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Implications of a polyglutamine tract in the function of the human androgen receptor

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Abstract

The androgen receptor (AR) is a ligand-dependent transcription factor and belongs to the nuclear receptor family. The AR gene contains a long polymorphic CAG repeat, coding for a polyglutamine tract. In the full size AR, the deletion of the polyglutamine tract results in an increase in the transactivation through canonical AREs. However, this effect is clearly dependent on the response elements, since it is not observed on selective elements. In our assays, a deletion of the repeat positively affected the interactions of the ligand-binding domain with the amino-terminal domain as well as the recruitment of the p160 coactivator SRC-1e to the amino-terminal domain of the AR. This is reflected by an enhanced coactivation of the AR by SRC-1e.

Keywords: Androgen receptor; CAG-repeat; Polyglutamine; Transcription; SUMO-1; ARE; Direct repeat; Coactivator

Androgens are important for the male sexual differentiation, testicular descent, and spermatogenesis. Testosterone and dihydrotestosterone are the two most important natural androgens, and their biological function is exerted through activation of the androgen receptor (AR). The AR is a ligand-dependent transcription factor [1].

The activated class I steroid receptors (AR, glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR)) recognize the same response elements organized as inverted repeats of 5′-TGTTCT-3′-like sequences. These elements are called canonical androgen response elements (AREs). In addition, the AR can bind to direct repeats of 5′-TGTTCT-3′-like sequences, a characteristic proposed to contribute to the androgen-specificity of in vivo transcriptional responses [2]. The latter elements are therefore called selective AREs.

Unlike the other nuclear receptors (NRs), the AR contains a weak activation function 2 (AF-2) in the ligand-binding domain (LBD). However, it is the amino-terminal domain (NTD) that is the most important and indispensable region for transcription activation of androgen-regulated genes. The AR-NTD has a strong LBD-dependent activation function (AF-1), also called Tau-1 (transcription activation unit 1) residing between aa 100–360 [3]. We proposed that the LKDIL-motif, located between aa 178 and 183, is important for AF-1 [4]. When the LBD is deleted, this activation domain shifts more C-terminally and is called Tau-5 (aa 360–485) [3].

Coregulators modulate the transactivating functions of nuclear receptors [5]. SRC-1, e.g., is a well-known coactivator that belongs to the group of the 160-kDa NR coactivators or p160 coactivators. They interact with the LBD or AF-2 via a centrally located NR-interacting region containing three highly conserved α-helical LxxLL motifs or NR signature motifs [6]. For the AR, this interaction is weak, which may be the explanation for the weakness of its AF-2 function. However, another recruitment surface for the p160s resides within the NTD of the AR. In the p160s, it is the glutamine rich region (Qr) (aa 989–1240) which forms the NTD-interacting surface

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[7,8]. The AR activity is also dependent on interactions between the amino- and carboxyterminal domains (N/C-interaction) for its full activity. This interaction is mediated by an amphipathic helix formed by the amino-terminal amino acids of the AR-NTD [9–12].

The AR gene has a CAG repeat encoding a polyglutamine (polyQ) tract at position aa 58 in its amino-terminus. Previous studies have shown that at least eight human neurodegenerative disorders like Huntington's disease and spinobulbar muscular atrophy (SBMA) or Kennedy's disease are linked to expanded CAG repeats. Individuals with SBMA have an expanded CAG tract of more than 40 repeats in the AR gene, while the normal repeat length varies between 9 and 36 repeat units. In contrast, a correlation between an increased risk for prostate cancer and a shorter polyglutamine repeat has been suggested [13–15].

The goal of the present study was to investigate the role of the polyglutamine tract in the AR function. Therefore we deleted the polyglutamine tract, studied the effects of it on AR activity, and compared the AF-1 activity on canonical and selective AREs. In addition, we have analysed the effect on the recruitment of SRC-1e to the AR-NTD and the coactivation by SRC-1e of the AR with or without the polyglutamine tract.

Materials and methods

Plasmid constructs. The expression vectors pSG5AR$_{538–919}$ (encoding the hAR-DBD-LBD), pSG5AR (expressing Flag-tagged full-length hAR), pSG5SRC-1e, the vector Qr-SRC-1 (expressing the Q-rich domain of SRC-1 fused to the Gal4-DBD), and the Gal4-DBD- and the VP16-fusion constructs with hAR$_{1–529}$ are described elsewhere [4,8,12,16]. The deletion of the polyQ tract was made by a PCR-based method by using the following pairs of primers: 5′-GGCAGA TCTCCATGGAAGTGCAGTTAGGGCTGGG-3′ and 5′-CTGCT GGAGACTAGCCCCAGGC-3′ and 5′-GGCTAGTCTCCAGCAGC AGCAGACTGGC-3′ and 5′-GGATCCTCAACGCATGTCCCCG TAAGGTCCGGA-3′. As template, the construct pSG5hAR$_{1–529}$ was used. This PCR-generated hAR-NTD fragment was inserted in-frame with the Gal4-DBD in the *Bam*HI restriction site of pABGal4 and in-frame with the VP16 activating domain in the *Bgl*II site of pSNATCH-II [4]. Expression vectors for the hAR carrying the polyQ deletion were made by insertion of a *Eco*52I/*Eco*81I-fragment of the PCR-products in the pSG5AR construct which was cut with the same restriction enzymes. Restriction and modifying enzymes were obtained from MBI Fermentas GmbH. The luciferase reporter constructs containing the isolated elements *slp*-HRE2, *sc*-ARE1.2, *slp*-HRE2 mut-4T-A; +2A-T and *sc*-ARE1.2 mut-4T-A are driven by the thymidine kinase (TK) minimal promoter and have been described elsewhere [17, and references therein]. The reporter construct driven by the E1b promoter and containing two copies of the rTAT-GRE was described previously [17]. The reporter construct (Gal4)$_5$-TATA-Luc, a kind gift from Dr. M.G. Parker (ICRF, London, UK), was used for measuring the AF-1 AR activity and QrSRC-1 interaction. The reporter plasmid pMMTV-luc was obtained from Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch Cedex, France). The pCMV-βGal vector was obtained from Stratagene. The expression plasmid for SUMO-1 was a kind gift from Dr. A. Dejean (Unité de Recombinaison et Expression Génétique, Institut Pasteur, France).

Transfections and preparation of COS-7 whole cell extracts. All transfections and reporter assays were done exactly as described earlier [12]. At 24 h after transfection, cells were stimulated with R1881 (10^{-8} M). The medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) (Gibco-BRL). The cells were collected in 1.5 ml of ice-cold PBS per dish and pelleted by centrifugation (1 min). The PBS was removed and the cells were resuspended in 60 μl of ice-cold PBS buffer containing protease inhibitors PMSF (10 mg/ml) and trasylol (Bayer AG, Germany). The pellet was collected and the supernatant was removed and stored at −80 °C. For SUMOylation of the AR, COS cells were transfected with pSG5AR constructs (1 μg) and cotransfected with SUMO-1 (1.5 μg). Extracts were prepared as described above with 20 mM *N*-ethylmaleimide (Pierce, Rockford, USA) to inhibit SUMO-1 deconjugation. For the detection of VP16AD or Gal4DBD fusion proteins, extracts were made in passive lysis buffer.

Western blots. For Western blotting, equal amounts of protein were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 6% (AR constructs) or an 8% gel (AR-NTD constructs) and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech.). The membranes were probed with a monoclonal M2 anti-Flag antibody (Stratagene) or with Gal4DBD antibody (Santa Cruz Biotechnology), and immunoreactive proteins were visualized with the chemiluminescence reagent plus (NEN Life science).

Results

Deletion of the polyQ tract

To investigate the effect of the polyQ tract on AR transactivation, transient transfections were performed in COS-7 cells with either human wtAR containing a tract of 19 glutamine residues or with ARΔQ (Fig. 1A). The ARΔQ construct lacks the CAG repeat, encoding the polyQ tract. As luciferase reporter construct, 2×rTAT-GRE was used and different concentrations of R1881 were tested. The transactivation assay shows that deletion of the polyQ tract in human AR results in a fourfold increased activity compared to the wtAR-activity. Western blot analysis of extracts containing wtAR or ARΔQ was performed using a monoclonal M2 anti-Flag antibody (Fig. 1B). No difference in protein expression levels was observed.

A similar experiment was performed on a series of selective and canonical androgen responsive luciferase reporter constructs (Figs. 2A and B). They all contain two copies of the isolated elements, cloned upstream of the thymidine kinase (TK) minimal promoter. As response elements, we used the selective *slp*-HRE2 and *sc*-ARE1.2 (Fig. 2A) and the canonical mutated forms *slp*-HRE2 mut-4T-A; +2A-T and *sc*-ARE1.2 mut-4T-A (Fig. 2B). On canonical AREs, ARΔQ shows an increased activity compared to the wild-type AR (Fig. 2B). This is also true for the canonical ARE 2×rTAT-GRE (Fig. 1A). On the selective AREs *slp*-HRE2 and *sc*-ARE1.2, however, no difference in activation is seen between the wtAR and the ARΔQ (Fig. 2A).

The deletion of the polyQ tract results in an increased transcriptional activity of the AR on canonical elements

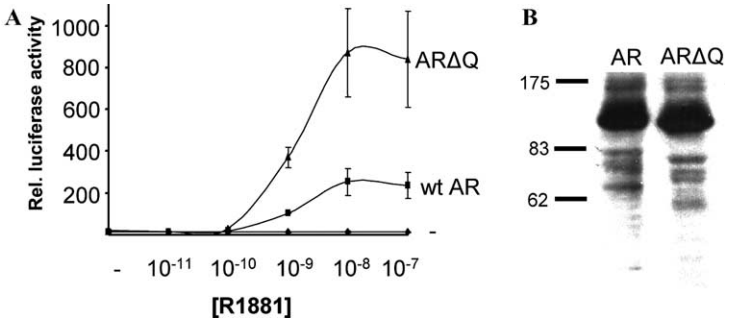

Fig. 1. (A) Transcriptional activation of the wtAR and ARΔQ. A reporter construct driven by the E1b promoter containing two copies of the rTAT-GRE (100 ng) was transiently transfected into COS-7 cells and cotransfected with 20 ng of empty vector, pSG5wtAR or pSG5ARΔQ as indicated on the right. Cells were incubated for 24 h without hormone or with different concentrations of hormone (R1881, 10^{-11}–10^{-7} M). *Squares* and *triangles* represent the transcriptional activities of the AR and ARΔQ, respectively. The activity of the AR in the presence of 10^{-9} M R1881 was set at 100. Luciferase activities were adjusted according to the β-galactosidase activity in the same sample. Results are averages of at least three separate experiments performed in triplicate, the error bars indicate the standard error of the mean (SEM) values. (B) Western blot analysis of cell extracts containing wtAR and ARΔQ using anti-Flag antibody was performed as described in the Materials and methods section.

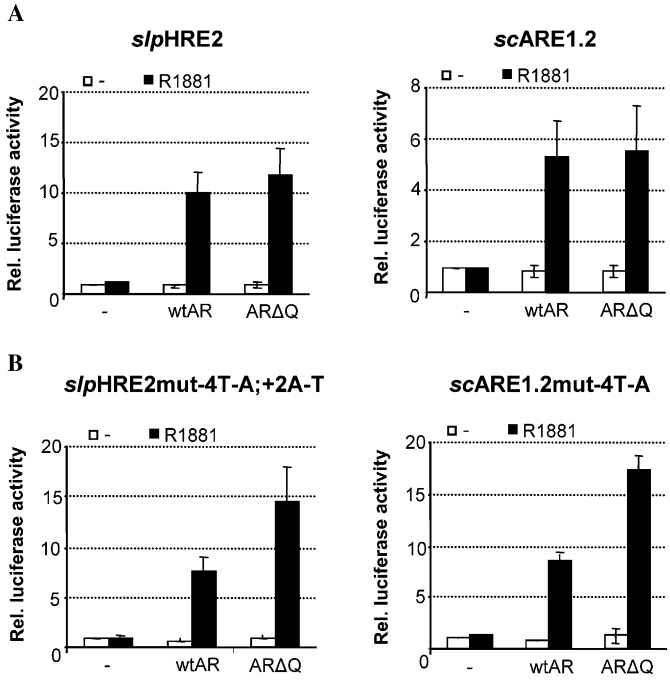

Fig. 2. Transcriptional activation of the wtAR and ARΔQ through selective versus canonical AREs. (A) Selective AREs. TK minimal promoter-driven luciferase reporter constructs containing two copies of either *slp*-HRE2 or *sc*-ARE1.2 motifs (100 ng) were transiently transfected into COS-7 cells and cotransfected with 20 ng of empty vector, pSG5wtAR or pSG5ARΔQ. Cells were incubated for 24 h without hormone (*open bars*) or with hormone (R1881, 10^{-8} M, *black bars*). *Bars* represent transcriptional activities of the AR constructs, relative to the activity of the construct containing the empty pSG5 vector in the absence of hormone, which was set at 1. Luciferase activities were adjusted according to the β-galactosidase activity in the same sample. Results are averages of at least three separate experiments performed in triplicate, the error bars indicate SEM values. (B) Canonical AREs. Transfections were performed as in Fig. 2A, using luciferase reporter constructs containing two copies of the mutated *slp*-HRE2 and *sc*-ARE1.2 motifs, namely *slp*-HRE2 mut-4T-A; +2A-T and *sc*-ARE1.2 mut-4T-A.

(Figs. 1A and 2B). Therefore, we tested whether the constitutive activation unit tau-5 is also influenced. COS-7 cells were transiently transfected with the AR-NTD constructs with or without the polyQ tract fused to Gal4DBD. As reporter construct, $(Gal4)_5$TATA-Luc was used (Fig. 3A). The small decrease observed in the functional assay probably correlates with the lower expression level of the NTDΔQ construct as demonstrated in the Western blot (Fig. 3B).

SUMOylation of the AR

Recently, an enhanced immunoreactivity for SUMO-1 (small ubiquitin-like modifier 1) has been reported in neurons of patients with polyglutamine diseases, like Huntington's disease, Machado–Joseph disease (MJD), spinocerebellar atrophy type-1 (SCA1), and dentatorubral pallidoluysian atrophy (DRPLA) [18]. SUMOylation is a posttranslational modification which resembles,

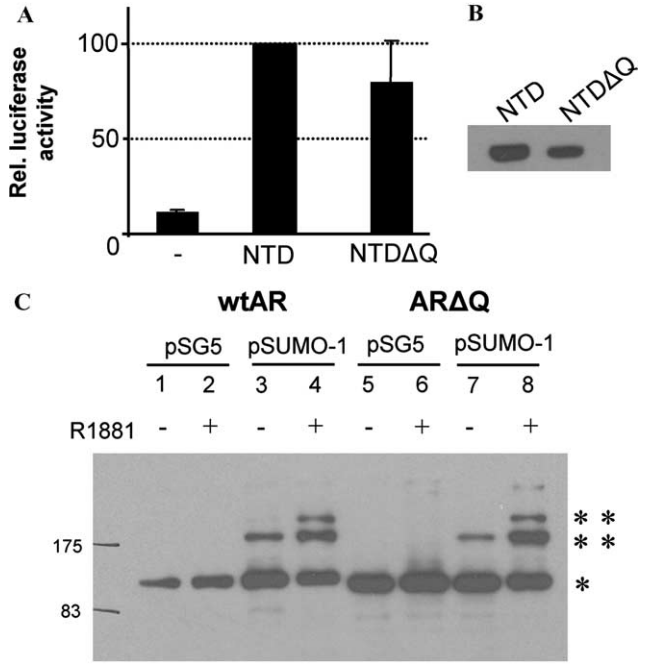

Fig. 3. (A) Study of the constitutive activation domain Tau-5 of the wtAR-NTD and NTDΔQ. COS-7 cells were transfected with 50 ng of the AR-NTD and AR-NTDΔQ fused to the Gal4-DBD, together with 100 ng of the luciferase reporter construct $p(Gal4)_5$-TATA-Luc. Activities are depicted relative to the activity of the wtAR-NTD construct, which was set to 100. Results are averages of at least three separate experiments performed in triplicate, the error bars indicate SEM values. (B) Western blot analysis of cell extracts containing wtAR-NTD and AR-NTDΔQ fused to the Gal4DBD was done as described in the Materials and methods section. (C) SUMO-1 conjugation to hAR and ARΔQ. COS-7 cells were transfected with Flag-tagged AR or ARΔQ (0.5 μg) and cotransfected with either empty vector pSG5 (lanes 1, 2 and 5, 6) or with pSG5SUMO-1 (lanes 3, 4 and 7, 8) (1 μg). Cells were incubated for 24 h without hormone or with hormone (R1881, 10^{-8} M) as indicated above the lanes. The Western blot was developed with the monoclonal M2 anti-Flag antibody. The non-modified AR-band and the SUMOylated AR bands are indicated on the right by an asterisk or a double asterisk, respectively. The position of molecular weight markers is indicated at the left.

but is distinct from, the ubiquitinylation system. The AR has two SUMO-1 attachment sites, one at lysine 385 and one at lysine 511 [19]. We tested whether the polyQ tract in the AR affects the efficiency of AR-SUMOylation. A Western blot was performed with Flag-tagged wtAR or ARΔQ with or without cotransfection with pSG5SUMO-1 or hormone (R1881, 10^{-8} M). It seems from the Western blot experiment in Fig. 3C that the deletion of the polyQ tract does not dramatically affect the conjugation of SUMO-1.

AR-N/C-interaction and AR-N/SRC-1-interaction

The first amino acids in the NTD of the AR are important for its interaction with the LBD [9–12]. Since the polyQ tract resides close to this site, a double hybrid assay was performed to compare the affinity of the ARΔQ construct and the wtAR-NTD for the AR-LBD. Clearly, the glutamine deletion positively affects the AR-N/C-binding (Fig. 4A). We and others proposed that the efficient recruitment of p160 coactivators by the native AR occurs primarily through the NTD [4,7,8]. We tested whether the activity of ARΔQ could result from an increased interaction of SRC-1Qr with AR-NTD (Fig. 4B). In a double hybrid assay, the twofold increase of the luciferase expression clearly indicates a stronger interaction between the AR-NTDΔQ and SRC-1Qr. Immunoblotting of the cell extracts shows that the increased interactions are not due to a higher expression level of the protein (Fig. 4C).

SRC-1e mediated coactivation of the AR

To determine whether the deletion of the glutamine tract has an effect on the SRC-1e mediated coactivation of the AR, transient cotransfections were performed (Fig. 5). Consistent with earlier observations, the coactivation of the AR by SRC-1e is only moderate. However, the ARΔQ construct is clearly more efficiently coactivated by SRC-1e. There is a more than twofold increase in activity.

Discussion

The polyQ tract in the human AR resides in the NTD of the receptor at position 58. Besides this repeat there are two small glutamine repeat tracts at position 83 (approximately six residues) and one at position 192. There is some degree of evolutionary conservation of these polyQ tracts. Mice and rats, for example, have only two glutamine residues in the first tract and two residues in the second tract, but have longer polyQ tracts in the third repeat (approximately 20 residues). The length of the repeats is highly variable because of slippage of DNA polymerase during replication on the multiple CAG nucleotides in the DNA template. The normal range of CAG repeat length in the human AR, which can vary somewhat with ethnicity and race, is between 9 and 36 repeats with a highest frequency of approximately 20 repeats [20,21].

Epidemiologic studies established an inverse correlation between the length of the polyQ tract and incidence of prostate cancer. An expansion of the polyQ tract of more than 40 residues, however, is thought to cause X-linked SBMA, a disease characterized by a loss of motor neurons in the brainstem and spinal cord. Males who suffer from this disease also show a mild form of androgen insensitivity, causing a decreased fertility.

The NTD of the AR is indispensable for its function as a transcription factor since it contains a strong transcription activation function (AF-1 or Tau-1) from position 100 to 360. The LKDIL-motif, located between

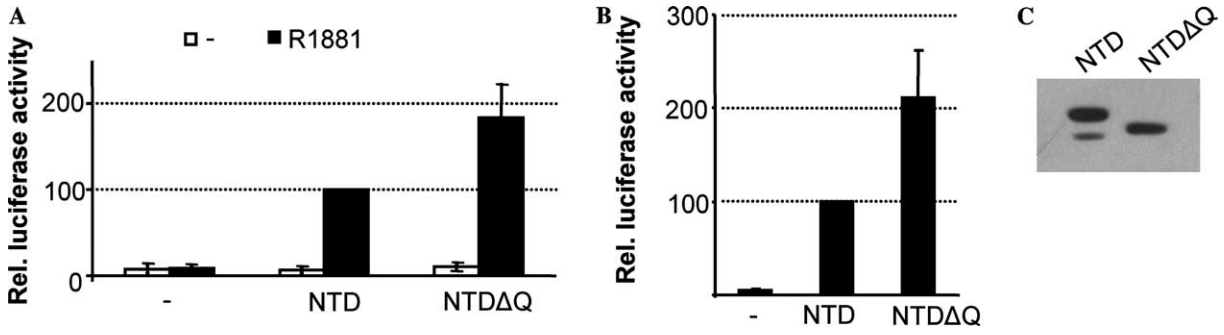

Fig. 4. (A) Two-hybrid assay for the interaction between AR-NTD and AR-LBD. pSG5AR-DBD-LBD (538–919) (50 ng/well) was coexpressed in COS-7 cells with either the empty pSNATCH-II expression vector or the same expression vector containing the wild-type NTD or the NTD without the polyQ stretch (50 ng/well). Assays were performed using the 2×TAT-GRE(E1b)-Luc reporter (100 ng) and the CMV-βGal reporter (5 ng/well) in the presence or absence of 10^{-8} M R1881. Activities are depicted relative to the activity of the wtAR-NTD construct, which was set to 100. The values shown are averages of at least three independent experiments performed in triplicate. Error bars indicate SEM values. (B) Two-hybrid assay for the interaction between AR-NTD and QrSRC-1. pABGal4-DBDQrSRC-1e (989–1240) (50 ng/well) was coexpressed in COS-7 cells with 50 ng of empty pSNATCH-II, pSNATCH-IIAR$_{1\text{-}529}$ or pSNATCH-IIAR$_{1\text{-}529}$ΔQ. Assays were performed using the (Gal4)$_5$-TATA-Luciferase reporter (100 ng). Activities are depicted relative to the activity of the wtAR-NTD construct, which was set to 100. The values shown are averages of at least three independent experiments performed in triplicate. Error bars indicate SEM values. (C) Western blot analysis of cell extracts containing wtAR-NTD and AR-NTDΔQ using the monoclonal M2 anti-Flag antibody.

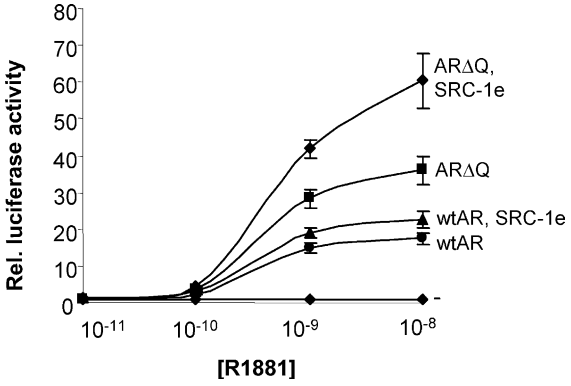

Fig. 5. Coactivation of wtAR and ARΔQ by SRC-1e. COS-7 cells were transfected with 20 ng of pSG5 with no insert or with wtAR and ARΔQ and cotransfected with 100 ng pSG5SRC-1e. As reporter construct, 100 ng of MMTV-Luc was used. Luciferase activities, obtained after stimulation with increasing concentrations of R1881, were adjusted according to the β-galactosidase activity in the same sample. Results are averages of at least three separate experiments performed in triplicate, the error bars indicate SEM values.

aa 178 and 183, plays a key role in this transcriptional activation function AF-1 [4]. In this study, we explore the effects of a deletion of the polyQ tract on AR activation function. The transfection assay in Fig. 1A shows that this deletion results in a fourfold increase of AR activity. Comparative Western blotting demonstrates that the enhanced AR activity is not explained by an increase in AR protein level (Fig. 1B). These results are consistent with the findings of several studies that the transactivation potential of the AR is inversely correlated to repeat length [22,23].

In our working hypothesis, selective AREs are direct repeats in contrast to canonical AREs which are organized as inverted repeats of the 5′-TGTTCT-3′ core sequence. This then suggests an alternative dimerization of the binding receptors. It is very likely that such dimerization not only affects the DNA-binding domains but also other receptor domains and their interactions. We therefore analysed the activity of ARΔQ on different responsive constructs. Surprisingly, we observed no change in the transcription activating capacity of ARΔQ compared to wtAR on selective AREs (Fig. 2A). However, when these AREs are mutated to become inverted, canonical AREs [2,17], AR transactivation was significantly affected by the polyQ deletion (Fig. 2B).

We also investigated the influence of a deleted polyQ tract on the constitutive activation domain Tau-5 (Fig. 3A). There is a small decreased Tau-5 activation by deleting the polyQ tract. We can conclude from this, together with the effect observed in the full size AR, that the deletion of the polyQ tract results in an increased AR-transactivation capacity by affecting the Tau-1 activation unit and not the Tau-5 activation unit.

In previous studies, a decrease in AR-transactivation capacity was observed when SUMO-1 was cotransfected [19]. In addition, there is strong evidence for an enhanced SUMO-1 conjugation in polyQ diseases [18]. However, we observed no effect of the presence or absence of the polyQ tract on SUMOylation of the AR (Fig. 3C). This is probably due to the fact that the polyQ tract in the AR tested was not expanded.

In our previous studies, we and others showed that an amphipathic helix in the AR-NTD at position 23 is involved in a N/C-interaction which is needed for optimal AR activity [10–12]. Deletion of the core amino acids FQNLF (ARΔFQNLF) in the amphipathic helix abolished the interaction between the NTD and the LBD. We observed a decrease in the transcription activating capacity of ARΔFQNLF, compared to wtAR on ca-

nonical AREs, while no changes were observed on the selective AREs.

Therefore, we tested whether the deletion of the polyQ repeat influences N/C-interaction or AR-N/SRC-1 interaction. We observed an almost 2-fold better N/C-interaction (Fig. 4A). We suggest that the AR activity on the canonical AREs increases due to a tighter N/C-interaction, while the AR activity on selective AREs is not influenced by deletion of the polyQ tract because the AR-transcriptional activity on these elements does not depend on N/C-interaction. In double hybrid assay, we analysed the interaction between the Qr (aa 989–1240) of SRC-1 and the AR-NTD. There is an enhanced NTD-p160 interaction (Fig. 4B). We therefore hypothesize that a shorter polyQ tract gives the AR a more accessible or stable surface for AR-interacting proteins, like SRC-1 or the AR-LBD.

It is thought that p160s play a key role in recruiting several factors to the receptor–coactivator transcriptional activation complex [24]. Some of these factors have histone-acetyltransferase activity like CREB-binding protein and its associated protein p/CAF [25]. In addition, p160s recruit a histone methyltransferase, ultimately resulting in a change in local chromatin structure and the assembly of transcriptional initiation complexes at the target promoter [26]. It was not known which of these processes is affected by the polyQ tract of the AR-NTD.

We therefore determined the effect of a deleted polyQ tract on SRC-1 mediated coactivation of the full size AR. When the polyQ tract in the AR is deleted, we see an increased coactivation (Fig. 5). This again indicates that coactivation of the AR by SRC-1 results from recruitment of SRC-1, and via SRC-1 the other components of the activation complex, principally via the NTD of the AR. This also correlates with the inverse finding that a longer polyQ tract inhibits p160-mediated coactivation [23].

Our data indicate that the polyQ tract at position 58 in the AR affects the activity of the AR on canonical AREs. It does so by influencing the N/C-interactions, as well as the p160 recruitment by the NTD. Taken together, our data corroborate the hypothesis that the AR transactivates its target genes via different mechanisms, some of which are more affected by N/C-interactions, by the presence of a polyQ tract, or by the nature of the response elements.

Acknowledgments

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