Truncated forms of the androgen receptor are associated with polyglutamine expansion in X-linked spinal and bulbar muscular atrophy

Rachel Butler*, P. Nigel Leigh, Michael J. McPhaul1 and Jean-Marc Gallo*

Department of Clinical Neurosciences, Institute of Psychiatry and King’s College School of Medicine and Dentistry, De Crespigny Park, London SE5 8AF, UK and 1Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235-8857, USA

Received September 5, 1997; Revised and Accepted September 29, 1997

X-linked spinal and bulbar muscular atrophy (SBMA) is a rare form of motor neuron degeneration linked to a CAG repeat expansion in the first exon of the androgen receptor gene coding for a polyglutamine tract. In order to investigate the properties of the SBMA androgen receptor in neuronal cells, cDNAs coding for a wild-type (19 CAG repeats) and a SBMA mutant androgen receptor (52 CAG repeats) were transfected into mouse neuroblastoma NB2a/d1 cells. The full length androgen receptor proteins, of 110–112 kDa and 114–116 kDa for the wild-type and mutant protein, respectively, were detected by Western blotting in transfected cells. In addition, the presence of an expanded polyglutamine tract in the SBMA androgen receptor appears to enhance the production of C-terminally truncated fragments of the protein. A 74 kDa fragment was particularly prominent in cells expressing the SBMA androgen receptor. From its size, it can be deduced that the 74 kDa fragment lacks the hormone binding domain but retains the DNA binding domain. The 74 kDa fragment may therefore be toxic to motor neurons by initiating the transcription of specific genes in the absence of hormonal control. Immunofluorescence microscopy on transfected NB2a/d1 cells showed that, after hormone activation, the wild-type androgen receptor translocated to the nucleus whereas the SBMA androgen receptor was mainly localized in the cytoplasm in the form of dense aggregates with very little androgen receptor protein in the nucleus. This could explain the reduction in transcriptional activity of the SBMA mutant as compared with wild-type androgen receptor.

INTRODUCTION

X-linked spinal and bulbar muscular atrophy (SBMA), or Kennedy’s disease, is a rare form of adult hereditary motor neuronopathy characterized by progressive muscle weakness and atrophy, usually beginning in the third to fifth decade of life (1). The main pathological feature of SBMA is a marked loss and atrophy of lower motor neurons, particularly of the anterior horn cells of the entire spinal cord (2–4). SBMA is associated with an expansion of a polymorphic CAG repeat sequence in the first exon of the androgen receptor gene coding for a polyglutamine tract in the N-terminal domain of the protein (5). In the normal population, the size of the CAG repeat region ranges from 12 to 30 repeats but, in patients with SBMA, the expanded domain varies in size between 40 and 72 repeats (5–9).

In addition to SBMA, a number of neurogenetic diseases are linked to trinucleotide repeat expansion mutations in the coding region of the gene involved. These include Huntington’s disease, spinocerebellar ataxias, dentatorubral-pallidoluysian atrophy and Machado-Joseph disease. These are all slow progressive neurodegenerative disorders affecting selective populations of neurons [for a review see (10)].

The striking similarity of these mutations suggests a common pathological mechanism leading to the modified protein having acquired a neurotoxic function. Expanded polyglutamine stretches in proteins could modify the affinity for interacting proteins, as shown for huntingtin, the product of the Huntington’s disease gene (11,12). Polyglutamine stretches have been shown to form stable β-pleated sheet or ‘polar zippers’ which enable protein–protein interaction (13,14). When the polyglutamine repeat tract expands beyond a certain number of glutamines it may become an avid substrate for transglutaminases leading to protein aggregates within the cell (15–17).

The androgen receptor is expressed in diverse neuronal populations within the brain and spinal cord, but is preferentially localized in motor nuclei of cranial nerves and in motor neurons of the spinal cord (18–20). Androgens influence the survival of facial and hypoglossal motor neurons and increase the rate of regeneration of their axons after axotomy (21–26). Androgens also have a trophic effect on the sexually dimorphic neurons of the spinal nucleus of the bulbocavernosus (27,28).

The capability of the SBMA mutant androgen receptor to transactivationally regulate gene expression in non-neuronal (29,30) or neuronal cells (31) is reduced in comparison with that...
of the normal androgen receptor and is inversely related to the length of the polyglutamine tract (32). This, together with the decrease in maximal androgen binding of the SBMA androgen receptor (33–35), would explain the mild androgen insensitivity symptoms often present in patients with SBMA, such as reduced fertility, gynaecomastia and feminized skin changes.

The reduced activity of the androgen receptor cannot, however, explain the specific cellular death of motor neurons in SBMA as there is no sign of motor impairment in complete androgen insensitivity syndromes caused by mutations in the androgen receptor. Therefore, the mutation associated with SBMA is likely to cause a gain, rather than a loss, of function of the androgen receptor, at least in motor neurons.

The aim of this work was to investigate the properties of the SBMA mutant androgen receptor in neuronal cells. Here we show that the presence of an expanded polyglutamine tract in the androgen receptor leads to the increased production of C-terminally truncated fragments of the protein, which could trigger the abnormal expression of specific genes. In addition the SBMA androgen receptor appears to have a propensity to aggregate in the cytoplasm of transfected cells rather than translocate to the nucleus after hormone stimulation.

RESULTS

Expression of the wild-type and mutant androgen receptors in cultured cells
cDNAs coding for a wild-type human androgen receptor containing 19 CAG repeats (hARWT) and a SBMA mutant androgen receptor with 52 CAG repeats (hARSBMA) were subcloned into the mammalian expression vector pCMV under the transcriptional control of the CMV promoter. These plasmids were tested for their ability to drive expression of the androgen receptor in mammalian cells by transiently transfecting COS-7 cells. Western blot analysis of transfected COS-7 cells with the pAnti-AR antibody revealed high levels of ectopic expression of the androgen receptor (Fig. 1A). The hARWT protein had the predicted molecular weight of 110–112 kDa (Fig. 1A, lane 1). In contrast, and as expected from the presence of the expanded polyglutamine tract, the electrophoretic mobility of the hARSBMA protein was slightly reduced, with a molecular weight of ∼114–116 kDa (Fig. 1A, lane 2). The endogenous androgen receptor in untransfected cells was below the level of detection.

The vectors coding for hARWT and hARSBMA were also used to transfected mouse neuroblastoma NB2a/d1 cells, in both non-differentiated and differentiated states. NB2a/d1 cells are a subclone of the C1300 cell line and have been extensively characterized (36,37). NB2a/d1 cells can be differentiated into a neuronal phenotype and high transfection efficiencies can be achieved in the differentiated state (38). As in COS-7 cells, immunoreactive protein bands were detected in transfected cells at ∼110 kDa and 114 kDa corresponding to the hARWT and hARSBMA proteins, respectively (Fig. 1B, C).

In addition to the bands corresponding to the full-length androgen receptor proteins, another immunoreactive band of ∼74 kDa was specifically observed in cell lysates from COS-7 cells and NB2a/d1 cells transfected with hARSBMA. Interestingly this low molecular weight form of the androgen receptor appeared to be less abundant in undifferentiated than in differentiated cells. A faint band of 93 kDa could also be observed in differentiated NB2a/d1 cells expressing hARSBMA (Fig. 1C, lane 2).

Characterization of the androgen receptor fragments

In order to determine whether androgen receptor species equivalent to the 74 kDa and 93 kDa forms observed in cells expressing hARSBMA were also generated in cells expressing hARWT, increasing amounts of cell lysates from transfected differentiated NB2a/d1 were analyzed by Western blotting with the antibody pAnti-AR (Fig. 2). A single fragment of ∼83 kDa was detected at high protein loading in cells expressing hARWT. By contrast, two fragments, of ∼93 kDa and 74 kDa, were observed in cells expressing hARSBMA. The band corresponding to the 93 kDa species appeared more intense than the 83 kDa species generated by the wild-type androgen receptor, which could only be visualized at the highest loadings (Fig. 2, lane 4). Quantification of the ratios of fragment/full-length protein proved to be difficult as the fragments only represent a small proportion of the full-length protein under the experimental conditions used.

The antibody used, pAnti-AR, was elicited against a domain of the androgen receptor corresponding to amino acids 331–572 (39). Thus, the low molecular weight immunoreactive species contains at least part of this domain. In order to elucidate which parts of the androgen receptor protein the 74 kDa, 83 kDa and 93 kDa fragments corresponded to, they were characterized using antibodies to the N- and C-terminal domains of the full-length protein. These antibodies recognize epitopes in the N-terminal (residues 1–21) and C-terminal (residues 898–917) regions of the protein (40). Western blot analysis using these two antibodies was carried out on cell lysates from differentiated NB2a/d1 cells transfected with hARWT and hARSBMA (Fig. 3). Both antibodies recognized the full-length androgen receptor, wild-type or SBMA mutant. In addition, the anti-N-terminal antibody detected another polypeptide at a molecular weight of ∼83 kDa in cells transfected with the wild-type androgen receptor and two polypeptides at ∼93 kDa and 74 kDa, in cells transfected with the...
SBMA androgen receptor (Fig. 3A). These fragments were identical in size to those visualized with the pAnti-AR antibody used previously and most likely represent the same molecular entities. The anti-C-terminal antibody detected the full-length androgen receptor, either wild-type or mutant, but failed to react with the smaller species revealed by the anti-N-terminal antibody (Fig. 3B). The faint bands in the 68–97.4 kDa region do not correspond to bands stained with the N-terminal-specific antibody. Molecular weights (kDa) are indicated on the right.

**Localization of wild-type and SBMA mutant androgen receptor in transfected NB2a/dl cells**

The subcellular distribution of the hARWT and hARSBMA proteins in transfected differentiated NB2a/dl cells were compared by immunofluorescence microscopy. Prior to fixation, the cells were treated for 1 h with 10 nM mibolerone, a non-metabolizable synthetic steroid producing permanent activation of the androgen receptor and inducing its translocation to the nucleus. The hARWT protein showed a clear pattern of nuclear staining with hardly any residual staining in the cytoplasm (Fig. 4A). In comparison, a striking difference in distribution was observed for the hARSBMA protein; after mibolerone treatment there was only weak staining in the nucleus, and the majority of the protein appeared to remain in the cytoplasm in the form of large aggregates (Fig. 4B). Aggregates were also sometimes observed in the nucleus. These patterns of androgen receptor staining were identical irrespective of the differentiation state of the cells or whether the N-terminal or C-terminal specific antibodies were used. Ectopic expression of the androgen receptor at high levels probably contributes to the formation of cytoplasmic aggregates as some were occasionally observed in cells expressing hARWT, however, this was always accompanied by a clear nuclear staining, which was only faint in cells expressing hARSBMA.

**DISCUSSION**

The main function of the androgen receptor is to control expression of target genes by binding to an androgen response element following interaction with the hormone. Expansion of polyglutamine tracts in the androgen receptor has been shown to result in a reduction in transcriptional activity in non-neuronal (29,30,41) and neuronal cellular systems (31,32). In the present study, we confirmed these findings by showing that an androgen receptor containing 52 CAG repeats has a transcriptional activity...
reduced by ~80% compared with the activity of an androgen receptor with 19 CAG repeats in transfected COS-7 cells.

As revealed by immunofluorescence microscopy, and as expected, the distribution pattern exhibited by the wild-type androgen receptor after activation was clearly nuclear with little or no residual staining in the cytoplasm. In contrast, after steroid treatment the SBMA androgen receptor protein was mainly localized in the cytoplasm in the form of dense aggregates with very little androgen receptor protein appearing in the nucleus. The clear nuclear staining as well as the high levels of transcriptional activity produced from the reporter plasmid show that the wild-type androgen receptor is functioning normally. The very low level of nuclear translocation achieved by the SBMA mutant androgen receptor and its accumulation as dense cytoplasmic aggregates correlates with its low transactivational capability. A possible explanation for the SBMA androgen receptor aggregation is that the protein is crosslinking with itself or other proteins that bind preferentially to polyglutamine tracts either by the formation of ‘polar zippers’ (13,14) or by becoming substrates for glutaminases (15–17). A fragment of MJD1, a protein involved in the translation of huntingtin with expanded polyglutamine stretches, has been shown to precipitate in the cytoplasm (42). Recently, huntingtin-containing neuronal intranuclear inclusions has been described in transgenic mice expressing exon 1 of the huntingtin gene (43,44). Similar protein aggregates can also be formed in vitro (44). However, we did not detect the presence of high molecular weight complexes of the androgen receptor in western blot analysis of cells transfected with the SBMA mutant androgen receptor.

Western blot analysis of transfected cells revealed that, in addition to the full length androgen receptor proteins, some smaller fragments were also generated. The SBMA androgen receptor gave rise to two fragments, of 93 and 74 kDa, the latter being particularly prominent. In cells transfected with the wild-type androgen receptor, a fragment of ~83 kDa was observed at high protein loadings, and occasionally a faint band of ~71 kDa was also detected, especially with the N-terminal specific antibody. The 93 and 74 kDa fragments in cells expressing the SBMA androgen receptor are likely to arise from the same mechanisms as the 83 and 71 kDa fragments from cells expressing the wild-type androgen receptor; the presence of the 33 extra glutamine residues would partly account for the molecular weight difference. Analysis with N- and C-terminal specific antibodies revealed that these fragments retained the N-terminus of the full-length protein but were C-terminally truncated. This confirms that the truncated fragments contain the polyglutamine tract, which starts at position 58.

The 83/93 kDa fragments would correspond in size to a form of the androgen receptor, termed the A-form, found in a number of human tissues, but not in brain (45,46). However, the A-form is N-terminally truncated, possibly as the result of alternative translation from the first internal methionine residue (Met188). Thus, the 83/93 kDa androgen receptor detected in the present study cannot correspond to the A-form as they retain an intact N-terminal domain.

Truncated fragments have not been reported in earlier studies involving the transfection of the SBMA androgen receptor in cultured cells (30,31,41,47). Analysis of the androgen receptor protein in spinal cord (48,49) or scrotal skin fibroblasts (45) from SBMA patients also failed to detect the presence of truncated fragments. This may be because the truncated forms only represent a small proportion of the total androgen receptor protein, and they can only be observed with high protein loadings on gels.

The C-terminally truncated androgen receptor fragments probably result from proteolytic cleavage enhanced by the presence of the extended polyglutamine tract. Indeed, proteins with expanded polyglutamine stretches appear to be good substrates for cysteinyl aspartate proteases (caspases). This has been shown for the product of another gene with CAG repeats, the 350 kDa huntingtin of Huntington’s disease. Normal length huntingtin is cleaved by caspase-3, or apopain, but the rate of cleavage is increased with the number of glutamine residues present in the protein (50,51). Larger polyglutamine tracts are thought to alter the conformation of proteins (50,52). This could enhance proteolysis by exposing sites which would normally be masked.

A similar mechanism of cysteine protease cleavage could be envisaged for the androgen receptor. It has been suggested that the cleavage of abnormal huntingtin may result in aberrant subcellu-
lar trafficking leading to the accumulation of truncated huntingtin in subcellular compartments (53). As the SBMA androgen receptor forms aggregates in the cytoplasm, it may be more prone to proteolysis resulting in the production of larger amounts of the truncated forms. Small androgen receptor fragments could be toxic either through general mechanisms of toxicity of proteins with long polyglutamine stretches (42,54,55), alternatively, truncated fragments may have specific properties.

From its size, it can be deduced that the 74 kDa fragment produced in cells expressing the SBMA androgen receptor lacks the hormone binding domain, which starts around residue 670, but retains the DNA binding domain. C-terminally truncated androgen receptors are constitutively active, but only have 10% of the transactivational ability of the intact protein (56,57). The 74 kDa fragment may therefore be toxic by initiating the transcription of specific genes in the absence of hormonal control. Identifying such genes will be a crucial importance in understanding the pathogenesis of SBMA.

In summary, this work provides further insight into the altered function of the androgen receptor in spinal and bulbar muscular atrophy leading to motor neuron cell death. The data presented show alterations in androgen receptor function as a result of the CAG repeat expansion in two ways: the mutation appears to interfere with the androgen receptor’s ability to translocate to the nucleus and enhances the production of potentially toxic androgen receptor fragments.

MATERIALS AND METHODS

Expression vectors
cDNAs coding for the wild-type (hARWT, 19 CAG repeats) and mutant (hARSMA, 52 CAG repeats) human androgen receptors (29) (obtained from Dr L. Pinsky, The Lady Davis Research Institute, Montreal) were excised by BamHI digestion from pSG5-based vectors (obtained from Dr S. Guidato, Institute of Psychiatry, London) and subcloned at the BamHI site in the vector pCMVneo (a gift from Dr L.-H. Tsai, Harvard Medical School). The resulting mammalian expression vectors were referred to as pCMVneo-hARWT and pCMVneo-hARSMA, respectively. The vector pMMTVCAT, coding for chloramphenicol acetyltransferase under the transcriptional control of the MMTV long terminal repeat (58), was a gift from Dr M. V. Govindan (Laval University, Quebec, Canada).

Antibodies
A polyclonal antibody against a fusion protein corresponding to amino acids 331–572 of the human androgen receptor, pAnti-AR (39) was purchased from Biosenesis (Poole, UK). Domain-specific anti-peptide antibodies to the N- and C-terminus of the human androgen receptor have been described previously (40).

Cell culture
NB2a/d1 cells (a gift from Dr T.B. Shea, University of Massachusetts at Lowell) and COS 7 cells were maintained in a humidified atmosphere of 95% air, 5% CO2 at 37 °C. COS 7 cells were transfected for 48 h by using the calcium phosphate transfection method. Liposome-mediated transfection was used for NB2a/d1 cells (Lipofectamine reagent, Gibco-BRL). In this case, the cells were exposed to the DNA/liposome complex for 5 h before being returned to normal growth medium. Cells were routinely analyzed 48 h after transfection. Transfection of differentiated NB2a/d1 cells was performed on the second day of dbcAMP treatment and the cells were maintained in differentiation medium for a further 24 h.

Assays for transactivational activity
Transactivational activity of the androgen receptor was determined in COS-7 cells by double transfection of 5 × 105 cells with 2.5 µg of each pMMTVCAT and 2.5 µg of pCMVneo-hARWT or pCMVneo-hARSMA. Transfected cells were cultured with or without 50 nM testosterone for 48 h in phenol-red free DMEM/10% (v/v) dextran-coated, charcoal-stripped HIFBS. Cell lysates were then prepared and the levels of CAT enzyme produced were measured using an ELISA technique (Boehringer Mannheim, Lewes, UK) according to the manufacturer’s instructions.

Western blotting
Prior to harvesting, cells were treated with 10 nM mibolerone and incubated for 1 h at 37 °C in order to activate the androgen receptor and promote translocation to the nucleus. After incubation, the cells were washed once with PBS at 4 °C and scraped into an appropriate volume of ice-cold TEDGP buffer [40 mM Tris, pH 7.4, 1 mM EDTA, 10% (w/v) glycerol, 10 mM DTT, 0.6 mM PMSF, 0.5 mM bacitracin]. Androgen receptor samples were stored at –70 °C. Proteins were separated in 8% (w/v) SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in PBS containing 5% (w/v) skimmed milk for 1 h at 37 °C. Subsequent antibody incubations were carried out in PBS containing 5% (w/v) skimmed milk and 0.5% (w/v) Tween-20. Membranes were routinely incubated with primary antibodies overnight at 4 °C (or for 1 h at 37 °C). Three 10 min washes in PBS containing 0.5% (w/v) Tween-20 were carried out between antibody steps, followed by incubation with the appropriate species-specific secondary antibody conjugated to horseradish peroxidase (Amersham, Little Chalfont, UK; 1:2000 dilution) for 1 h at room temperature. Immunoreactivity was detected using the enhanced chemiluminescence development method (Amersham).

Immunofluorescence microscopy
Cells, grown on coverslips, were rinsed in PBS at 37 °C and fixed in methanol at –20 °C for 5 min. Following fixation, cells were rehydrated in PBS for 10 min and all antibody incubations were carried out in PBS. Cells were incubated at room temperature with anti-androgen receptor antibodies for 30 min, followed by FITC-conjugated anti-rabbit antibody (Vector Laboratories, Canada).
Peterborough, UK: 1:250 dilution) for 30 min. Between incubations, cells were washed in PBS over 20 min. Coverslips were mounted in VectaShield™ (Vector Laboratories).

ACKNOWLEDGEMENTS

We thank Drs Govindan, L.-H. Tsai and L. Pinsky for providing vectors used in this study and Dr T.B. Shea for the NB2a/d1 cell line. Special thanks to Dr S. Guidato for the pSGAR vectors and vectors used in this study and Dr T.B. Shea for the NB2a/d1 cell line.

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