

Akt blocks ligand binding and protects against expanded polyglutamine androgen receptor toxicity

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Spinal and bulbar muscular atrophy (SBMA) is a progressive neurodegenerative disease caused by an expansion of the polyglutamine tract in the androgen receptor (AR). Here, we investigated the regulation of AR phosphorylation in order to understand factors that may modify SBMA disease progression. We show that expanded polyglutamine AR is phosphorylated by Akt. Substitution of the AR at two Akt consensus sites, S215 and S792, with aspartate, which mimics phosphorylation, reduces ligand binding, ligand-dependent nuclear translocation, transcriptional activation and toxicity of expanded polyglutamine AR. Co-expression of constitutively active Akt and the AR has similar consequences, which are blocked by alanine substitutions at residues 215 and 792. Furthermore, in motor neuron-derived MN-1 cells toxicity associated with polyglutamine-expanded AR is rescued by co-expression with Akt. Insulin-like growth factor-1 (IGF-1) stimulation, which activates several cell survival promoting pathways, also reduces toxicity of the expanded polyglutamine AR in MN-1 cells, in a manner dependent upon phospho-inositol-3-kinase. IGF-1 rescue of AR toxicity is diminished by alanine substitutions at the Akt consensus sites. These results highlight potential targets for therapeutic intervention in SBMA.

INTRODUCTION

Expansion of CAG trinucleotide repeats encoding polyglutamine tracts are responsible for Huntington's disease, dentatorubropallidolusian atrophy, six forms of spinocerebellar ataxia and spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease (1,2). In each disease, different populations of neurons are affected, despite the widespread distribution of the polyglutamine proteins throughout the central nervous system, implying that not only the mutation, but also the flanking sequences are important for pathogenesis.

Expansion of the polyglutamine tract in the androgen receptor (AR) causes SBMA (3). SBMA patients have selective loss of motor neurons in the brainstem and spinal cord, leading to slowly progressive weakness, atrophy and fasciculation in bulbar and limb muscles (4). SBMA patients also have signs of androgen insensitivity, such as gynecomastia, testicular atrophy and decreased fertility, due to partial loss of AR

function (5). However, patients with androgen insensitivity syndrome caused by other loss of AR function mutations do not show signs of neurodegeneration. Expansion of the polyglutamine tract is thus likely to confer a novel toxic gain of function to the AR (5).

Like other nuclear hormone receptors, the AR is normally present in the cytosol in an inactive state. Upon androgen binding, the AR is actively transported to the nucleus to activate transcription. In animal models, reduction of serum androgen levels by castration (6,7) or leuporelin treatment (8) decreases nuclear AR, mitigates the behavioral defect and improves survival in transgenic SBMA mice. Also, testosterone administration to SBMA transgenic females, which do not show signs of disease, results in neuronal degeneration (7,9). In humans, homozygous carrier females are generally asymptomatic (10). It follows that decreasing the effect of ligand on the AR could reduce disease progression.

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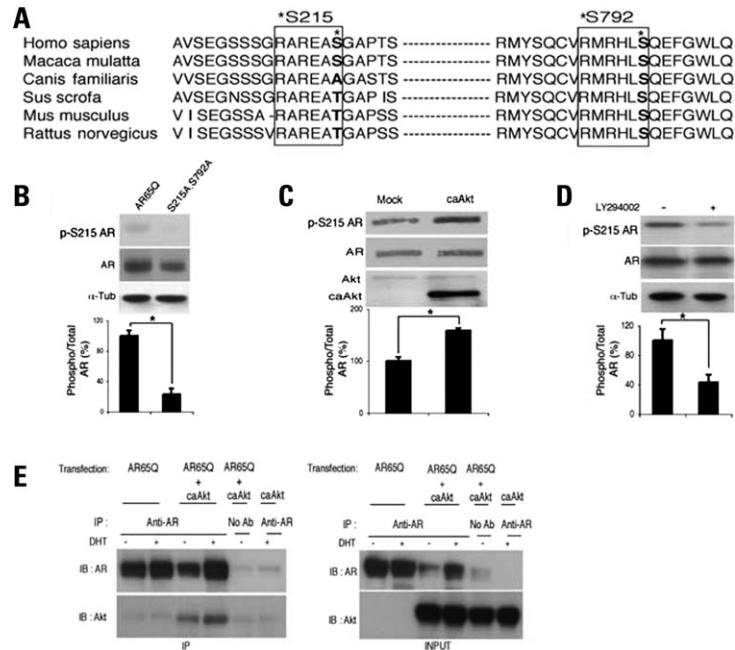


Figure 1. PI3K-Akt signaling augments serine 215 phosphorylation of AR. (A) Alignment of amino acid sequences around serine 215 (S215) and serine 792 (S792) of the AR, which conform to the Akt consensus site (RXXRXXS/T*), shows that the two serines are conserved in mammals, with the exception of S215 in *Canis familiaris*. (B) Cos1 cells were transfected with pCMV-AR65Q or pCMV-AR65Q with alanine substitutions at S215 and S792. Phosphorylation of S215 and protein expression were monitored by western blotting with anti-phospho-S215 and anti-AR antibodies. α -Tubulin immunoreactivity is shown as loading control. The change in S215 phosphorylation is represented as the ratio between the signals detected with the phospho-S215 and anti-AR antibodies. The first sample is represented as 100%, and the mean \pm SEM values from three independent experiments are shown. (C) Cos1 cells were transfected with pCMV-AR65Q together with empty vector or myristilated constitutively active Akt (pCMV-caAkt). Phosphorylation of S215 and protein expression were monitored with phospho-S215 antibody, anti-AR and anti-Akt antibodies. Endogenous Akt is found above caAkt on the blot. (D) Cos1 cells were transfected with pCMV-AR65Q and treated with the PI3K inhibitor LY294002 or vehicle. Phosphorylation of S215 and protein expression were analyzed by western blotting. α -Tubulin immunoreactivity is shown as loading control. * $P < 0.02$ (Student's *t*-test in B, C and D). (E) HEK 293T cells were transfected with pCMV-AR65Q and pCMV-caAkt as indicated and processed for immunoprecipitation (IP) with anti-AR antibody. Immunoprecipitates and fractions of the input were analyzed by western blotting with specific anti-AR and anti-Akt antibodies (IB).

Here, we show that phosphorylation of the AR at the Akt consensus sites serine 215 (S215) and serine 792 (S792) regulates AR function. Phosphomimetic mutation of S215 and S792 with aspartate prevents ligand-dependent nuclear translocation, transactivation and toxicity of the expanded polyglutamine protein as a consequence of reduced ligand binding. A constitutively active mutant of Akt reduces activation of the AR by androgen, but have diminished effect on the AR with non-phosphorylatable alanine substitutions at S215 and S792, suggesting that Akt represses AR function through phosphorylation at these sites. Importantly, we demonstrate that activation of phospho-inositol-3-kinase (PI3K)-Akt signaling by insulin-like growth factor-1 (IGF-1) protects motor neuron-derived cells against toxicity of the mutant protein. There is currently no effective therapy for Kennedy's disease. Our results point to the PI3K-Akt as an important signaling pathway for development of SBMA therapeutic targets and to IGF-1 as potential therapy for SBMA.

RESULTS

AR65Q is phosphorylated by Akt

In the AR, S215 and S792 conform to the Akt consensus site (RXXRXXS/T*) (11). With the exception of the S215 site in

Canis familiaris, these Akt consensus sites are conserved in mammals, suggesting an important role in AR function (Fig. 1A). The non-expanded AR (AR24Q) has been shown to be a substrate of Akt in prostate cancer and Cos1 cells (12). To determine whether the AR with an expanded polyglutamine tract (AR65Q) is phosphorylated at S215 under normal growth conditions, Cos1 cells, which lack endogenous AR (not shown), were transfected with an AR65Q expression vector, and phosphorylation was monitored by immunoblotting with a specific AR phospho-serine 215 antibody (Fig. 1B). The specificity of the antibody was confirmed using an artificial phosphorylation site mutant of the AR, in which S215 and S792 were replaced by non-phosphorylatable alanine residues (S215A, S792A). The relative amount of S215-phosphorylated AR was represented as the ratio of phosphorylated to total AR (Fig. 1B, bottom panels). We detected S215 phosphorylation with non-substituted AR65Q, but not the alanine-substituted receptor.

To investigate whether Akt regulates AR65Q phosphorylation, we co-expressed a myristilated constitutively active Akt (caAkt) (13) (Fig. 1C). AR65Q phosphorylation at S215 was augmented by $58 \pm 5\%$ with co-expression of caAkt. Since Akt is known to be activated downstream of PI3K, we examined whether S215 phosphorylation is dependent on PI3K, by treating AR65Q-expressing Cos1 cells with the

PI3K inhibitor LY294002 (13) (Fig. 1D). We observed a reduction in S215 phosphorylation ($57 \pm 10\%$) after LY294002 treatment, indicating that PI3K activity contributes to phosphorylation of AR65Q at S215.

Kinases occasionally form stable complexes with their substrates, which is thought to promote specificity. We investigated whether Akt interacts with AR by immunoprecipitating AR from cell lysates of HEK 293T cells overexpressing AR65Q plus caAkt (Fig. 1E). AR65Q co-immunoprecipitated with caAkt independent of androgen stimulation, indicating that the two proteins might be in a complex. In addition, we show that AR65Q and Akt are present in the same sub-cellular compartments in motor neuron-derived MN-1 cells (Supplementary Material, Fig. S1). Together, our results show that Akt and AR65Q interact and that AR65Q phosphorylation is stimulated by PI3K signaling.

Androgen receptor protein stabilization is prevented by substitution of serines 215 and 792 with aspartate

The AR protein is stabilized by ligand (14). To investigate whether Akt-consensus phosphorylation sites play a role in androgen-dependent AR protein stabilization, we compared the effects of serine to alanine substitutions, which prevent phosphorylation (S215A, S792A), and serine to aspartate substitutions, which mimic constitutive phosphorylation (S215D, S792D) (Fig. 2A). The protein levels of the alanine-substituted versions of AR24Q and AR65Q were increased 2–3-fold by 5 α -dihydrotestosterone (DHT), as are non-substituted AR24Q and AR65Q, when expressed in HEK 293T cells. In contrast, the protein levels of the aspartate-substituted receptors were not affected by DHT. Similar results were obtained in Cos1 and MN-1 cells (not shown). The mRNA levels of the AR constructs were not altered by androgens (not shown). Thus, we hypothesized that substitution of S215 and S792 to aspartate prevents ligand-dependent protein stabilization.

To test this hypothesis we performed pulse-chase analysis (Fig. 2B). We found that the half-lives of AR24Q and AR65Q were 5 and 3.5 h, respectively, and increased to longer than 6 h after DHT treatment, as previously reported (14). Similar results were obtained with the receptors in which the S215 and S792 were replaced by alanine (not shown). Notably, the half-lives of the aspartate-substituted receptors were 3.5 and 2 h, respectively, and did not change with DHT stimulation. However, accumulation of the aspartate-substituted versions of AR24Q and AR65Q was apparent when the cells were treated with the proteasome inhibitor MG132 (Fig. 2C). These data indicate that mutations mimicking constitutive phosphorylation prevent androgen-dependent protein stabilization, and result in protein degradation through the ubiquitin–proteasome system.

Androgen-dependent nuclear translocation of the androgen receptor is prevented by phosphomimetic mutations of S215 and S792

To determine whether the phosphorylation sites S215 and S792 play a role in regulating androgen-dependent nuclear translocation of the AR, we analyzed the subcellular

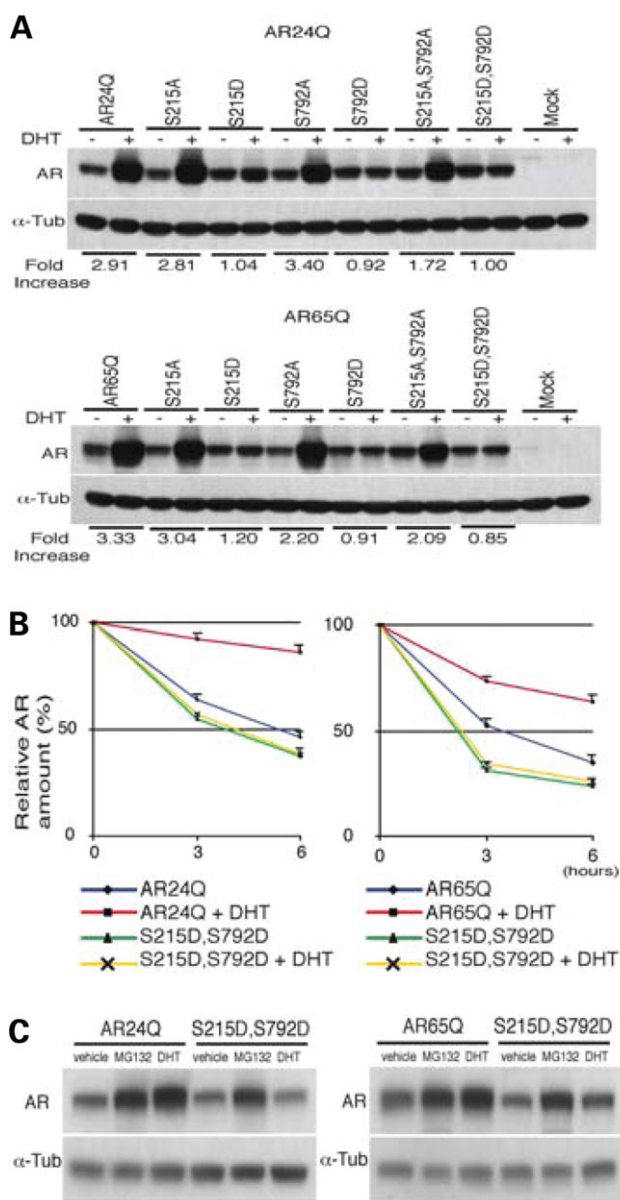


Figure 2. Ligand-dependent AR protein stabilization is reduced by substitution of S215 or S792 with aspartate. (A) HEK 293T cells were transfected with the indicated AR expression vectors, and treated with DHT or vehicle for 48 h. AR protein expression was monitored by western blotting with anti-AR antibody. Protein fold increase was measured as the ratio between the amount of AR in the absence and presence of DHT. Representative experiments from three repetitions are shown. α -Tubulin immunoreactivity is shown as a loading control. (B) Cos1 cells were transfected with pCMV-AR24Q, pCMV-AR65Q or the aspartate-substituted versions of AR24Q and AR65Q, and the half-lives of the AR proteins were monitored by pulse-chase analysis in the presence and absence of DHT. (C) HEK 293T cells transfected with pCMV-AR24Q, pCMV-AR65Q or the aspartate-substituted versions of AR24Q and AR65Q were treated with MG132 or DHT, and AR protein expression level was monitored by western blotting. α -Tubulin immunoreactivity is shown as a loading control.

localization of the alanine- and aspartate-substituted versions of the AR in the presence or absence of DHT in Cos1 cells (Fig. 3A and B). In unstimulated cells, $16 \pm 3\%$ and $7 \pm 4\%$ of the AR24Q and AR65Q-expressing cells showed

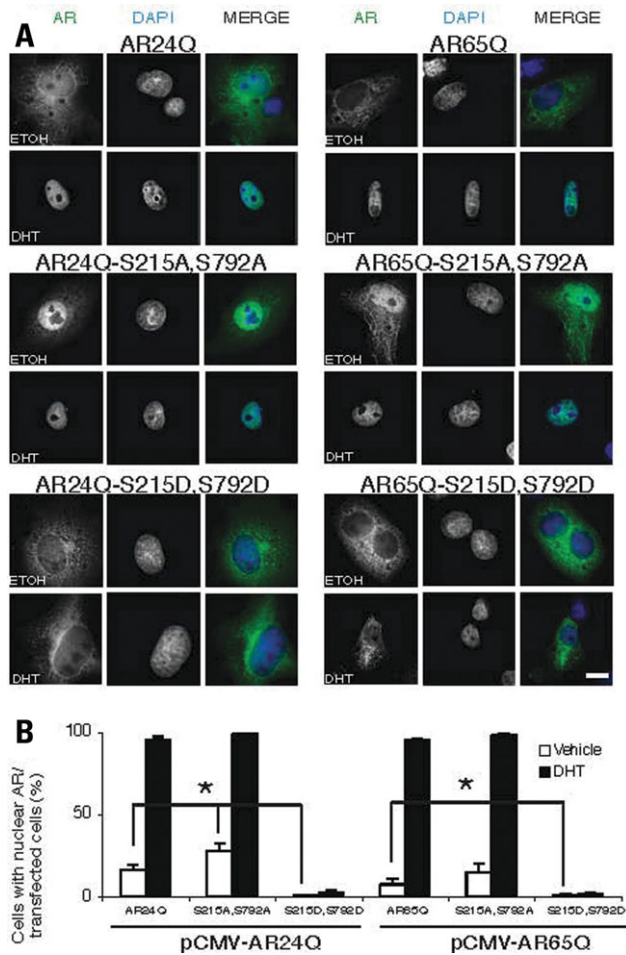


Figure 3. Phosphomimetic substitution of S215 and S792 with aspartate reduces androgen-induced nuclear translocation of the AR. (A) Cos1 cells transfected with the indicated AR constructs were treated with vehicle or DHT and processed for immunocytochemistry. AR was detected with anti-AR antibody (green), and nuclei were stained with DAPI (blue). In the absence of DHT, the AR was present in the cytosol of transfected cells. After 4 h of DHT treatment, the receptor translocated to the nucleus. The receptors with alanine substitutions at S215 and S792 also translocated to the nucleus. The receptors with aspartate substitutions were found in the cytosol in the presence or absence of DHT. Bar, 10 μ m. (B) The percentage of transfected cells with a greater concentration of AR in the nucleus than the cytoplasm was determined for AR24Q and AR65Q before and after DHT treatment. Bars, mean \pm SEM, * P < 0.05 (n = 400, Student's t -test).

the receptor in the nucleus, respectively. The percentage of cells with the alanine-substituted receptors in the nucleus in the absence of DHT was increased to $27 \pm 5\%$ and $15 \pm 5\%$ for the normal and the expanded polyglutamine AR, respectively. The percentage of cells expressing the aspartate-substituted mutants in the nucleus in the absence of DHT was decreased to $0.5 \pm 0.5\%$ for AR24Q and $0.6 \pm 0.6\%$ for AR65Q. After DHT treatment, more than 95% of the cells expressing AR24Q, AR65Q and the corresponding alanine mutants showed the receptor in the nucleus. In contrast, only $2.4 \pm 1\%$ and $1.3 \pm 1\%$ of the cells expressing the aspartate-substituted AR24Q and AR65Q, respectively, showed the receptors in the nucleus in the presence of DHT. Single alanine or aspartate mutations

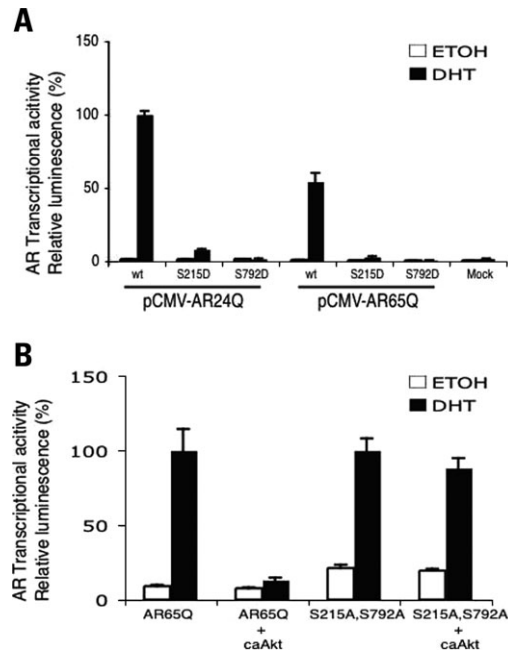


Figure 4. Phosphorylation of the AR by Akt results in a loss of AR transactivation function. (A) HEK 293T cells were transfected with the indicated AR24Q and AR65Q vectors together with both the luciferase pARE-E1b-Luc and the β -galactosidase pCMV β reporter constructs. AR transactivation was measured in the presence and absence of DHT by luciferase assay and normalized to β -galactosidase activity. Data shown are representative of three independent experiments. Vertical bars are mean \pm SEM of three independent values. (B) HEK 293T cells were co-transfected with pCMV-AR65Q, the alanine-substituted receptor, empty vector and caAkt as indicated together with pARE-E1b-Luc and pCMV β reporters. AR transactivation was measured as in (A). In (A) the activity of the AR24Q in the presence of DHT is set as 100. In (B) the activity of the AR65Q and AR65Q-S215A, S792A is set as 100.

of AR24Q and AR65Q had similar effects to the respective compound mutants (not shown). These results indicate that mutations mimicking constitutive phosphorylation at S215 and S792 decrease nuclear translocation of the AR in response to DHT.

Phosphorylation at the Akt consensus sites results in loss of androgen receptor function

The AR activates transcription of the specific AR reporter pARE-E1b-Luc in response to DHT in HEK 293T (Fig. 4A). Substitution of S215 and S792 with alanine in AR24Q and AR65Q did not affect the ability of the AR to activate transcription (not shown). In contrast, substitution of either S215 or S792 or both with aspartate repressed transactivation (Fig. 4A and data not shown). We verified that the transfection efficiency was similar among the samples by co-transfecting a plasmid encoding green fluorescent protein and analyzing protein levels by western blotting (not shown).

To investigate whether Akt phosphorylation of AR65Q has the same inhibitory effect on transactivation, we co-expressed AR65Q with caAkt (Fig. 4B). We found that caAkt repressed AR65Q transactivation to levels similar to those observed for the aspartate AR mutants. To examine whether this inhibition is caused by direct phosphorylation of AR at S215 and S792,

we co-expressed the caAkt and AR with alanine substitutions at the phosphorylation sites. CaAkt was unable to repress the transcriptional response to DHT of the alanine mutant, suggesting that Akt phosphorylation of AR65Q results in loss of AR transactivation function.

Androgen binding is sensitive to phosphorylation at serines 215 and 792

The observation that phosphomimetic mutations of the S215 and S792 sites affect AR response to the ligand led us to examine whether they also decrease the affinity of the AR for androgen. We measured the ability of the aspartate-substituted AR to bind to ^3H -R1881, a non-metabolizable AR agonist (Fig. 5A, see Materials and Methods). We found significantly less labeled R1881 bound to the double aspartate-substituted receptors than to non-substituted AR65Q. Substitution of a single site, either S215 or S792, was also sufficient to reduce ligand binding.

To confirm that Akt phosphorylation inhibits binding of the AR to androgen, we co-expressed AR65Q and caAkt (Fig. 5B). The affinity of AR65Q for the ligand was reduced by caAkt. To determine whether caAkt inhibits ligand binding through phosphorylation at S215 and S792, we measured the ability of caAkt to repress binding of androgen to the alanine-substituted AR. The affinity of the alanine-substituted receptor was slightly increased by co-expression of caAkt, perhaps due to other effects of mutant Akt on the AR with alanine substitutions at S215 and S792. We verified that protein expression levels of AR and Akt were similar in all the samples (Fig. 5A and B, bottom panels). These results indicate that Akt phosphorylation and phosphomimetic mutations at S215 and S792 reduce binding of the AR to its ligand.

Phosphorylation at serines 215 and 792 modifies toxicity of the expanded polyglutamine androgen receptor

The finding that phosphorylation at S215 and S792 affects androgen binding prompted us to determine whether it also protects against toxicity of the expanded polyglutamine AR. Cell viability correlates with the rate of reduction of the tetrazolium salt XTT to an orange formazan dye, a process that only occurs in metabolically active cells. By this measure, we found that in the presence of DHT cell viability was decreased in HEK 293T cells expressing AR65Q when compared with cells expressing AR24Q (Fig. 6A). Importantly, aspartate substitution at S215 and S792 partially reduced the toxicity of the expanded AR in cells treated with DHT.

IGF-1 activates Akt. Since aspartate substitution at the Akt phosphorylation sites rescued the toxicity of AR65Q, we reasoned that IGF-1 might protect against mutant AR toxicity. To test this, we used motor neuron-derived MN-1 cells stably transfected with AR24Q and AR65Q (15). We found that cell viability was lower in AR65Q-expressing cells when compared with AR24Q-expressing cells (Fig. 6B). Importantly, cell survival was increased by IGF-1 in AR65Q-expressing cells. In addition, we measured cell death by propidium iodide incorporation, and we found that IGF-1 reduces cell death in the AR65Q-expressing cells (not shown). To determine whether IGF-1 decreases toxicity of the expanded AR

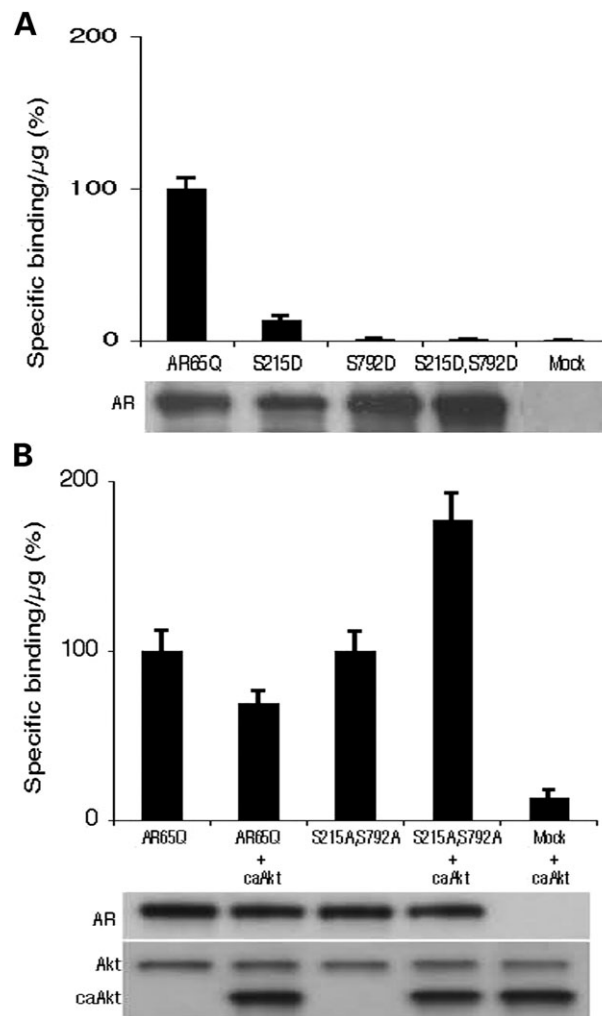


Figure 5. Phosphomimetic substitution of S215 and S792 with aspartate or co-expression of the AR and caAkt reduce the affinity of the AR for androgen. (A) HEK 293T cells transfected with the indicated AR65Q constructs or empty vector were incubated with the non-metabolizable androgen analog ^3H -R1881, and the ability of the AR to bind androgen was measured (see Materials and Methods). (B) HEK 293T cells were transfected with either pCMV-AR65Q, the alanine-substituted receptor or empty vector together with caAkt as indicated, and the ability of the AR to bind to androgen was monitored by radio-ligand binding assay. AR and Akt protein expression was monitored by western blotting with anti-AR and anti-Akt antibodies. Bars, mean \pm SEM.

through phosphorylation at S215 and S792, we used parental MN-1 cells transiently expressing either non-substituted AR65Q or the alanine-substituted version of the receptor and measured cell viability in the presence of DHT (Fig. 6C). The pro-survival effect of IGF-1 was lower in cells expressing the alanine-substituted AR65Q when compared with non-substituted AR65Q-expressing cells, suggesting that phosphorylation at S215 and S792 is important for IGF-1 to protect against the toxicity of the expanded polyglutamine AR. The finding that IGF-1 decreased toxicity of truncated expanded polyglutamine AR lacking Akt phosphorylation sites suggests that the protective effect of IGF-1 is also attributable to activation of other pathways (Supplementary Material, Fig. S2).

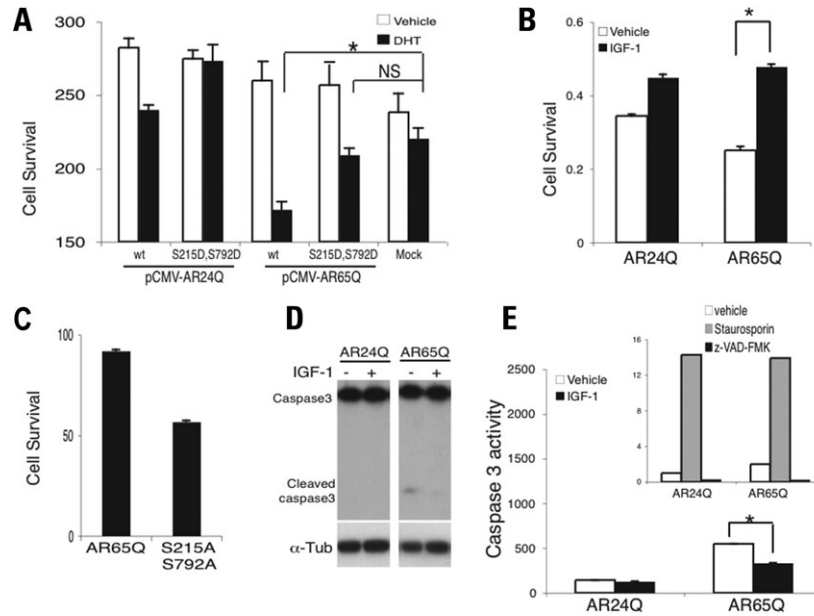


Figure 6. IGF-1 modulates the toxicity of polyglutamine-expanded AR in cell. (A) HEK 293T cells were transiently transfected as indicated and treated with DHT or vehicle. Cell survival was measured by XTT assay. Bars, mean \pm SEM. $*P < 0.01$ ($n = 3$, Student's *t*-test). (B) MN-1 cells stably transfected with pCMV-AR24Q and pCMV-AR65Q constructs were treated with or without IGF-1 and processed for XTT assay. Bars, mean \pm SEM. $*P < 0.05$ ($n = 3$, Student's *t*-test). (C) MN-1 cells transiently transfected with pCMV-AR65Q vector or the alanine-substituted AR construct were treated with IGF-1 and DHT and processed for XTT assay. Increased cell viability is shown as the mean IGF-1-specific increase in cell survival \pm SEM. (D) MN-1 cells stably expressing AR24Q and AR65Q were treated with IGF-1. Caspase 3 activation was monitored by western blotting with anti-caspase 3 antibody. α -Tubulin immunoreactivity is shown as loading control. (E) MN-1 cells stably expressing AR24Q and AR65Q were treated with IGF-1, and caspase 3 activity was measured by a fluorometric assay. Cells were incubated with the fluorescent caspase 3 substrate DEVD-AFC and emission of free AFC was quantified. As control, cells were treated with the caspase activator staurosporin and the caspase inhibitor z-VAD-FMK (inset). Bars, mean \pm SEM. $*P < 0.05$ ($n = 3$, Student's *t*-test).

Expression of the expanded polyglutamine AR in MN-1 and HEK 293T cells has been previously shown to result in caspase 3 activation, which precedes apoptotic cell death (16). Activation of caspase 3 involves its proteolytic cleavage into 17 and 19 kDa fragments. We found that expression of AR65Q also resulted in caspase 3 cleavage in stably transfected AR65Q MN-1 cells (Fig. 6D). IGF-1 decreased caspase 3 processing to levels nearly undetectable by western blotting. We verified that IGF-1 reduced the AR65Q-associated increase in caspase 3 activation by a fluorometric assay of caspase 3 activity (Fig. 6E). As positive and negative controls, we measured caspase 3 activity in the presence of the caspase activator staurosporin and the caspase inhibitor z-VAD-FMK (Fig. 6E, inset). We verified that IGF-1 stimulation of cells did not affect AR mRNA or protein levels by real-time PCR and western blotting (not shown). Together, our results show that IGF-1 decreases expanded AR toxicity, at least in part through phosphorylation of the AR at S215 and S792.

Insulin-like growth factor-1 rescue of AR65Q toxicity requires the phospho-inositol-3-kinase pathway

The two major signal transduction pathways activated by IGF-1 are PI3K-Akt/TOR and MEK-ERK (Fig. 7A). We investigated whether either or both of these pathways are required for the IGF-1-stimulated reduction in caspase 3 activity in AR65Q-expressing cells (Fig. 7A). Treatment of the cells with PD98059, a specific MEK inhibitor, decreased

activation of ERK1,2, as measured by phosphorylation at threonine 202/tyrosine 204. As expected, this treatment had no effect on Akt phosphorylation at serine 473 downstream of IGF-1 signaling (Fig. 7B, bottom panel). PD98059 treatment slightly increased caspase 3 activation in the absence of IGF-1 in AR65Q-expressing cells (Fig. 7B, upper panel), suggesting a contribution of this pathway in modulating expanded AR toxicity, as previously shown (17). However, the effect of IGF-1 on caspase 3 activation was not affected by PD98059, indicating that MEK-ERK signaling is not required for the IGF-1 amelioration of AR65Q toxicity.

To assess the role of PI3K, we incubated the cells with increasing amounts of the PI3K inhibitor LY294002 and measured caspase 3 activation (Fig. 7C). In AR24Q-expressing cells, LY294002 led to a slight increase in toxicity. In contrast, treatment of AR65Q-expressing cells with LY294002 led to a marked dose-dependent increase in caspase 3 activation. The ability of IGF-1 to decrease caspase 3 activation in AR65Q-expressing cells was progressively inhibited and completely lost at the highest doses of LY294002 used. The inhibitory effect of LY294002 on PI3K was measured as a reduction in phosphorylation of Akt at serine 473 (Fig. 7C, bottom panel). Similar results were obtained with Wortmannin, another PI3K inhibitor (not shown). To further examine the role of PI3K, we transfected cells with dominant negative mutant PI3K (dnPI3K). Expression of dnPI3K blocked the IGF-1-mediated decrease in caspase 3 activation (Fig. 7D). We analyzed the role of Akt by transiently transfecting the MN-1 cells with caAkt.

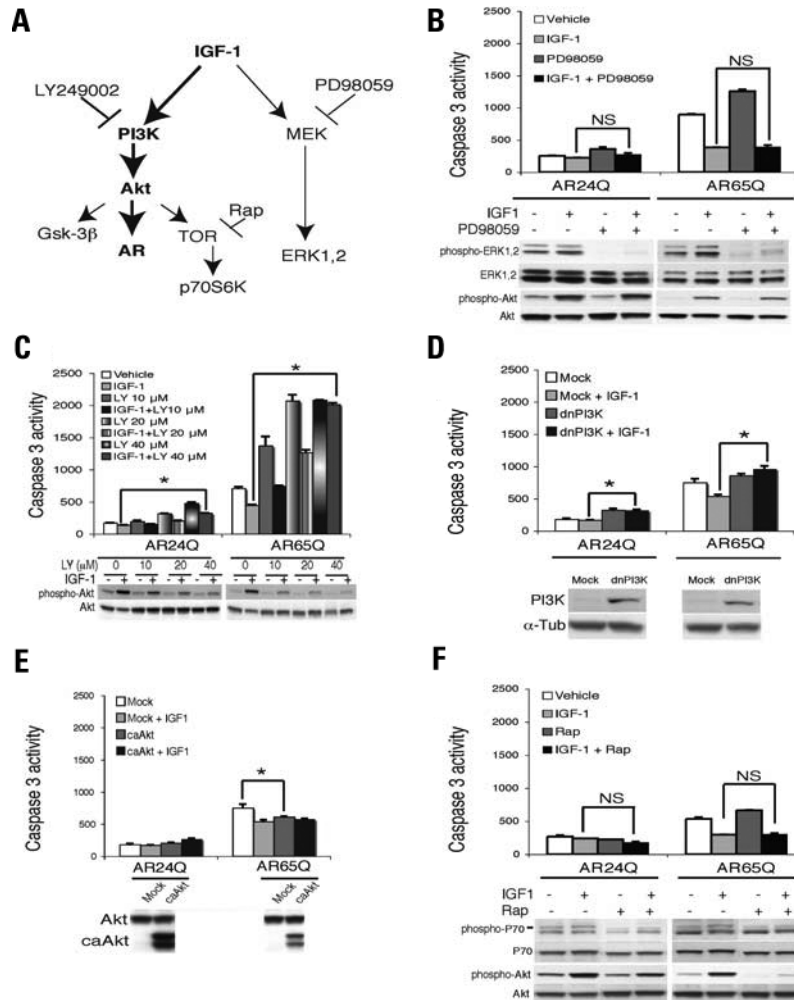


Figure 7. PI3K, but not MEK, signaling is required for IGF-1 to reduce caspase 3 activation in AR65Q cells. (A) Schematic representation of the signaling pathways activated by IGF-1. The pharmacological inhibitors of PI3K (LY249002), MEK (PD98059) and TOR (Rapamycin, or Rap) are indicated. (B) MN-1 cells stably transfected with pCMV-AR24Q and pCMV-AR65Q vectors were treated with IGF-1 and PD98059 as indicated. Caspase 3 activity was monitored by measuring emission of the free AFC fluorophore released from the caspase 3 substrate DEVD-AFC upon caspase 3 activation. Phosphorylation and protein expression level of ERK1,2 and Akt were monitored by western blotting of cells treated in the same conditions used to measure caspase 3 activity using anti-phospho-threonine202/tyrosine204 ERK1,2, anti-ERK1,2, anti-phospho-serine473 Akt and anti-Akt antibodies. (C) MN-1 cells stably expressing AR24Q and AR65Q were treated with IGF-1 and LY249002 (LY) as indicated and processed for caspase 3 assay. LY249002 treatment increased caspase 3 activation, and reduced IGF-1 ability to rescue caspase 3 activation in AR65Q-expressing cells. Akt phosphorylation and protein expression were monitored by western blotting with anti-phospho-serine473 Akt and anti-Akt antibodies. (D) MN-1 cells stably expressing AR24Q and AR65Q were transiently transfected with either an empty (mock) or PI3K dominant negative (dnPI3K) vector and treated with IGF-1 or vehicle. Caspase 3 activity was measured by fluorometric assay. PI3K protein expression was monitored by western blotting. α -Tubulin immunoreactivity is shown as a loading control. (E) MN-1 cells stably expressing AR24Q and AR65Q were transiently transfected with either an empty (mock) or caAkt vector and treated with IGF-1 as indicated. Caspase 3 activity was measured by fluorometric assay. CaAkt protein expression was monitored by western blotting. Endogenous Akt immunoreactivity is shown as a loading control. (F) MN-1 cells stably expressing AR24Q and AR65Q were treated with rapamycin (Rap) and IGF-1. Caspase 3 activity was measured by fluorometric assay. Phosphorylation and expression level of p70 S6 kinase and Akt were monitored by western blotting using anti-phospho-threonine389 p70 S6 kinase, anti-p70 S6 kinase, anti-phospho-serine473 Akt and anti-Akt antibodies. Staining with anti-phospho p70 S6 kinase antibody revealed a specific upper band and a lower band that is non-specific. Bars, mean \pm SEM. * P < 0.05; NS, non-significant (n = 3, Student's t -test).

CaAkt significantly decreased caspase 3 activation in the presence and absence of IGF-1 in MN-AR65Q cells, consistent with a role for Akt in mediating the IGF-1 effect (Fig. 7E). These results suggest that IGF-1 protects AR65Q-expressing cells through activation of the PI3K pathway, and likely through Akt activation.

Akt activates many downstream effectors, including TOR, which is associated with cell survival (18). Incubation of cells with the selective TOR inhibitor rapamycin completely

abolished phosphorylation of the substrate P70 S6 kinase in the presence and absence of IGF-1 (Fig. 7F, bottom panel). Interestingly, in AR65Q cells, IGF-1-induced phosphorylation of Akt was dramatically reduced in the presence of rapamycin, suggesting that in this cell line Akt phosphorylation is sensitive to prolonged exposure of rapamycin, as previously shown in other cell types (19). However, rapamycin did not block the effect of IGF-1 on caspase 3 activation (Fig. 7F, upper panel). Together, these data indicate that activation of

PI3K and Akt, but not MEK and TOR signaling, is required for IGF-1 to reduce AR65Q toxicity in MN-1 cells.

DISCUSSION

In this study, we demonstrate that phosphorylation or phosphomimetic mutations of the AR at Akt consensus sites reduce androgen binding, decrease ligand-induced protein stabilization, nuclear translocation and transcriptional activation, and protect against the toxicity of the expanded polyglutamine AR in a cell model of SBMA. IGF-1 and constitutively active Akt reduced the toxicity of the mutant AR in motor neuron-derived cells. The IGF-1 effect was decreased in cells expressing the AR with alanine substitutions at S215 and S792. PI3K, but not MEK, was required for the IGF-1 rescue of toxicity from expanded polyglutamine AR. These results show that phosphorylation of the AR at the Akt consensus sites modifies the biology of the AR and mitigates the toxicity of the expanded polyglutamine AR.

Phosphorylation at the Akt consensus sites alters the ability of the expanded androgen receptor to bind androgens, translocate to the nucleus and induce cell death

A relationship between toxicity and nuclear localization of the polyglutamine proteins has been shown in Huntington's disease (20–22), and spinocerebellar ataxia 1 (23) as well as SBMA (6–9,24). Nuclear localization of the AR is regulated by binding with the androgen ligands, testosterone and DHT. The observation that ligand binding and nuclear translocation of the polyglutamine-expanded AR are critical for toxicity indicates that androgen reduction is a potential therapy for SBMA (8). We propose that IGF-1 stimulation of Akt-induced AR phosphorylation may be an alternative or synergistic approach to reduce AR binding to androgen.

Akt phosphorylates other polyglutamine proteins, including ataxin 1 (25) and huntingtin (26). However, Akt activity is not universally beneficial. In the case of ataxin 1, phosphorylation at the Akt consensus site serine 776 is pathogenic in mouse and *Drosophila* (25,27). On the other hand, phosphorylation of expanded huntingtin at serine 421 by Akt has been shown to decrease toxicity in cultured striatal neurons. How phosphorylation of huntingtin by Akt reduces toxicity of mutant huntingtin has not yet been clarified. The AR has been previously shown to be phosphorylated by stress kinases at serine 650, which leads to nuclear-cytoplasmic shuttling (28), and by Akt at S215 and S792, but the consequence of this phosphorylation was previously unknown (12). The crystallographic structure of the ligand-binding domain of the AR shows that S792 is near, but not within the ligand-binding pocket, and phosphorylation or substitution with aspartate is not predicted to interfere directly with ligand binding (structure 1XQ3, <http://www.rcsb.org/pdb/home/home.do>). The structure does not include S215, which is in the N-terminal region. The work presented here is the first demonstration that phosphorylation at S215 and S792 by Akt regulates ligand binding, and the subcellular distribution of the receptor. Moreover, using pulse-chase analysis we provide evidence that substitution of S215 and S792 with aspartate prevents

ligand-dependent AR protein stabilization leading to increased protein clearance through the proteasome. The turnover of the expanded polyglutamine AR with aspartate substitutions is increased when compared with the aspartate-substituted normal receptor. Phosphorylation may therefore play a larger role in the clearance of the mutant receptor. Furthermore, using a cell viability assay (XTT assay), we show that phosphomimetic substitution of S215 and S792 reduces the toxicity associated with the expanded polyglutamine AR in cells treated with DHT.

Insulin-like growth factor-1 as a potential therapy for spinal and bulbar muscular atrophy

IGF-1 counteracts toxicity of the expanded polyglutamine AR in cell models of SBMA through activation of PI3K-Akt signaling. IGF-1 promotes neuronal survival in several neurodegenerative disorders (29,30), including Huntington's disease (26) and amyotrophic lateral sclerosis (31,32). In amyotrophic lateral sclerosis, viral-mediated delivery (32) and specific skeletal muscle expression (31) of IGF-1 enhance motor neuron survival and ameliorate disease manifestation in transgenic mice. However, the mechanism through which IGF-1 rescues amyotrophic lateral sclerosis motor neurons from death has not yet been clarified.

IGF-1 has been shown to work as a neuronal pro-survival factor through activation of anti-apoptotic pathways including the PI3K-Akt pathway (29,33). PI3K-Akt signaling leads to activation or inhibition of a number of downstream Akt effectors, such as Gsk-3 β , TOR, FOXO and Bad, which are known to regulate apoptotic cell death. We show that IGF-1 is a pro-survival factor for motor neuron-derived cells that express the expanded polyglutamine AR, that this occurs through activation of the PI3K pathway and that the same effect can be accomplished by expression of caAkt. Although we show that TOR is not required for rescue, complete resolution of PI3K components involved in the rescue requires further investigation. It is clear, however, that phosphorylation of the AR at the Akt consensus sites contributes to the rescue. IGF-1 has been previously reported to induce AR phosphorylation (12). By a variety of different approaches (phosphorylation mutants, constitutively active Akt and pharmacologic inhibition of the IGF-1-activated signaling pathways), we have now demonstrated that PI3K-Akt signaling is required for IGF-1 to modulate AR function and toxicity in cell culture.

Our data represent a novel mechanism that regulates androgen binding to the AR. Here, we have shown that phosphorylation of the AR at the Akt consensus sites S215 and S792 reduces ligand binding, blocks ligand-dependent activation of the AR and rescues motor neuron cells from the toxicity of the expanded AR. To our knowledge, this is the first demonstration that binding of androgen to the AR can be reduced by phosphorylation. It is predicted that IGF-1 treatments may reduce the ligand binding function of the AR and could cause AR loss of function (which may be deleterious), however the extent to which it does so *in vivo* remains to be empirically determined. In addition to its effects on the AR, IGF-1 promotes pro-survival signals in a number of cell types. It will therefore be of interest to determine whether therapy based on activation of the IGF-1-PI3K-Akt pathway is effective in

mitigating disease manifestations in SBMA transgenic mice and SBMA patients.

MATERIALS AND METHODS

Plasmids

The caAkt, dnPI3K (p85 α regulatory subunit mutant) and pARE-E1b-luc expression vectors were a kind gift of Drs R.S. Freeman (University of Rochester, NY, USA), Murakami (Aichi Cancer Center, Japan) and C. Smith (Baylor College of Medicine, Huston, TX, USA), respectively; pCMV β and pcDNA3.1 vectors were obtained from Clontech and Invitrogen, respectively. The human AR substitutions S215 and S792 (numbers refer to NM_000044) were obtained by site-directed mutagenesis (SeqWright, TX, USA), using the following primers: GAGAGCGAGGGAGGCCGCGGGGG CTCCCACTTC (S215A-F) and GAAGTGGGAGCCCCCG CGCCTCCCTCGCTCTC (S215A-R); GA GAGCGAGGG AGGCCGACGGGGCTCCCACTTC (S215D-F) and GAAG TGGGAGC CCCGTCGGCCTCCCTCGCTCTC (S215D-R), GTCCGAATGAGGCACCTCGCTCA AGAGTTTGGATGG C (S792A-F) and GCCATCCAAACTCTTGAGCGAGGTG CCTC ATTCGGAC (S792A-R), and GTCCGAATGAGG CACCTCGACCAAGAGTTTGGATGG C (S792D-F) and GCCATCCAAACTCTTGGTCGAGGTGCCTCATTCGGAC (S792D-R).

Cell cultures and transfections

MN-1 (34), Cos1 (ATCC, CRL-1650) and HEK 293T (ATCC, CRL-1573) cells were cultured as previously described (15,16). All the experiments were carried out in complete medium containing 10% charcoal dextran stripped-fetal bovine serum (CDS) (HyClone), when not indicated otherwise. MN-1 and Cos1 cells (1×10^6 cells) were transiently transfected using the Cell Line Nucleofector kit V (Amaxa Biosystem) with 5 and 2 μ g of DNA, respectively. HEK 293T (2×10^5 cells) were transfected with 2 μ g of DNA, using lipofectamine/Plus reagent (Invitrogen). MN-1 cells were treated for 48 h with R1881 (10 nM, Sigma), DHT (10 nM, Sigma), IGF-1 (100 ng/ml), LY294002 (10–40 μ M), PD98059 (50 μ M, Calbiochem), rapamycin (100 nM, Calbiochem), staurosporin (1 μ M, Calbiochem), MG132 (1 μ M, Calbiochem), z-VAD-FMK (10 μ M, BD Biosciences).

Immunocytochemistry and microscopy

Immunofluorescence was performed as previously described (35). Antibodies: primary antibody AR(N20) (1:250, sc-816, Santa Cruz); secondary antibody Alexa Fluor 488-Goat Anti-Rabbit IgG antibody (A-11008, Molecular Probes). Images were acquired digitally with a DeltaVision microscope and deconvolved with the softWoRx algorithm (Applied Precision). For the graph in Figure 3B, transfected cells were classified into five groups: cells with AR in the nucleus (1), more in the nucleus than in the cytosol (2), equally divided between nucleus and cytosol (3), more in the cytosol (4) or only in the cytosol (5). Data in Figure 3B represent groups 1 and 2 expressed as a percentage.

Caspase 3, XTT, transcriptional activity and ligand-binding assays

Caspase 3 activity, cell proliferation and viability (XTT assay) and transcriptional activity were measured using the ApoTarget fluorometric assay (Biosource International), Cell Proliferation Kit II (Roche Diagnostics) and LucLite Luminescence Reporter Gene Assay System (Perkin-Elmer), respectively, according to manufacturer's instructions. To measure cell viability by XTT assay, cells were serum-starved and treated with aphidicolin (400 ng/ml, Calbiochem) for 48 h. In the ligand-binding assay, HEK 293T cells were treated with 10 nM [³H]-R1881 (72.0 Ci/mmol, Perkin-Elmer) for 1 h in 0.5% bovine serum albumin in Dulbecco's Modified Eagle's medium. Triamcinolone acetonide (10 μ M, Sigma) was added to the samples to prevent binding with corticoid and progesterin receptors. The cells were washed twice, for 5 min each, in phosphate-buffered saline (PBS), harvested in PBS, mixed with Optiphase Supermix Cocktail (Perkin-Elmer) and counted in a Wallac Microbeta TriLux scintillator (Perkin-Elmer). Protein concentration was estimated on parallel samples by bicinchoninic acid protein assay method (Pierce). Specific binding was determined as $100 \times$ (³[H]-R1881 binding – ³[H]-R1881 binding in the presence of 100-fold molar excess cold R1881)/micrograms of protein in 100 μ l of extract.

Western blotting, immunoprecipitation, pulse-chase analysis

Cells were washed twice in ice-cold PBS and scraped in 50 μ l lysis buffer (150 mM NaCl, 6 mM Na₂HPO₄, 12 mM NaH₂PO₄, 5 mM ethylenediaminetetraacetic acid, 1% Na-deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate) plus protease inhibitor cocktail (Roche Diagnostics) and serine/threonine protein phosphatase inhibitor Cocktail 1 (Sigma). The lysate was sonicated, and centrifuged at 15 700 g for 10 min at 4°C. Cells lysates were denatured at 95°C in 5 \times sample buffer (60 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, 20% β -mercaptoethanol) and processed for 7.5–10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and electro-transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Immunoblotting was done in 5% non-fat dry milk in Tris-buffered saline for all antibodies as follows: AR(N20) (sc-816, Santa Cruz); caspase 3 (9662), total-Akt (9272), phospho-Akt (9271), total-ERK1,2 (9102), phospho-ERK1,2 (9101), total-GSK3 β (9332), phospho-GSK3 β (9336), total-p70S6 kinase (9202) and phospho-p70S6 kinase (9205) (Cell Signaling); α -tubulin (T6199) and Flag (F7425, Sigma); phospho-S215-AR (IMG-561, Imgenex). Immunoreactivity was detected using peroxidase-conjugated AffiniPure Goat Anti-Rabbit or Anti-Mouse IgG (Jackson ImmunoResearch), and visualized using LIGHTNING chemiluminescence reagent (Perkin-Elmer) following the manufacturer's instructions.

All immunoprecipitation procedures were carried out at 4°C. HEK 293T cells were washed twice with PBS, scraped in 500 μ l IP buffer (50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) plus protease inhibitor cocktail (Roche) and sonicated. Cleared lysate was immunoprecipitated with 10 μ g of AR(441) antibody (sc-7305, Santa Cruz)

for 3 h. Fifty microlitre of beads (Protein A/G PLUS-Agarose Immunoprecipitation Reagent, sc-2003, Santa Cruz) were added to each sample overnight. Precipitated proteins were then washed three times in IP buffer, resuspended in sample buffer, boiled and subjected to SDS-PAGE.

Pulse-chase analysis was performed as previously described (14). In brief, COS 1 cells were incubated in cysteine/methionine-free media (Sigma) for 2 h followed by incubation in cysteine/methionine-free media containing 100 μ Ci 35 S-labeled cysteine/methionine (GE Healthcare, Piscataway, NJ, USA) for 1 h. After labeling, cells were washed once with culture media containing 10-fold excess of unlabeled methionine and cysteine (5 mM each) and incubated further in the same medium for the indicated times. Cells were collected at the indicated time points, and processed for immunoprecipitation with anti-AR antibody. Immunocomplexes were separated on 6% SDS-PAGE. Gels were dried and exposed to phosphorimager screen for visualization on a STORM phosphorimager system (Molecular Dynamics, Piscataway, NJ, USA). Densitometric analysis of protein bands was carried out using Imagequant PhosphorImager software (Molecular Dynamics).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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