Altered transcriptional regulation in cells expressing the expanded polyglutamine androgen receptor

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Kennedy's disease is a degenerative disease of motor neurons in which the causative mutation is expansion of a CAG/polyglutamine tract near the 5' end of the androgen receptor gene. The mutant protein misfolds, aggregates, and interacts abnormally with other proteins, leading to a novel, toxic gain of function and an alteration of normal function. We used a cell culture model to explore the mechanisms underlying the alterations in androgen receptor function conferred by the mutation. Here we show that cells expressing the wild-type androgen receptor with 24 CAG repeats respond to ligand by showing trophic effects including prolonged survival in low serum, whereas cells expressing the mutant receptor with 65 CAG repeats do not show a robust trophic response. This partial loss of function correlates with decreased levels of the mutant protein due to its preferential degradation by the ubiquitin–proteasome pathway. Expression analysis using oligonucleotide arrays confirms that the mutant receptor has undergone a partial loss of function, and fails to regulate a subset of genes whose expression is normally affected by ligand activation of the wild-type receptor. The mutant receptor has also undergone several functionally important post-translational modifications in the absence of ligand that the wild-type receptor undergoes in the presence of ligand, including acetylation and phosphorylation. These modifications correlate with a ligand-independent gain of function exhibited by the mutant receptor in expression analysis. Our findings suggest that polyglutamine expansion alters androgen receptor function by promoting its degradation and by modifying its activity as a transcription factor.

INTRODUCTION

Kennedy's disease, or X-linked spinal and bulbar muscular atrophy, is a chronic, progressive neuromuscular disease characterized by proximal muscle weakness, atrophy, and fasciculations. The neurological manifestations of Kennedy's disease correlate with a loss of motor neurons in the brainstem and spinal cord (1). Affected males may also show signs of androgen insensitivity, including gynecomastia, testicular atrophy and decreased fertility (2,3). The cause of Kennedy's disease is expansion of a CAG repeat in the first exon of the androgen receptor gene on the X chromosome at Xq11–12 (4).

Since the identification of the androgen receptor mutation in Kennedy's disease, eight other inherited neurodegenerative diseases have been identified that are caused by expansions of CAG/polyglutamine [poly(Q)] tracts (5). Shared features among these disorders suggest that there is a common mechanism underlying neuronal dysfunction and degeneration. Expanded poly(Q) tracts lead to protein misfolding and aggregation in a repeat length-dependent manner. There is a remarkable correlation between the repeat length threshold for aggregation in cell-free systems and the repeat length that leads to human disease (6,7). Poly(Q) tracts may self-aggregate as antiparallel β-strands linked by hydrogen bonds, forming so-called ‘polar zippers’ (8,9). Aggregation may be further facilitated by transglutamination (10,11), a process that when inhibited decreases the aggregation and toxicity of expanded poly(Q)-containing proteins in cell culture (12). Misfolded proteins are targeted for degradation primarily through the ubiquitin–proteasome pathway (13). Neuronal inclusions of mutant protein in mouse models and human tissue stain positively for components of this pathway and functionally

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related molecules, including ubiquitin, chaperones and proteasome components (14–16). Most strikingly, modulating the activity of this pathway modifies the phenotype in cell culture, Drosophila and mouse models of poly(Q) disease (14,17–24). These and other studies (25,26) have dissociated cell dysfunction, as evidenced by behavioral abnormalities in mice, from the presence of visible protein aggregates and neuron loss.

Even in the absence of visible inclusions, the misfolded, mutant proteins may aberrantly interact with and sequester additional proteins, particularly those that contain poly(Q) tracts (27). Among this group of proteins are factors that regulate transcription (28). At least a dozen different transcription factors and co-activators have been implicated in the pathogenesis of the poly(Q) diseases, many of which acetylate histones or transcription factors (21,23,29–38). Several of these co-activators, including cAMP response element-binding protein (CREEB-binding protein (CBP), steroid receptor co-activator-1 (SRC-1), TAFII130 and TATA-binding protein, co-localize to aggregates. The sequestration of TAFII130, p53 and CBP may interfere with factor-mediated transcription, and overexpression of TAFII130 and CBP decreases cell death in transiently transfected cells (30,31,35,37). Altered expression of other transcriptional co-activators modifies the disease phenotype in Drosophila (21). That sequestration of co-activators with acetyltransferase activity may contribute to disease pathogenesis is supported by the diminished toxicity of expanded poly(Q) tracts in cell culture, yeast and Drosophila models following treatment with deacetylase inhibitors (21,39–41). Taken together, these findings are consistent with prior studies that showed the importance of nuclear localization for toxicity of proteins with expanded poly(Q) tracts (26,42). The data are also consistent with evidence of transcriptional dysregulation in cell culture and mouse models of poly(Q) disease (43–45).

Here we have used a cell culture model of Kennedy’s disease to study the effects of the mutation on androgen receptor function. This model takes advantage of the well-defined function of the androgen receptor as a transcription factor to explore effects of the mutation on transcriptional regulation. Our system models the trophic actions of the wild-type androgen receptor on motor neurons and the partial loss-of-function that results from expansion of the poly(Q) tract. These data show that our model will enable us to study the molecular basis of the alteration in function that the androgen receptor undergoes as a result of poly(Q) expansion.

**RESULTS**

**A cell culture model of Kennedy’s disease**

We have developed a cell culture model of Kennedy’s disease that will allow us to study the function of the wild-type and expanded poly(Q) androgen receptors in motor neuron-like cells (46,47). This model uses the mouse motor neuron–neuroblastoma hybrid cell line MN-1 (48). While these cells exhibit several features of motor neurons, they do not express androgen receptor protein at levels detectable by either western blot or radiolabeled ligand binding (46,47). This enabled the establishment of several independent clonal lines constitutively expressing either the full-length wild-type androgen receptor with 24 glutamines (Q24) or the full-length mutant receptor with 65 glutamines (Q65) (46,47). This mutation represents the upper end of the expansion length identified in Kennedy’s disease patients. Immunofluorescence using an antibody against an N-terminal epitope reveals that the wild-type and mutant receptors are distributed similarly (Fig. 1A). In the absence of ligand, both forms of the androgen receptor are diffusely distributed throughout the cytoplasm. Upon addition of the synthetic ligand R1881, both the wild-type and mutant receptors translocate to the nucleus. This indicates that both forms of the androgen receptor appropriately interact with the cellular machinery that regulates the receptor’s intracellular distribution. We have not observed intracellular aggregates of androgen receptor protein by immunofluorescence or western blot analysis in cells expressing either the wild-type or mutant receptor.

**This system models the in vivo trophic effects of androgens on motor neurons, and the loss of receptor function conferred by poly(Q) expansion**

MN-1 cells expressing the wild-type androgen receptor show a trophic response in the presence of ligand (46). This response is readily observed when cells are stressed by culturing them in media containing a low concentration of serum, and occurs only in lines expressing the androgen receptor and not in negative controls (46). Under these conditions, androgens promote the survival of cells expressing the wild-type androgen receptor (Fig. 1B). This ligand-dependent increase in cell survival is shown for two independent clonal lines expressing the wild-type receptor, designated Q24-1 and Q24-2, as measured by a metabolic assay in which the substrate MTT is reduced. Similar results have been obtained by directly counting dead cells that uptake propidium iodide (data not shown), and by qualitatively assessing cell morphology (Fig. 1C). These data indicate that androgens exert a direct trophic effect on MN-1 cells expressing the wild-type androgen receptor. In contrast, there is a diminished response to ligand in cells expressing the mutant androgen receptor (designated Q65), suggesting that poly(Q) expansion has caused a partial loss of receptor function. Thus, our system models both the trophic effects mediated by activation of the wild-type receptor and the partial loss-of-function that results from expansion of the poly(Q) tract. These data show that our model will enable us to study the molecular basis of the alteration in function that the androgen receptor undergoes as a result of poly(Q) expansion. Since the wild-type lines Q24-1 and Q24-2 behaved similarly in these and all other biochemical analyses, subsequent results will be shown for line Q24-2 only, although similar data have been obtained from line Q24-1.

**The mutant androgen receptor has a shorter half-life than the wild-type receptor owing to its degradation by the ubiquitin–proteasome pathway**

The diminished trophic effect mediated by activation of the mutant receptor correlates with lower steady-state levels of...
androgen receptor protein as determined by western blot and Scatchard analysis. Although the wild-type and mutant receptors have similar ligand-binding affinity in the cell lines used in these studies, cells expressing the mutant receptor only have about half the number of ligand-binding sites (46, 47). Similar results have been obtained from radiolabeled ligand-binding studies using scrotal fibroblasts derived from some but not all Kennedy’s disease patients (49). As seen in Figure 2A, mutant protein is expressed at lower levels than the wild-type protein in the absence of ligand. Ligand increases the steady-state levels of both the wild-type and mutant receptors. In contrast to the differences in steady-state protein levels, androgen receptor mRNA levels are quite similar between cells expressing the wild-type and mutant receptors. These data suggest that the exogenous mutant protein is more rapidly degraded than the wild-type protein.

To directly test this hypothesis, we performed pulse-chase analysis to measure the half-lives of the wild-type and mutant androgen receptor proteins (Fig. 2B). In the absence of ligand (shown in blue), the mutant receptor is degraded significantly more rapidly than the wild-type receptor \( (P < 1 \times 10^{-3} \text{ by ANOVA}) \), with a half-life of about 1.5 hours. In contrast, the half-life of the wild-type receptor is about 3 hours. Both the wild-type and mutant receptors are stabilized by ligand (shown in red). Similar data characterizing the half-life of the wild-type androgen receptor and its ligand-dependent stabilization have been reported previously (50). Treatment with the proteasome inhibitor lactacystin resulted in marked accumulation of both forms of the androgen receptor (Fig. 2C), indicating that degradation of the wild-type and mutant receptors occurs through the ubiquitin–proteasome pathway. Collectively, these data suggest one possible mechanism by which poly(Q) expansion causes decreased levels of soluble androgen receptor protein, as seen in patients with Kennedy’s disease.

**Expansion of the poly(Q) tract causes a partial loss of androgen receptor function**

We next used our cell culture model and oligonucleotide arrays to compare the function of the wild-type and mutant androgen receptors as ligand-activated transcription factors. The Affymetrix arrays used in these studies have tiled on them ~11,000 named mouse genes and expressed sequence tags.
For these experiments, cells were treated with ligand or vehicle control for 24 hours. Expression analysis was performed on duplicate samples using Affymetrix GeneChip software. This software package provides a qualitative measure of expression differences between samples (increased, decreased or unchanged). As duplicate experiments allowed us to perform a four-way comparison, our criteria for calling a gene up- or downregulated were that it must be called increased or decreased in three out of four comparisons. These same criteria have been used previously, and were found to yield >90% concordance with northern blot or S1 protection assay (44). We have found similarly high concordance by confirmatory northern blots on a subset of a dozen genes (data not shown).

We used the oligonucleotide arrays and cells expressing either the wild-type or mutant receptor to answer two questions. First, we sought to determine the effect on baseline gene expression caused by the chronic production of the mutant protein. It is our expectation that this approach will facilitate the identification of compensatory pathways that have enabled cell survival despite the expression of a toxic protein. Second, we sought to determine the differences in function between the wild-type and mutant androgen receptor. Since the receptor is a ligand-activated transcription factor, expression analysis should provide a robust readout of its function.

A comparison of baseline gene expression between cells producing the wild-type or mutant receptor revealed differential expression of ~3% of genes tiled on the arrays. Among the upregulated genes in cells that chronically express the mutant protein are several components of the ubiquitin–proteasome pathway and a molecular chaperone. This group of differentially expressed genes includes the chaperone HSP22, two inducible components of the 20S proteasome (Lmp7 and MECL1), and a proteasome activator (PA28) (data not shown). These data correlate well with our observation that the mutant protein has a shortened half-life due to its degradation via the ubiquitin–proteasome pathway. It also complements studies in several cell culture and animal models showing that this pathway is an important modifier of the toxicity of proteins with expanded poly(Q) tracts (14,17–24).

To compare the functions of the wild-type and mutant forms of the androgen receptor, we treated cells for 24 hours with or without ligand, isolated RNA, and then used oligonucleotide arrays to identify androgen-responsive genes. This strategy enables us to assess androgen receptor function on endogenous promoters, where activity is dependent upon the sum of several factors, including chromatin structure and interactions with transcriptional co-activators or co-repressors. The identified androgen-responsive genes are shown in Figure 3. In cells expressing the wild-type androgen receptor, ligand activation altered the expression of 54 genes, the vast majority of which were upregulated by receptor activation (Fig. 3: third column). In contrast, ligand activation of the mutant receptor altered the expression of only 17 genes (Fig. 3: forth column). The mutation caused a partial, not complete, loss of androgen receptor function, in that there was a subset of androgen-responsive genes that were regulated by both the wild-type and mutant receptors. This loss of function affected the regulation of genes that were both activated and repressed by the wild-type receptor. These results of expression profiling are consistent with clinical symptoms of androgen insufficiency...
in patients with Kennedy’s disease and with our functional assay that quantifies the trophic effects mediated by the wild-type and mutant androgen receptors, shown in Figure 1B.

**Aberrant post-translational modifications of the mutant androgen receptor**

We next sought to identify other mechanisms by which the expanded poly(Q) tract influences androgen receptor function. Ligand-dependent activation of the wild-type androgen receptor is accompanied by several post-translational modifications.

In some cases, these modifications have well-characterized effects on androgen receptor function as a transcription factor (e.g. acetylation and sumoylation) (51–53). In other instances, the effect of the modification on androgen receptor function or half-life is less clear, although similar modifications have been implicated in regulating the half-lives of other steroid hormone receptors (e.g. phosphorylation) (54). To determine whether the wild-type and mutant androgen receptors interact similarly with proteins that regulate receptor function, we compared the phosphorylation, acetylation and sumoylation status of the Q24 and Q65 receptors in the absence and presence of ligand. We found that the mutant receptor shows aberrant acetylation and phosphorylation (Fig. 4), but not altered sumoylation (data not shown).

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**Figure 3.** Androgen-responsive genes in cells expressing the Q24 or Q65 androgen receptors. Cells were treated in duplicate with 10 nM R1881 or vehicle control for 24 h, and androgen-responsive genes were identified using oligonucleotide arrays. The first and second columns show the GenBank number and gene name. The third and fourth columns show androgen-responsive genes in Q24 and Q65 cells, respectively. The fifth column shows a comparison of baseline gene expression levels in mutant versus wild-type cells. Increased expression is shown in red, decreased in green and no change in black. Expression differences called in 4/4 comparisons are shown by dark shades, and those called in 3/4 comparisons are shown by light shades.

**Figure 4.** Altered post-translational modifications of the Q65 androgen receptor. (A) The mutant receptor is acetylated in the absence of ligand. Androgen receptor was immunoprecipitated from cells expressing wild-type or mutant forms following treatment with 10 nM R1881 (+) or vehicle control (−) for 6 h in the presence of 5 mM sodium butyrate. Half of the immunoprecipitate was analyzed by western blot for acetylated lysine, and a portion of the remainder was analyzed for androgen receptor. (B) Altered phosphorylation of the mutant androgen receptor. Cells expressing wild-type or mutant receptor were treated with 10 nM R1881 (+) or vehicle control (−) for 6 h. Androgen receptor was immunoprecipitated and then incubated with alkaline phosphatase (lanes 5–8) or buffer alone (lanes 1–4) for 30 min at 37°C. Androgen receptor protein was visualized by western blot using an anti-androgen receptor antibody.
The wild-type androgen receptor is acetylated in a ligand-dependent manner (51). Acetylation of the androgen receptor may be mediated by interaction with transcriptional co-activators p300 or P/CAF. These co-activators possess both histone and factor acetyltransferase activity, and acetylate transcription factors, including the androgen receptor. This modification is associated with ligand-dependent activation of the receptor in reporter gene assays. To determine whether the wild-type and mutant receptors are similarly acetylated, we immunoprecipitated the androgen receptor from cells treated with ligand or vehicle control and then evaluated acetylation status using an anti-acetyl-lysine antibody (Fig. 4A). While the wild-type receptor shows ligand-dependent acetylation, the mutant receptor is acetylated even in the absence of ligand. These data indicate that the mutant receptor is hyperacetylated in the absence of ligand, perhaps owing to abnormal interactions with the transcriptional co-activators that are responsible for this modification.

The wild-type androgen receptor is a phosphoprotein that undergoes further phosphorylation in the presence of ligand (50,55). This ligand-dependent phosphorylation of the wild-type androgen receptor results in decreased electrophoretic mobility that is apparent on lower percentage gels after prolonged electrophoresis (Fig. 4B: lane 2 versus lane 1). That this shift in mobility is due to ligand-dependent phosphorylation of the receptor is confirmed by treatment with alkaline phosphatase (Fig. 4B: lanes 5 and 6). In contrast, the mutant receptor migrates as several bands even in the absence of ligand and does not show a ligand-dependent alteration in its migration pattern (Fig. 4B: lanes 3 and 4). Notably, the mutant receptor migrates as two bands after alkaline phosphatase treatment. This may reflect phosphorylation at resistant sites, or another currently undefined post-translational modification. These data suggest that, like the acetylation status, the phosphorylation status of the mutant receptor is altered compared with that of the wild-type receptor.

**Poly(Q) expansion also confers a gain of function upon the mutant androgen receptor**

These altered post-translational modifications suggest that the mutant receptor interacts aberrantly with proteins that normally influence the activity of the wild-type receptor as a ligand-dependent transcription factor. That these interactions may have important functional consequences is supported by additional data derived from expression analysis. When comparing expression levels in the absence of ligand of genes previously identified as androgen-responsive, we found that many of these genes were differentially expressed in cells producing the mutant receptor versus cells producing the wild-type receptor. As shown in Figure 3 (far-right column), 11 of these 57 genes showed increased or decreased expression in the absence of ligand in the mutant compared with the wild-type cells. This represents ~19% of all androgen-responsive genes identified, and is a far greater number of differences than one would have expected if only by chance (\( P < 0.001 \) by chi-squared analysis). These data suggest that expansion of the poly(Q) tract leads to ligand-independent activation of the receptor, resulting in a gain of function. This gain of function may be mediated by aberrant interaction with transcriptional co-activators or other proteins that normally regulate the function of the wild-type androgen receptor.

**DISCUSSION**

We have used a cell culture model of Kennedy’s disease to explore the effects of the expanded poly(Q) tract on androgen receptor function. Our data demonstrate that the mutation confers both a loss and a gain of function. The soluble mutant receptor is targeted for degradation by the ubiquitin–proteasome pathway, resulting in lower levels of expressed protein. A similar decrease in androgen receptor protein levels has been documented in some patients with Kennedy’s disease (49). The shortened half-life of the mutant receptor fits with a partial loss of function seen both in patients with Kennedy’s disease (i.e. signs of androgen insensitivity) and in our model. In the latter, it is manifest by a diminished trophic effect exerted by ligand activation of the mutant receptor, and by the failure of the mutant receptor to regulate a subset of genes responsive to ligand activation of the wild-type receptor. Stable expression of the mutant protein also resulted in the upregulation of several components of the ubiquitin–proteasome pathway and a molecular chaperone. Interestingly, some of these same components are also upregulated in a mouse model of Huntington’s disease (44), and may represent an important aspect of the stress response triggered in cells that express proteins with expanded poly(Q) tracts. These data are complementary to work by Bailey et al. (56) showing that the ubiquitin–proteasome pathway degrades truncated fragments of the androgen receptor, and that molecular chaperones shorten the half-lives of soluble proteins containing expanded poly(Q) tracts.

Our data support and extend prior studies that showed that the expanded poly(Q) tract causes a partial loss of androgen receptor function. Earlier analyses used reporter gene assays to demonstrate that the mutant androgen receptor has diminished activity as a ligand-activated transcription factor (47,55,57–60). Here we used oligonucleotide arrays to examine the function of the wild-type and mutant receptors. This strategy enabled us to assess androgen receptor function on endogenous promoters, where activity is dependent upon the sum of several factors, including chromatin structure. Our data confirm the partial loss of function due to the mutation. While the results presented here and in previous studies (47,55,57–60) argue that the expanded poly(Q) tract alters intrinsic activity of the androgen receptor, it is also possible that the some of these affects on transcriptional regulation may be secondary to the toxicity of the mutant receptor. Our analysis also led to the identification of aberrant activity of the mutant receptor. Approximately 19% of the androgen-responsive genes that we detected were differentially expressed in the baseline state between the wild-type and mutant cells, as compared with 3% of non-androgen responsive genes (\( P < 0.001 \) by chi-squared analysis). These data suggest that the mutant receptor may have ligand-independent activity. We also identified a small set of genes responsive to ligand activation of the mutant receptor but not the wild-type receptor. These data further support our interpretation that the mutation alters normal function of the androgen receptor as a ligand-activated transcription factor.
This altered activity may be mediated by the mutant receptor's aberrant interaction with transcriptional co-activators. The expanded poly(Q) androgen receptor sequesters several co-activators, including CBP and SRC-1 (23,37). These interactions may contribute to the mutant receptor's ligand-independent hyperacetylation and activation, and may play an important role in its toxicity.

There is much compelling evidence that poly(Q) expansion causes an important toxic gain of function. There is also emerging evidence, including the data presented here, that the mutation affects the normal function of mutant proteins. These alterations in normal function may also contribute to disease pathogenesis. For example, the protein huntingtin is antiapoptotic (61,62), and its conditional knockout in mice causes progressive neurodegeneration (63). Poly(Q) expansion causes a loss of huntingtin-mediated brain-derived neurotrophic factor (BDNF) transcription, possibly resulting in a loss of trophic support (64). In SCA1, increasing the poly(Q) tract length in ataxin-1 decreases the protein's ability to bind RNA (65). And in Kennedy's disease, the mutation causes partial androgen insensitivity, and a loss of ligand-dependent androgen receptor function (47,55,57–60,66). This partial loss of function may deprive motor neurons of trophic support normally received from activation of the wild-type receptor. Combined with the ligand-independent activity of the mutant receptor, this altered function may play an important contributory role in the cell-type specificity of Kennedy's disease.

The mechanism by which activation of the wild-type androgen receptor triggers a trophic response in motor neurons is poorly understood. The set of androgen-responsive genes identified here demonstrates that the activated receptor alters expression of a diverse set of genes whose protein products impact many biochemical pathways. Of the 48 named genes identified in this study, 9 were shown previously to have their expression modulated by androgens in other systems (67–75). In one case, the gene's promoter has been studied and an authentic androgen-responsive element identified (73). Among the earliest gene expression changes that we detected following the addition of ligand was increased expression of the transcription factor C/EBPβ (data not shown).

These data, and our prior results showing that ligand activation of the wild-type receptor upregulates the expression of the RNA-binding proteins tra-2α and fxh (76), suggest that androgen receptor activation influences gene expression both by regulating the activity of hormone-responsive promoters and by influencing the expression of other components of the transcriptional and splicing machinery. Understanding how androgens exert trophic effects on motor neurons may provide important insights into treatment strategies for motor neuron disease.

MATERIALS AND METHODS

Cell lines and reagents

Mouse MN-1 cells stably expressing the full-length human androgen receptor with 24 or 65 glutamines were previously described (46,47). The synthetic androgen R1881 was obtained from New England Nuclear. Androgen receptor antibody N-20, recognizing the receptor's N terminus, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For western blot analysis, equivalent protein loading was confirmed with an anti-actin antibody (Sigma, St Louis, MO). Matrigel was obtained from Becton Dickinson (Bedford, MA). The androgen receptor probe for northern blot was generated from an 817 bp fragment isolated from the full-length cDNA following digestion with PstI. Calf intestinal alkaline phosphatase was obtained from New England BioLabs (Beverly, MA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Deconvolution microscopy

Cells were grown on Matrigel-coated chambered slides (Nalge, Nunc International, Naperville, IL) in media supplemented with 10% charcoal and dextran-striped fetal bovine serum (HyClone, Logan, UT), then treated with 10 nM R1881 or vehicle control. Following 24 h incubation, cells were fixed with methanol and stained with an anti-androgen receptor antibody. Staining was visualized using a FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Images were deconvolved using DeltaVision software (Applied Precision, Issaquah, WA).

Cell viability assay

20 000 cells/well were plated on Matrigel-coated 96-well dishes in DMEM containing 2% charcoal- and dextran-striped fetal bovine serum, 0.4 μg/ml aphidicolin and 1 mM sodium butyrate, as described in (46). Medium was supplemented with 10 nM R1881 or vehicle control. At the indicated time point, 25 μl MTT diluted to 5 mg/ml in PBS was added to each well. Following 2 h incubation at 37°C, crystals were dissolved in lysis buffer containing 10% SDS and 50% dimethylformamide (pH 4.7), and the optical density at 540 nm was determined. Androgen-specific increased survival was calculated as 100 × (survival with ligand — survival without ligand)/survival without ligand.

Pulse-chase analysis

Cells were rinsed twice and then incubated in methionine-free DMEM (Life Technologies) containing 10% dialyzed charcoal- and dextran-striped fetal bovine serum (pulse medium) for 15 min. Medium was then removed, and pulse medium supplemented with 0.25 mCi/ml [35S]methionine was added for 30 min at 37°C. Cells were then rinsed and harvested at the end of the pulse, or following 3 or 6 h chase in pulse medium supplemented with 15 mg/l methionine and either 10 nM R1881 or vehicle control. The androgen receptor was immunoprecipitated from equivalent amounts of protein lysates, and the amount of radiolabeled receptor present in each sample was determined using a phosphoimager.

Expression analysis

Cells grown in DMEM containing 10% charcoal- and dextran-striped serum were treated with 10 nM R1881 or vehicle
control for 24 h in duplicate. Total cellular RNA was isolated using RNeasy (Qiagen, Valencia, CA) following the manufacturer’s protocol. Biotinylated cRNA was prepared as described in (44) and hybridized to Mu11K A and B oligonucleotide arrays according to the manufacturer’s recommendations (Affymetrix). The arrays were scanned with a Hewlett Packard GeneArray scanner, and the scanned images were analyzed using GeneChip software (Affymetrix) as previously described (44). We used the default parameters in the GeneChip software to call mRNAs increased, decreased or not changed. We selected genes for inclusion in this report that were called as increased or decreased in at least three of four comparisons from duplicate samples. These criteria have been previously used and have been found to show >90% concordance between array calls and northern or S1 analysis (44).

We have observed a similarly high rate of concordance in one dozen confirmatory northern blots on samples (data not shown).

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