Spinal and bulbar muscular atrophy (SBMA, Kennedy's disease) is one of a group of progressive neurodegenerative diseases resulting from a polyglutamine repeat expansion. In SBMA the polymorphic trinucleotide CAG repeat in exon 1 of the androgen receptor (AR) gene is increased, resulting in expansion of a polyglutamine tract. Patient autopsy material reveals neuronal intranuclear inclusions (NII) in affected regions that contain only amino-terminal epitopes of the AR. Cell models have previously been unable to produce intranuclear inclusions containing only a portion of the AR. We report here the creation of an inducible cell model of SBMA that reproduces this important characteristic of disease pathology. PC12 cells expressing highly expanded AR form ubiquitinated intranuclear inclusions containing amino-terminal epitopes of the AR as well as heat shock proteins. Inclusions appear as distinct granular electron-dense structures in the nucleus by immunoelectron microscopy. Dihydrotestosterone treatment of mutant AR-expressing cells results in increased inclusion load. This model mimics the formation of ubiquitinated intranuclear inclusions containing the amino-terminal portion of AR observed in patient tissue and reveals a role for ligand in the pathogenesis of SBMA.

X-linked spinal and bulbar muscular atrophy is a progressive neuromuscular disorder that is one of a group of neurodegenerative diseases, including Huntington’s disease, Dentatorubral-pallidoluysian atrophy, and several spinocerebellar ataxias (SCA1, -2, -3, -6, -7, -17), caused by expansion of a trinucleotide CAG repeat in exon 1 of the androgen receptor (Ref. 1, reviewed by Refs. 2 and 3). The pathophysiology of SBMA is characterized by the loss of motor and sensory neurons (7) and by the finding of AR-containing ubiquitinated neuronal intranuclear inclusions (NII) in spinal motor neurons (8). Ubiquitin-positive intranuclear inclusions also appear in affected neurons in Huntington’s disease, SCA3 (Machado-Joseph disease), SCA7, SCA17, and dentatorubral-pallidoluysian atrophy (1, 9–13), indicating that expanded polyglutamine proteins abnormally accumulate in these diseases.

Cell culture and transgenic mouse studies of SBMA and other polyglutamine diseases indicate that these diseases result from toxic properties of the mutant expanded polyglutamine protein, part or all of which accumulates in NII caused in part by its reduced turnover (14). Whether the development of inclusions represents one of the toxic properties of expanded polyglutamines is unclear. Inclusion formation was not associated with toxicity in several model systems (15–17). Although NII may be neither necessary nor sufficient for neuronal dysfunction and death, their presence signals the inefficient clearance of the mutant protein.

Previous models of SBMA have revealed the formation of NII and the sequestration of a variety of proteins involved in protein degradation and AR transcriptional function. Transiently transfected HeLa cells expressing full-length expanded AR treated with ligand show an accumulation of AR into cytoplasmic aggregates (with rare NII) that contain Hsp70, Hsp90, NEDD8 (a ubiquitin-like protein), PA700 (26 S proteasome cap), SRC-1 (steroid receptor coactivator 1), and mitochondria (18). In addition, histological studies of mice expressing a truncated, highly expanded form of AR revealed intranuclear inclusions positive for ubiquitin, the molecular chaperones Hsp42 and Hsc70, components of the 26 S proteasome, and CBP (CREB-binding protein) (19). The sequestration of molecular chaperones and subunits of the proteasome (18–22) suggests that neurons are unable to efficiently fold and degrade expanded polyglutamine proteins.

Of the various cell culture models developed to study the pathogenesis of SBMA (17, 18, 23–28), none have succeeded in reproducing the nuclear inclusions containing only amino-terminal epitopes of the AR protein seen in SBMA patients (8). Therefore, we created an inducible PC12 cell model of SBMA in which full-length highly expanded AR112 is expressed under control of a tetracycline-inducible promoter. Treatment with dihydrotestosterone (DHT) greatly enhanced the formation of cells induced to express full-length AR112. These inclusions contained only amino-terminal epitopes of AR. Ubiquitin and the molecular chaperone Hsc70 co-localized with AR in these NII, consistent with the mutant protein being
targeted for degradation. Analysis by electron microscopy revealed a granular rather than fibrillar appearance to the NII as observed in patient tissue (8).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies to the AR (N20, AR441, C19), actin, p53, CBP (C1), SRC-1, and cyclin D1 were purchased from Santa Cruz Biotechnology. Anti-ubiquitin antibody was obtained from DAKO. Antibodies to the 20 S proteasome core, 19 S ATPase subunit TP7, and subunit PA28g of the 11 S proteasome regulator were purchased from StressGen Biotechnologies. Other antibodies include H222 (NeoMarkers Inc.), AR (AR315) (DNAtech), Hsp90 (Neomarkers), Hsp70 (Affinity BioReagents), and IC2 (Chemicon International).

Establishment of Inducible PC12 Cell Lines—PC12 Tet-On cells (Clontech) were transfected using LipofectAMINE Plus (Invitrogen) with pTRE-AR112 or pTRE-AR10 constructs and a plasmid conferring hygromycin resistance (pTRE-hygromycin, a kind gift from K. Fischbeck). Full-length AR cDNA containing 112 CAG repeats (pTRE-AR112) was obtained from K. Fischbeck (National Institutes of Health) and was created by cloning the insert from pSP64-AR112 (a kind gift from Y. Kobayashi) into the pTRE vector (Clontech). An AR cDNA plasmid containing 10 CAG repeats was derived from pTRE-AR112 by successive transformation. Stable transformants were selected with 200 μg/ml hygromycin. Single colonies were isolated and expanded, induced with 10 μg/ml doxycycline, and screened for transgene expression by Western blot using the N20 antibody. Cells were maintained in normal growth media (Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% heat-inactivated horse serum (Invitrogen), 5% Tet-approved fetal bovine serum (Clontech), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin/streptomycin (Invitrogen), 200 μg/ml hygromycin (Invitrogen), and 100 μg/ml G418 (Mediatech)) at 37°C, 10% CO2.

Biochemical Analysis—To determine expression levels, cells were plated onto collagen-coated dishes (BD Biosciences) and grown for 24 h with or without 10 μg/ml doxycycline (Sigma). Cells were lysed in RIPA assay buffer (1% Igepal, 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 100 μg/ml phenylmethylsulfonyl fluoride) and sonicated. Proteins were separated on 10% Novex Tris/glycine polyacrylamide gels (Invitrogen) and transferred to Immobilon-P membrane using a semidry transfer apparatus (Fisher Scientific). Western development was performed as described (14).

Immunofluorescence—Cells were seeded onto collagen-coated dishes (BD Biosciences) and allowed to adhere overnight. Cells were then differentiated by growth in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated horse serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin/streptomycin (Invitrogen), 200 μg/ml hygromycin (Invitrogen), and 100 μg/ml G418 (Mediatech). Cells were incubated with AR antibody (N20) at 1:1000 in 0.1% bovine serum albumin/PBS and developed with H2O2 and diaminobenzidine by incubation with a secondary antibody conjugated to horseradish peroxidase (horse- radish peroxidase-conjugated secondary antibody at 1:100 in 0.1% bovine serum albumin/PBS) overnight at 4°C followed by horse-radish peroxidase-conjugated secondary antibody at 1:100 in 0.1% bovine serum albumin/PBS and developed with H2O2 and diaminobenzidine. Diaminobenzidine was enhanced using a modified Rodriguez silver/gold enhancement method (29). Cells were then fixed in 2% glutaraldehyde/PBS overnight, dehydrated in a graded ethanol series, and embedded in Epon. Epon blocks were cut on an ultra microtome, stained with 1% uranyl acetate, and analyzed using a JEOL 1010 transmission electron microscope.

Cell Survival—Cells that were differentiated for 2 days were treated with 10 μg/ml doxycycline and 50 μM dihydrotestosterone for times as indicated. 100 μg/ml nerve growth factor was added daily to maintain cells in a differentiated state. Cells were maintained for a total of 6 days in culture. One day of treatment on the graph represents 2 days of differentiation followed by differentiation with the addition of ligand on the last of the 6 days in culture. Cells were all harvested on the same day to eliminate differences in viability based on the number of days in culture. A cytotoxicity assay was performed for cell survival using the Cytotoxicity 96 kit (Promega).

**RESULTS**

Expression of AR in PC12-inducible Cell Lines—We established inducible PC12 cell lines that express normal human AR with 10 glutamines (Q10) or a highly expanded mutant AR with 112 glutamines (Q112). Western analysis revealed expression of the AR protein with 10 or 112 glutamates at the expected molecular weight (Fig. 1, lanes 2 and 4). The PC12 Tet-On parental cell line showed no endogenous AR protein expression with the AR antibody (N20), which recognizes rat and human forms of the androgen receptor (data not shown). All clones displayed low levels of transgene expression in the absence of induction (Fig. 1, lanes 1 and 3). Clones with the highest ratio of induced to uninduced expression were chosen for further analysis.

Androgen Enhances the Formation of Intranuclear Inclusions—We analyzed the subcellular localization of the mutant and normal AR and the effect of ligand binding on this distribution using an antibody to the amino terminus of the AR (N20). Upon induction of AR expression and treatment with DHT, we observed a granular rather than fibrillar appearance to the NII (Fig. 1, data not shown), indicating that both normal and mutant proteins were able to translocate into the nucleus following hormone treatment. After 3 days of induction in the absence of ligand, a small number of cells contained multiple intranuclear inclusions containing the mutant AR112 protein (Fig. 2C). Treatment with DHT increased the frequency of inclusions nearly 7-fold (Fig. 2C). In addition, the percentage of cells with NII increased with the length of exposure to ligand (Fig. 2A and data not shown), indicating that both normal and mutant proteins were able to translocate into the nucleus following hormone treatment. After 3 days of induction in the absence of ligand, a small number of cells contained multiple intranuclear inclusions containing the mutant AR112 protein (Fig. 2C). Treatment with DHT increased the frequency of inclusions nearly 7-fold (Fig. 2C). In addition, the percentage of cells with NII increased with the length of exposure to ligand (Fig. 2A and B). AR-positive NII were not detected in PC12 parent cells (data not shown) or in AR10-expressing cells following 14 days of ligand treatment (Fig. 2A).

The formation of NII was not completely dependent upon ligand treatment. Cells given doxycycline alone showed inclusion formation at 3 and 7 days of treatment (Fig. 2C). However, ligand treatment greatly enhanced NII formation at both time points. Cells treated with DHT alone also showed a small number of NII, consistent with the somewhat leaky expression of this inducible gene. To determine whether the formation of NII observed without hormone treatment resulted from serum-derived hormones, cells were grown in hormone-free charcoal
Testosterone in SBMA

The Androgen Receptor

The observation that NII in SBMA—shown here. AR10- and AR112-expressing cell lines to confirm the findings. All experiments were carried out in at least two additional formations with induction/ligand treatment (data not shown). In undifferentiated cells resulted in similar rates of inclusion formation. AR antagonist flutamide resulted in both the nuclear translocation of the AR and the formation of NII (Fig. 2A). Treatment with the AR and the formation of NII in AR112-expressing cells (Fig. 2A).

The formation of NII did not depend upon differentiation of cells to create a neuronal phenotype. Experiments carried out in undifferentiated cells resulted in similar rates of inclusion formation with induction/ligand treatment (data not shown). All experiments were carried out in at least two additional AR10- and AR112-expressing cell lines to confirm the findings shown here.

Intranuclear Inclusions Contain Amino-terminal Epitopes of the Androgen Receptor—The observation that NII in SBMA patient tissue were exclusively detected with amino-terminal antibodies (8) prompted us to determine the regions of the AR detected in PC12 NII. NII were observed with the amino-terminal antibodies AR318 (data not shown). However, nuclei containing NII were not visible with antibodies that detect amino acids 299–315 (AR441) (Fig. 3A, panels e and f) or the carboxyl terminus (C19) (Fig. 3A, panels g and i). Antibodies 441 and C19 revealed a diffuse nuclear distribution of the AR protein in cells without NII (Fig. 3A, panels e and g). However, nuclei containing NII showed a complete lack of staining with AR441 and C19 (Fig. 3A, panels d–i), indicating that most or all of the AR protein in cells with inclusions has been proteolytically processed and sequestered into NII.

Intranuclear Inclusions Sequester Ubiquitin and Heat Shock Proteins—Molecular chaperones and proteasome components as well as transcriptional regulators have been observed in NII of other polyglutamine models. We performed immunofluorescence studies to determine whether any of these proteins are sequestered into AR112 NII, particularly those involved with protein folding, degradation, and AR function. Nuclear inclusions of AR112 protein contain ubiquitin (Fig. 3A), only amino-terminal epitopes are detected within NII. AR112-expressing PC12 cells treated with DHT for 7 days show NII detected by the amino-terminal antibodies N20 (a) and AR318 (b) (merged image in c). NII detected by N20 (d) were not detected by AR441 (e), an antibody that detects amino acids 299–315 (merged image in f). NII visualized with the amino-terminal antibody AR318 (h) were not detected by the carboxyl-terminal antibody C19 (g) (merged image in i). B, ubiquitin and Hsc70 co-localize with AR in intranuclear inclusions. Ubiquitin antibody (a) detects NII shown with AR318 (b) (merged image in c). Hsc70 antibody (c) detects NII shown with N20 (d) (merged image in f).

Intranuclear Inclusions Are Electron-dense Granular Structures—Immunoelectron microscopy with the amino-terminal antibody N20 revealed immunogold labeling of granular ele-
tron-dense structures in the nucleus distinct from the nucleolus (Fig. 4). These findings are similar to electron microscopy studies from SBMA patient tissue (30) in which granular AR-positive inclusions were found within the nucleus.

**DISCUSSION**

The creation of cellular and animal models that reproduce symptoms of SBMA is critical for understanding the molecular and cellular pathways that mediate disease pathogenesis. Cell models of SBMA that replicate the altered mutant AR metabolism and NII formation observed in patients have been difficult to develop. Previous cell models of SBMA created by transient transfection of full-length expanded AR have produced predominantly cytoplasmic aggregates containing the entire AR protein (17, 18, 25, 28). These models have revealed a role for ligand binding in promoting an altered conformation of the mutant AR leading to aggregate formation, but they did not reproduce the nuclear inclusions found in patients with SBMA.

We have developed a cell model in which cells expressing AR112 form AR-containing, ubiquitinated intranuclear inclusions. These NII are detected exclusively with antibodies to amino-terminal epitopes of the AR protein, as are the inclusions observed in patient tissue (8). Ligand treatment leads to increased NII formation, consistent with a role for nuclear localization, altered conformation, and aberrant proteolysis in the development of AR NII.

The expression of high levels of mutant AR protein in inducible PC12 clones resulted in nuclear inclusion formation, whereas expression of protein by cells transiently transfected with mutant AR has failed to reproduce this aspect of pathology. This phenomenon may be related to the temporal expression of mutant protein in a stable inducible cell line versus a transient transfection system. It may also be specific to PC12 cells. The type of cell used for transient transfection experiments with a truncated expanded AR altered the location of inclusions within the cell (26). Expression of truncated expanded AR in COS-7 cells produced cytoplasmic aggregates, whereas the same construct expressed in MN-1 cells produced NII. Furthermore, studies have shown that the context of proteins in a particular cell line can alter where expanded full-length AR forms aggregates, whether in the cytoplasm or nucleus (31). Therefore, it is possible that mutant AR adopts an altered conformation that results in protein associations unique to PC12 cells leading to the formation of NII. PC12 cells might thus exhibit properties in common with motor neurons that reproduce the environment necessary for the formation of NII in SBMA.

The neuronal inclusions found in SBMA patient tissue are detected exclusively by antibodies to amino-terminal epitopes (8). The inclusions shown here in AR112-expressing PC12 cells mimic this specific aspect of SBMA pathology. AR112-containing NII are also ubiquitinated, as seen in patient tissue. Whereas previous cellular and transgenic models of SBMA have revealed Hsp70, Hsc70, Hdj2, Hsp90, components of the 26 S proteasome, SRC-1, and CBP within AR-containing aggregates (18, 19), the NII in AR112-expressing PC12 cells contain only Hsc70. These cells may require longer periods of treatment than our growing conditions would allow for certain proteins to accumulate in the inclusions. In addition, there may be other unknown proteins necessary for neuronal survival and function sequestered in these NII that we have yet to identify.

The presence of amino-terminal epitopes in NII suggests that AR112 is proteolytically processed prior to or during aggregate formation. This may be a common pathogenic mechanism in many polyglutamine diseases, because amino-terminal epitopes of expanded polyglutamine protein have been detected in patient tissue in Huntington’s disease (10), dentatorubral-pallidoluysian atrophy (32), and SBMA (8). Biochemical evidence for such polyglutamine-containing fragments has been demonstrated for Huntington’s disease (10, 33), dentatorubral-pallidoluysian atrophy (32), and an inducible cell model expressing full-length expanded huntingtin (34). Whereas we have been unable to identify a protein fragment that is enriched in the nuclear fraction using biochemical approaches, immunofluorescence studies indicate that AR112 is indeed processed to form a fragment that aggregates in the nucleus in this cell system.

The formation of NII in AR112-expressing PC12 cells was not entirely ligand-dependent, as these structures were observed at low frequency in the absence of dihydrotestosterone in charcoal-stripped serum (Fig. 2C). The formation of NII in the absence of administered ligand may result from low levels of nuclear AR being present, even in the absence of exogenous ligand (data not shown), or from the incomplete removal of hormones from the charcoal-stripped serum. Nonetheless, administration of DHT substantially enhanced NII formation as did the antagonist flutamide. Treatment with the antagonist flutamide resulted in the nuclear translocation of AR112 and led to the formation of NII at a similar frequency (data not
shown). These results suggest that the localization of the AR to the nucleus is critical to NII formation. Moreover, these findings suggest that hormone levels are critical for the development of this pathologic feature of SBMA and provide a molecular explanation for the lack of symptoms in female carriers of the disease. Indeed, females are protected by the inactivation of the mutant AR in approximately half of their cells; our data indicate that they are also protected by low levels of circulating androgens.

DHT treatment of AR112-expressing cells resulted in progressive cell death. However, a similar loss of cell survival was not seen in AR10-expressing cells. Differentiation was found to make these cells more susceptible to AR-mediated toxicity, as seen in AR10-expressing cells. Differentiation was found to be a late event in the progression of disease pathology.

We have developed a model that reproduces several characteristics of SBMA pathology previously lacking in other cellular models (16, 17). Furthermore, in mouse models of SBMA (19) severe neurological dysfunction was found in the absence of detectable neuronal loss, suggesting that cell death may be a late event in the progression of disease pathology.

We have developed a model that reproduces several characteristics of SBMA pathology previously lacking in other cellular models, and we have demonstrated a role for ligand in the development of cellular pathology. This model will prove invaluable for biochemical studies of altered mutant AR metabolism and proteolysis and of cellular dysfunction resulting from the expression and abnormal accumulation of the mutant AR protein.

Acknowledgments—We thank Kenneth Fischbeck, Addis Taye, Paul Taylor, and Yasushi Kobayashi for the TRE-AR112 construct and Huiyi Wang and Seth Gilbert for technical support. We also thank Christina Wilson, Neelima Shah, and Kevin Yu for help with electron microscopy and Michael King for the use of the Leica microscope.

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Ligand Promotes Intranuclear Inclusions in a Novel Cell Model of Spinal and Bulbar Muscular Atrophy
Jessica L. Walcott and Diane E. Merry

J. Biol. Chem. 2002, 277:50855-50859. doi: 10.1074/jbc.M209466200 originally published online October 17, 2002

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