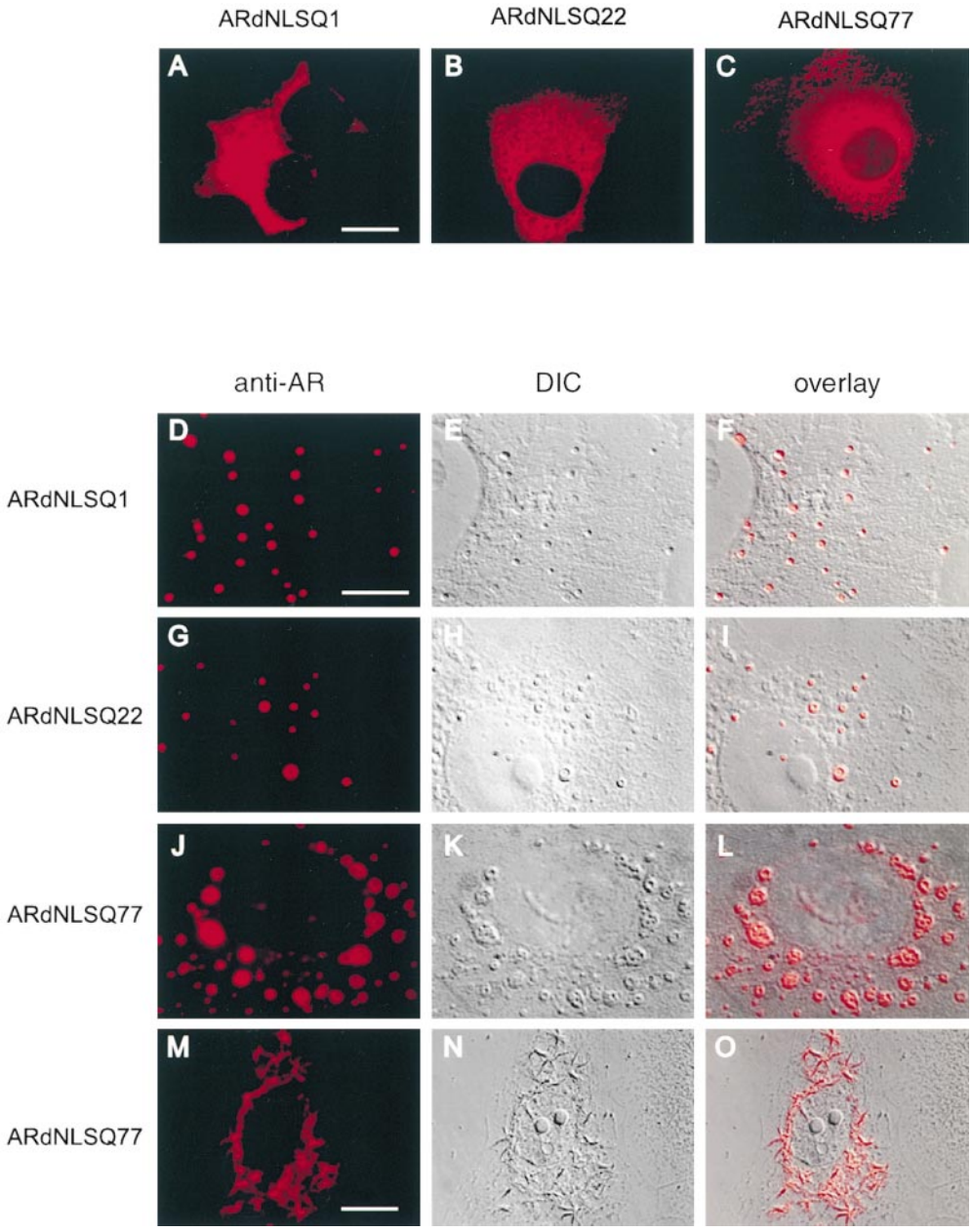


Figure 3. Detection of cytoplasmic inclusions formed by ARdNLSQ1, ARdNLSQ22, and ARdNLSQ77 in COS-7 cells. COS-7 cells were transfected with the indicated receptor constructs lacking the NLS, treated with (D–O) or without (A–C) DHT, and the cellular localization of the receptors assessed by fluorescence microscopy. Shown are the different types of aggregates formed as detected by immunofluorescence microscopy, DIC images, and an overlay of the two pictures. Bars: 15 μm (A–C); 30 μm (D–L); and 15 μm (M–O).

(ARdNLSQ1, ARdNLSQ22, and ARdNLSQ77). In the absence of hormone, all three mutant receptors showed a diffused cytoplasmic expression that was detected by fluorescence microscopy (Fig. 3, A–C). In the presence of androgen, these receptors formed inclusions that were permanently cytoplasmic (Figs. 3, D–O). The inclusions formed by the ARdNLSQ1 and ARdNLSQ22 were smaller, rounded, and on the whole similar in structure (Fig. 3, D–I). Thus, the ARQ1 and ARQ22 formed aggregates in the cytoplasm when forced to reside in this cellular compartment in the presence of hormone. These inclusions were most likely unrelated to the polyglutamine stretch, as similar structures were observed for both ARdNLSQ1 and ARdNLSQ22. In contrast, inclusions formed by ARdNLSQ77 were clearly distinct. They were larger and possessed a characteristic clustered morphology (Fig. 3, J–L). In addition, ~10% of the cells transfected with this mutant receptor formed fibrillary perinuclear star-like aggregates that were not observed in cells transfected with either the ARdNLSQ1 or ARdNLSQ22 constructs (Fig. 3, M–O).

The fibrillary aggregates formed by ARdNLSQ77 were sometimes as large as 6 μm, but it is uncertain whether they originated from the large clustered aggregates or were formed independently. From their structure, they are reminiscent of scrapie prions and β-amyloid fibrils in Alzheimer's disease (Koo et al., 1999). Upon staining with Congo red dye, a characteristic red-green birefringence in polarized light indicative of amyloidogenic inclusions was not obtained (Glenner, 1980) (data not shown). However, treatment of the transfected cells with the transglutaminase inhibitor cystamine drastically reduced the formation of the fibrillary aggregates without affecting the formation of the other aggregates (data not shown). This indicates a role of a transglutaminase reaction in the formation of the fibrillary structures. In this connection, it is worth noting that transglutaminase cross-linking and aggregation of Huntingtin fragment containing polyglutamine residues did not stain positive for amyloid (Karpuj et al., 1999), and filamentous aggregates sensitive to transglutaminase inhibitors were identified as pathognomonic of dentatorubral-pallidoluysian atrophy (Igarashi et al., 1998). The fibrillary structures described in this work may not only be formed by ARdNLSQ77 but possibly also during the delayed transport of the ARQ77 into the nucleus, and may constitute the toxic component in SBMA.

As the aggregate formation was greatly enhanced by the delayed nuclear transport of the AR, we performed the converse experiment in which rapid nuclear transport was induced to gain further information on aggregate formation. This was achieved by deletion of the ligand binding domain (LBD) of ARQ22 and ARQ77. As the LBD is known to keep the AR in the cytoplasm, these deletion mutants were constitutively nuclear but they did not form aggregates, despite the presence of the Q22 and Q77 stretches (Fig. 4). This demonstrates an important role of the LBD in the formation of aggregates.

To determine the contribution of the LBD to aggregate formation without necessarily deleting this region of the receptor, different androgen antagonists were used that are known to affect the conformation and the rates of cytoplasmic–nuclear transport of the receptors (Veldscholte et al. 1992; Kuil and Mulder, 1994; Kuil et al., 1995). In these experiments, the steroidal antagonist cyproterone acetate most efficiently promoted nuclear translocation of the receptors (Fig. 5, A and B), but not the nonsteroidal antagonists hydroxyflutamide or casodex (Fig. 5, I–L). Nonetheless, hydroxyflutamide and casodex did not induce aggregate formation by the wild-type ARQ22 and ARQ77 or by the NLS-defective mutant ARdNLSQ77 (Fig. 5, I–N). Cyproterone acetate, on the other hand, produced very tiny inclusions (Fig. 5, E and F) but these were nowhere near the large aggregates produced by DHT (Fig. 5, C and D). The difference in the action of the antagonists may be related to their structure and to their affinity to the LBD. Cyproterone acetate, a steroidal compound, binds slightly better to the AR than the nonsteroidal antagonists, hydroxyflutamide and casodex (Culig et al., 1993). In competition assays, the clustered aggregates and fibrillary depositions formed by the ARdNLSQ77 in the presence of DHT (Fig. 5, C and D) were drastically reduced by cyproterone acetate as well as by hydroxyflutamide and casodex (Fig. 5, G and H; data not shown). The biological significance of the destruction of aggregate formation by antiandrogens and the role of the cytoplasmic aggregation in SBMA in general are to be investigated in the future in transgenic mouse models. In this connection, cytoplasmic aggregate formation brought about by the ARdNLSQ77 will be targeted to specific areas of the brain of the mouse to find out whether they give rise to the neurodegenerative disorders described in SBMA.

Taken together, our results show that although cytoplasmic localization of the AR with polyglutamine stretch am-

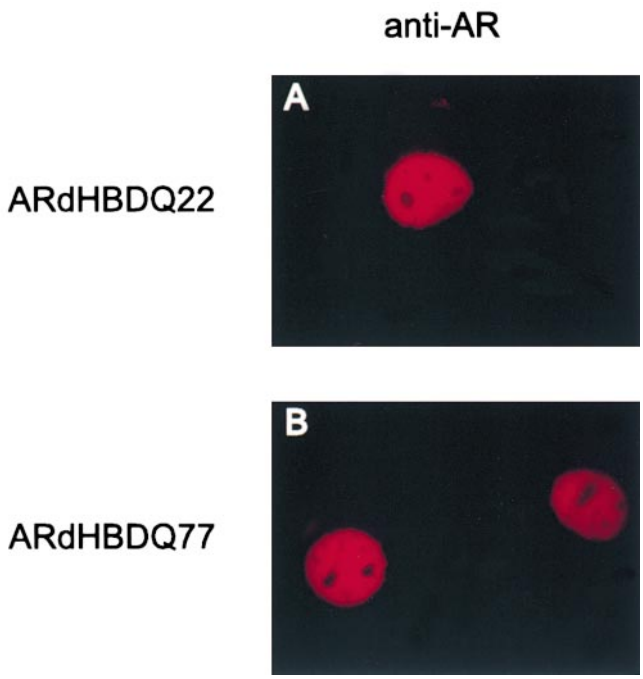


Figure 4. Androgen receptor lacking the LBD does not form aggregates. COS-7 cells were transfected with ARQ22 and ARQ77 lacking the LBD (ARdHBDQ22, ARdHBDQ77) and cellular localization of the receptor was determined by fluorescence microscopy.

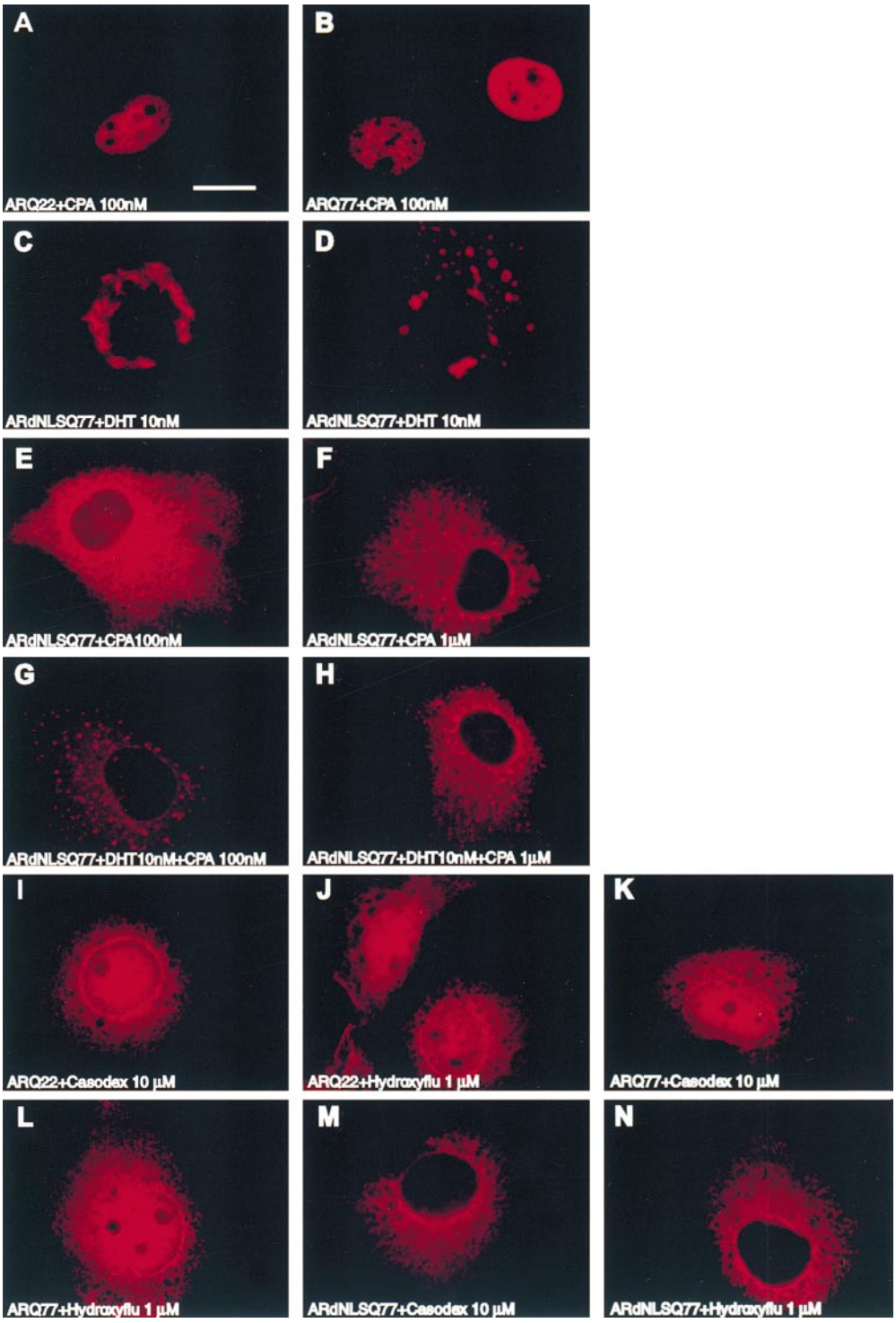


Figure 5. Cytoplasmic aggregation of the AR is not induced by antiandrogens. COS-7 cells were transfected with the receptor constructs ARQ22, ARQ77, and their counterparts lacking the NLS (ARdNLSQ22, ARdNLSQ77). The transfected cells were treated with the indicated concentrations of DHT and the antiandrogens cyproterone acetate (CPA), hydroxyflutamide (Hydroxyflu) or casodex for 3 h. The effect of these ligands on aggregate formation was detected by immunofluorescence microscopy. Bar, 15 μm.

plification may play a crucial role in the formation of aggregates, the LBD and the type of ligand bound are essential in this process. This latter point is important in view of the fact that, in the past, androgens were used for the treatment of SBMA, but rather unsuccessfully (Danek et al., 1994; Neuschmid-Kaspar et al., 1996). The ineffectiveness of the androgen therapy could be mechanistically accounted for by the induction of aggregate formation by these ligands. Our findings that antiandrogens inhibited aggregate formation would therefore form a useful starting point for determining the contribution of these structures to SBMA, and could provide the basis for a possible therapeutic intervention of this disorder.

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