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Androgen Receptor Acetylation Sites Differentially Regulate Gene Control

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Abstract Androgen receptor (AR) function is modulated by post-translational modifications such as acetylation, ubiquitylation, sumoylation, and phosphorylation. Concerning acetylation, three lysines residues located in a consensus KxKK motif of the AR hinge domain have been identified. For a better evaluation of the role of this modification, the activity of AR modified at different acetylation sites was determined by comparing the effects on natural and synthetic promoters. We found that mutation of AR acetylation sites affected both potency and efficacy of androgen-dependent response. Remarkably, elimination of all three acetylation sites was still compatible with strong AR activity on the PSA and MMTV promoters, but not on the Pem promoter. This differential effect was seen at various wild-type (wt) to mutant AR receptor ratios and at changing hormone concentrations. Subcellular localization studies showed that both mutated and wt AR efficiently translocated into the cell nucleus. Plasmid immunoprecipitation revealed comparable binding of both receptor forms to the Pem promoter. The differential effects observed for the Pem promoter were partially due to an androgen response element (ARE) named ARE-1 which was only poorly stimulated by the AR acetylation site mutant. Finally, AR mutants impaired in their N/C interaction elicited intact stimulation of the Pem promoter, suggesting that AR acetylation was not influenced by this inter-domain communication. The promoter-selective effects seen for the AR acetylation site mutants strongly suggest this post-translational modification to be important in the fine-tuning of the effects of androgens on different target genes. J. Cell. Biochem. 104: 511–524, 2008.

Key words: androgen receptor; acetylation; promoter; nuclear localization

The impact of post-translational modifications on protein function is documented by numerous studies. Among these, protein acetylation represents a frequent event that affects the ε-amino group of lysine residues in a reversible way [Kouzarides, 2000]. Acetylation of histone tails has been extensively studied and is instrumental in regulating gene transcription by controlling the local chromatin structure and thereby the access of regulatory proteins to promoter regions [Fraga and Esteller, 2005; An, 2007]. Recent work shows that acetylation of non-histone proteins also plays an important regulatory role, affecting processes such as intracellular localization, protein-protein interactions and protein stability [Glozak et al., 2005]. Many transcription factors including p53, c-MYC, E2F family members and HIF-1α undergo acetylation, leading to dramatic changes in their biological properties.

Steroid receptors represent a special class of ligand-dependent transcription factors, for which many post-translational changes have been described [Faus and Haendler, 2006]. Acetylation of the androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR) has been reported [Fu et al., 2003b, 2004b; Popov et al., 2007]. It affects lysines located in the respective hinge domains and belonging to the consensus acetylation motif KXKK/RXKK which is also found in the progesterone receptor (PR) [Faus and Haendler, 2006]. A potential acetylation site has also been postulated in the mineralocorticoid receptor (MR) [Pascual-Le Tallec and Lombes, 2005]. Mutagenesis studies show that, depending on the receptor targeted, the effects of acetylation differ. Concerning the AR, lysine residues located at positions 630, 632 and 633 have been

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identified as acetylation sites. They play a role in the modulation of transcriptional activity by favoring nuclear translocation and by shifting the balance between coactivator and corepressor binding [Fu et al., 2000, 2002]. Interestingly, the K630T modification which mimics the acetylation mark has been identified in a prostate cancer patient [Shi et al., 2002]. A xenograft bearing this mutation proliferates faster on nude mice and is resistant to apoptosis and anti-androgen treatment, suggesting that acetylation of K630 activates the AR [Fu et al., 2003a]. Acetylation has dual effects on the ERα since it reduces ligand-dependent activity on one hand but also stabilizes the protein [Wang et al., 2001a; Kim et al., 2006]. Two factors that impair acetylation and transcriptional activity of ERα have been identified, which further underlies the importance of this post-translational modification [Loven et al., 2003, 2004]. Interestingly, the ERα K303R mutation which increases the sensitivity to estrogen [Mishra et al., 2003; Cui et al., 2004] has been detected in breast pre-malignant lesions and at low frequency in invasive tumors [Conway et al., 2005].

The existence of a crosstalk between different post-translational modifications has furthermore been evidenced, indicating that intricate regulatory mechanisms exist. For instance, the mutation of acetylation sites changes the pattern of AR phosphorylation [Fu et al., 2004a]. Concerning the ERα, it has been found that besides acetylation the K266 and K268 residues may also undergo sumoylation by PIAS1 and PIAS3, which is needed for full activity [Sentis et al., 2005]. The other ERα acetylation sites, K302 and K303, may also be subject to mono-ubiquitylation by the BRCA/BARD complex [Eakin et al., 2007]. Far less information is available for the GR but one report documents that deacetylation is important for binding by NF-κB and repression of downstream target genes [Ito et al., 2006].

Several of the enzymes involved in the regulation of steroid receptor acetylation have been identified. They include members of the histone acetyltransferase (HAT) family such as CBP, p300, PCAF, and TIP60 [Fu et al., 2000; Gaughan et al., 2001, 2002; Gong et al., 2006], and of the histone deacetylase (HDAC) family, HDAC1, HDAC2, and SIRT1 [Gaughan et al., 2005; Fu et al., 2006; Tao et al., 2006; Dai et al., 2007]. Studies with HDAC inhibitors from different structural classes show that they inhibit the proliferation of prostate and breast tumor cell lines, more so when they express the AR or ERα [Margueron et al., 2004; Fronsdal and Saatcioglu, 2005; Rokhlin et al., 2006; Marrocco et al., 2007; Pledgie-Tracy et al., 2007]. On the other hand, reporter assays indicate that AR and ERα function is stimulated by various HDAC inhibitors. This was for instance seen with trichostatin for the androgen-dependent mouse mammary tumor virus (MMTV), prostate-specific antigen (PSA) and probasin promoters [Fu et al., 2000, 2002, 2003a; Gaughan et al., 2002; Korkmaz et al., 2004], and for the estrogen-dependent ERE minimal promoter [Wang et al., 2001b]. Another example is the stimulation of PSA expression by the SIRT1 inhibitor splitomycin in the absence of dihydrotestosterone [Fu et al., 2006]. Conversely, the HDAC inhibitors LAQ824 and sulforaphane impair the function of AR and ERα, respectively [Chen et al., 2005; Pledgie-Tracy et al., 2007]. In these cases a reduction of steroid receptor protein levels was noted. These data indicate that the impact of HDAC inhibitors on steroid receptor function is complex which can be explained by the fact that receptor acetylation, expression levels and histone acetylation may all be affected. In addition, proteins that directly interact with steroid receptors such as Hsp90 may also undergo acetylation [Fiskus et al., 2007; Scroggins et al., 2007].

For a more precise understanding of the role of AR acetylation in regulating gene transcription, we compared the response of target promoters to AR mutated at different acetylation sites. We found that the stimulation of the Pem, PSA and MMTV promoters varied, depending on the mutations introduced. Abrogation of all three AR acetylation sites still allowed robust stimulation of the PSA and MMTV promoters, but not of the Pem promoter. The absence of response of the Pem promoter was not due to reduced AR protein levels, to altered ligand recognition, to diminished AR nuclear translocation or to impaired promoter binding, suggesting that differential recruitment of cofactors may be involved.

MATERIALS AND METHODS

Materials

R1881 (methyltrienolone) was from Dupont NEN (Boston, MA). RPMI 1640, OPTI-MEM,

FCS, streptomycin, penicillin and L-glutamine were obtained from Gibco BRL Life Technologies (Eggenstein, Germany). The NuPAGE gels and the polyvinylidene fluoride membranes were from Invitrogen (Karlsruhe, Germany). Protein A agarose/salmon sperm DNA was from Upstate/Millipore (Schwalbach, Germany). FuGENE 6, Nonidet P40, benzonase and the complete protease inhibitor mix were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The M-PER reagent was from Perbio Science (Bonn, Germany). RNase H, proteinase K and the DNeasy Blood and Tissue kit were from Qiagen (Hilden, Germany).

The anti-AR antibody C-19 (sc-815) was from Santa Cruz Biotechnology (Santa Cruz, CA), the anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH-100) from Advanced ImmunoChemical (Long Beach, CA) and the anti-RNA polymerase II antibody (ab5408) from Abcam (Cambridge, UK). Secondary antibodies, the Western Lightning Chemiluminescence and the SteadyLite Plus reagents were from PerkinElmer (Rodgau-Jügesheim, Germany). Membrane stripping was performed with the Re-Blot Plus Strong Solution from Chemicon/Millipore (Schwalbach, Germany).

Plasmid Construction

The plasmid vectors were purchased as follows: pSG5 from Stratagene (Amsterdam, The Netherlands), pGL3-Basic and pGL3-Promoter from Promega (Mannheim, Germany). The generation of pGL3-based reporter constructs containing the mouse Pem promoter, four copies of ARE-1 or four copies of ARE-2 upstream of the luciferase (Luc) reporter as well as the pSG5-AR plasmid have been described before [Barbulescu et al., 2001; Geserick et al., 2003]. Site-directed mutagenesis of AR was performed with the QuickChange kit (Stratagene) following the manufacturer's instructions. The following mutating oligonucleotides and corresponding reverse complementary sequences were used: 5′-CTCTGGGAGCCCGGGCGCTGAAGAAACTTGG-3′ for the AxKK mutation; 5′-CTCTGGGAGCCCGGAAGCTGGCAGCGCTTGGTAATCTGAAACTACAGGAG-3′ for the KxAA mutation, 5′-GATGACTCTGGGAGCCCGGGCGCTGGCGGCACTTGGTAATCTGAAACTAC-3′ for the AxAA mutation, 5′-for: CCGAGGAGCTTTCCAGAATGCAGCTCAGAGCGTGCGCGAAGTG-3′ for the FxAA mutation and 5′- for: CTTCCTCATCCTGGCACACTGCAGCTACAGCCGAAGAAGGCCAGTTG-3′ for the WxxAA mutation. The complete AR coding region was resequenced to confirm the presence of the mutation and the exactness of the remainder of the sequence.

Cell Culture and Transfections

PC-3 and CV-1 cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

For the transactivation assays, the cells were seeded into 96-well plates at a concentration of 7,000 cells/100 μl per well in RPMI 1640 medium without phenol red, supplemented with 5% charcoal-stripped FCS and 2 mM L-glutamine. Transfections were carried out 18–19 h later using FuGENE 6 in OPTI-MEM. Expression plasmids for wild-type (wt) AR or mutant forms (50 ng, unless indicated otherwise) were cotransfected together with a Luc-based reporter vector harboring different natural or synthetic promoters (50 ng). Induction was performed 4 h later by adding 1 nM of R1881. Measurement of Luc activity was carried out after 24 h in a Victor multilabel counter (PerkinElmer), following the addition of 100 μl of SteadyLite Plus reagent. The activity of a constitutively active Luc vector was determined to assess transfection efficiency. For all points the average value of six wells treated in parallel was taken. The experiments were repeated at least three times independently.

Preparation and Analysis of Protein Extracts

Total protein extracts were prepared from transfected cells using M-Per reagent supplemented with protease inhibitor mix and benzonase.

For the separation between the cytoplasmic and nuclear fractions, PC-3 cells were grown in six-well plates, transfected and stimulated or not with 1 nM R1881. After 24 h, the cells were harvested and treated with lysis buffer (50 mM Tris pH 7.5, 10 mM NaCl, 5 mM EDTA, 0.5% Nonidet P40, protease inhibitor tablets). Following centrifugation for 5 min at 13,000*g*, the supernatant was kept as cytoplasmic fraction and the pelleted nuclei were lysed with M-Per buffer supplemented as above.

For Western blot analysis the protein extracts were separated using NuPAGE gradient gels

and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked and incubated overnight with the specified antibodies diluted 1:1,000 (anti-AR), 1:10,000 (anti-GAPDH) or 1:2,000 (anti-RNA polymerase II) before being developed with the appropriate secondary antibodies using the Western Lightning chemiluminescence reagent.

Plasmid Immunoprecipitation

PC-3 cells were plated on 10 cm-dishes and transfected with either 5 μg of pSG5-ARwt or pSG5-AR-AxAA together with 1 μg of the Pem reporter plasmid. Four hours later, the cells were treated with 1 nM R1881. After 24 h, the cells were cross-linked with 1% formaldehyde for 5 min and the reaction was stopped by adding glycine to a final concentration of 125 mM. Cells were harvested, resuspended in lysis buffer (50 mM Tris pH 8, 10 nM EDTA, 1% SDS, protease inhibitor mix) and sonicated on ice four times during 15 s at 70% power using a Sonopuls HD2070 device (Bandelin, Berlin, Germany). Cell lysates were diluted in dilution buffer (20 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100, protease inhibitor mix), precleared and incubated at 4°C overnight with the anti-AR antibody C-19 or without antibody as mock control. Protein A agarose/salmon sperm DNA was then added and the incubation was continued for 2 h. Washing was performed with buffer I (20 mM Tris pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), buffer II (20 mM Tris pH 8, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS) and buffer III (10 mM Tris pH 8, 1 mM EDTA, 250 mM LiCl, 1% Nonidet P40, 1% Sodium deoxycholate) followed by two washes with TE (10 mM Tris pH 8, 1 mM EDTA).

The immunocomplexes were eluted with 0.1 M $NaHCO_3$, 1% SDS and the cross-link was reverted at 65°C for 5 h. The eluates were sequentially treated with RNase H (1 h) and proteinase K (1 h). The DNA was then purified using the DNeasy kit. For the PCR amplification 5 μl of each sample were used as template. This was performed at an annealing temperature of 55°C for 35 cycles using primers designed to amplify the whole Pem promoter fragment cloned in the reporter plasmid. Primer sequences were: 5′-TGCCAGAACATTTCTC-TATCG-3′ (forward) and 5′-CTTTATGTTTT-TGGCGTCTTCC-3′ (reverse).

RESULTS

Mutation of AR Acetylation Sites Has Promoter-Selective Effects

Three lysine acetylation sites belonging to the consensus motif KLKK exist in the AR hinge region, just C-terminal of the DNA-binding domain (DBD) [Fu et al., 2000, 2002]. They were substituted by an alanine residue to generate loss-of-function mutants. Expression constructs for K630A (single mutant AxKK), K632A/K633A (double mutant KxAA) and K630A/K632A/K633A (triple mutant AxAA) were generated by site-directed mutagenesis (Fig. 1).

Cell-based transactivation assays were performed by transfecting the corresponding expression vectors into PC-3 cells, which do not express endogenous AR, in order to determine the impact of these changes on AR function. As reporter vectors we used constructs containing the mouse Pem promoter which is selectively responsive to androgens [Barbulescu et al., 2001; Geserick et al., 2003], the promoter of the gene for human PSA, an often used biomarker for prostate cancer [Cleutjens et al., 1996], and the highly androgen-responsive MMTV promoter [Cato et al., 1987]. When testing the Pem promoter (Fig. 2A), a similar androgen-dependent stimulation was observed after treatment with 1 nM R1881 in presence of wt AR and the KxAA form, and slightly less activity was seen in presence of the AxKK mutant. Conversely, no stimulation was seen for the AxAA triple mutant. The situation was not the same when using the PSA promoter as reporter (Fig. 2B). Here the AxKK and especially the

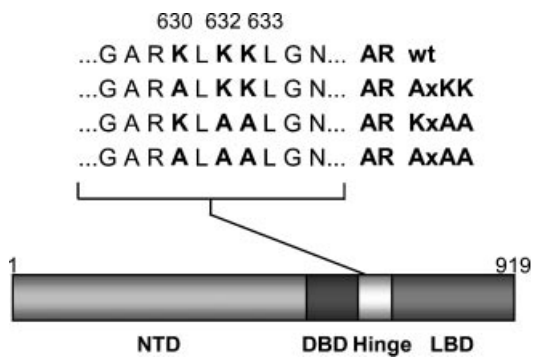
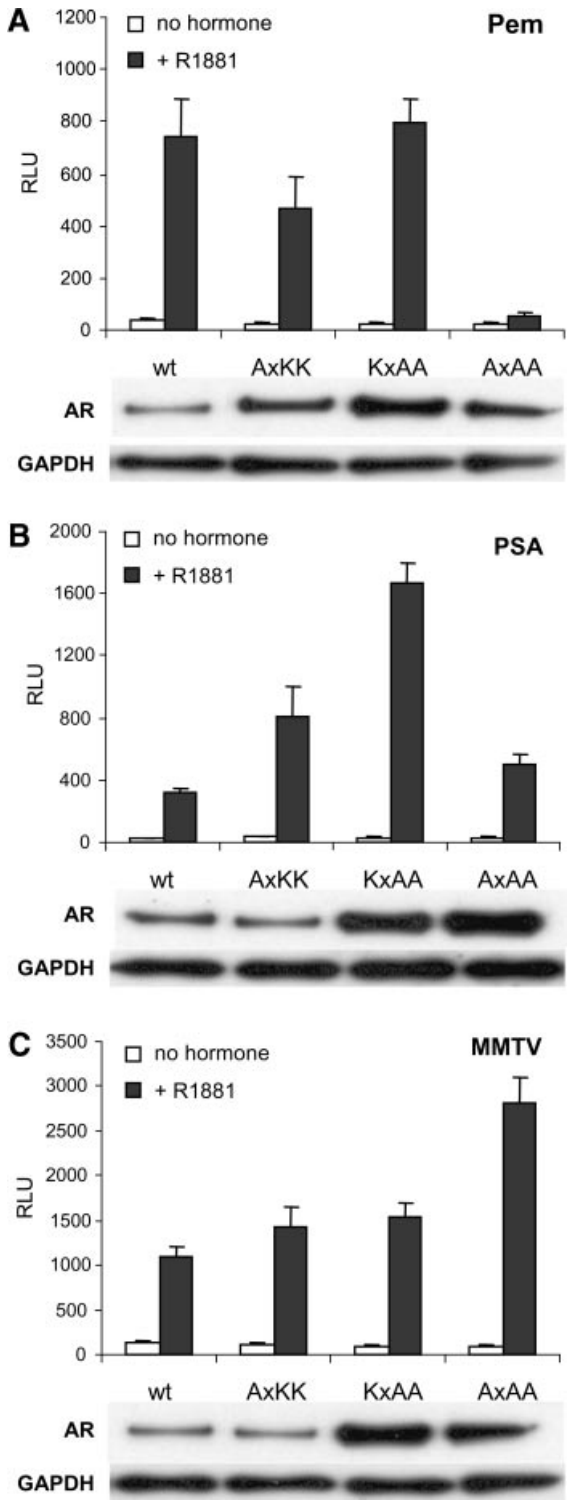


Fig. 1. Schematic representation of the human AR and localization of the motif involved in acetylation. The four AR domains are indicated: N-terminal domain (NTD), DNA-binding domain (DBD), hinge and ligand-binding domain (LBD). The amino acid residues that were modified in the mutant forms are highlighted in boldface in the sequence.

KxAA mutant had stronger activity than wt AR whereas the triple mutant was as active. Next, we tested the MMTV promoter (Fig. 2C). Here, wt AR, the AxKK and KxAA forms had similar activities whereas the AxAA mutant conveyed the strongest effect. Western blot analysis showed that the KxAA and AxAA forms were generally expressed at higher levels than wt AR or the AxKK form, but this did not parallel the differences seen in transactivation efficiencies at the different promoters (Fig. 2A–C). GAPDH levels were furthermore determined to ensure that comparable amounts of protein extracts had been loaded onto the gel (Fig. 2A–C).



These experiments point out that elimination of the three AR acetylation sites has promoter-selective effects. Remarkably, the AR still retains transactivation potential on some promoters despite elimination of its different acetylation sites.

Differential Activity of AR Acetylation Mutants Is Seen at Various Hormone Concentrations

For a more precise comparison of the activities of the different AR forms, we varied the androgen concentrations used for stimulation. When giving increasing R1881 amounts to PC-3 cells transfected with the Pem promoter-based reporter vector (Fig. 3A), reporter gene activity was measurable for wt AR starting at 0.01 nM and reaching a maximum at 1 nM. For the KxAA mutant, a stronger stimulation was seen at 0.1 nM and higher concentrations. For the AxKK and more so the AxAA form, little or no stimulatory effects of androgen were observed, even at the highest hormone concentrations used. Concerning the MMTV reporter (Fig. 3B), comparable induction profiles were seen in presence of wt AR and of the AxKK mutant, with 0.01 nM R1881 being sufficient to reach the plateau. Higher induction values were seen for the KxAA and more so for the AxAA mutants. The higher efficacy seen for the triple mutant was accompanied by a loss of potency, as an approximately tenfold higher hormone concentration was needed to achieve the EC_{50} value. Interestingly, androgen stimulation was

Fig. 2. Differential effects of acetylation site mutations on AR function. PC-3 cells were transfected with an androgen-dependent reporter vector and a pSG5-based expression plasmid for wt AR or a mutant form. Treatment was with 1 nM R1881 (black bars) or with vehicle (white bars). The reporter activity measured is given in relative light units (RLU). The results are a representative of three separate experiments and the bars are the mean ± SD of sextuplicate values. Western blot analysis of AR and GAPDH levels is shown for each experiment. **A**: Transfection with pSG5-AR (wt or mutant) and the mouse Pem promoter reporter. **B**: Transfection with pSG5-AR (wt or mutant) and the human PSA promoter reporter. **C**: Transfection with pSG5-AR (wt or mutant) and the MMTV promoter reporter.

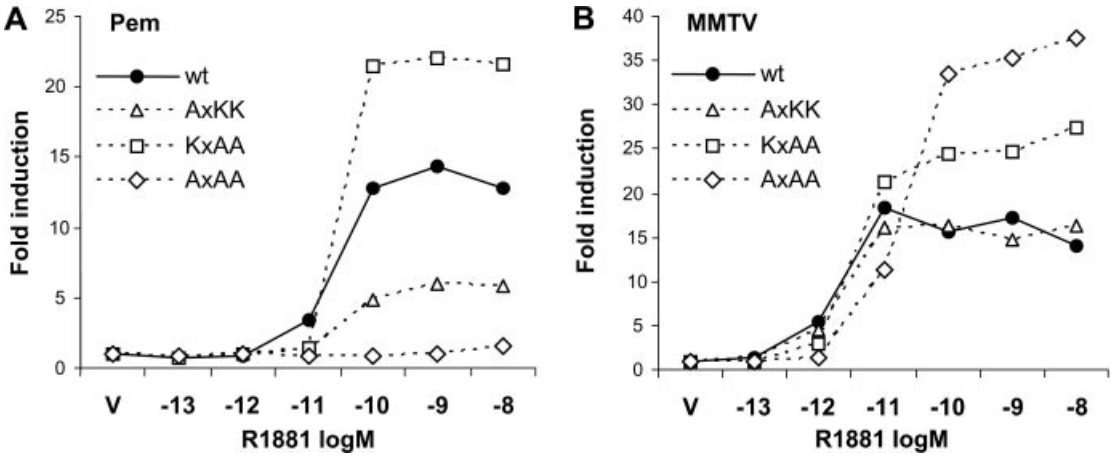


Fig. 3. Hormone-dependent activity of AR mutants. PC-3 cells were transfected with an androgen-dependent reporter vector and a pSG5-based expression plasmid for wt AR or a mutant form. Treatment was with different R1881 concentrations, as indicated. The reporter activity measured is given as fold induction. The results are a representative of three separate experiments. **A**: Transfection with pSG5-AR wt or mutant forms and the mouse Pem promoter reporter. **B**: Transfection with pSG5-AR wt or mutant forms and the MMTV promoter reporter.

already observed in presence of wt AR for both promoters at doses at which the triple mutant form was entirely inactive.

The treatment with different androgen concentrations confirms that the acetylation site mutants behave differently in presence of various promoters. It furthermore confirms that the acetylation-defective AR triple mutant has lost its transactivation potential for the Pem promoter. Conversely, this mutant is more active than wt AR in the presence of the MMTV promoter.

The Relative Wild-Type and Mutated AR Levels Are Determinant for the Promoter-Specific Effects

In order to further substantiate our findings, we incrementally varied the ratio between the wt AR and triple mutant form in our transactivation experiments. Decreasing amounts of plasmids coding for wt AR and increasing amounts of the construct expressing the AxAA mutant were transfected into PC-3 cells, while maintaining the total DNA concentration constant. In presence of the Pem promoter (Fig. 4A), increasing the AxAA/ARwt ratio led to a complete loss of androgen-dependent activity. The situation was different for the MMTV promoter (Fig. 4B). Here, the hormone-dependent reporter activity remained the same, regardless of the AxAA/ARwt ratio.

In order to find out if this result could be extended to another cell line, we performed similar experiments with CV-1 cells, which do not express the AR. Here also, increasing the amounts of the AxAA form led to a complete loss of androgen stimulation of the Pem promoter (Fig. 4D). Conversely, the response of the MMTV promoter was not affected by the AxAA/ARwt ratio (Fig. 4E). A comparison of the respective inductions (Fig. 4C,F), clearly showed that in both the PC-3 and the CV-1 cells, the Pem promoter was not responsive at all to the AxAA mutant. Conversely, the MMTV promoter, despite giving comparable signals for all tested mutant to wt AR ratios, was actually more strongly induced by the AxAA form. This was due to the lower basal activity of the MMTV promoter in presence of the AxAA form.

When determining the total AR protein levels we found some variation, but this could not explain the differences in activities seen. For instance, in CV-1 cells no stimulation of the Pem promoter was observed in presence of the AxAA form, even though this mutant was expressed at higher levels than wt AR in the same experiment (Fig. 4D).

These results further document that the Pem and MMTV promoters differentially respond to obliteration of the AR acetylation sites.

Mutation of AR Acetylation Sites Is Compatible With Nuclear Translocation

The KLKK motif is part of the bipartite AR nuclear targeting signal and inactivating

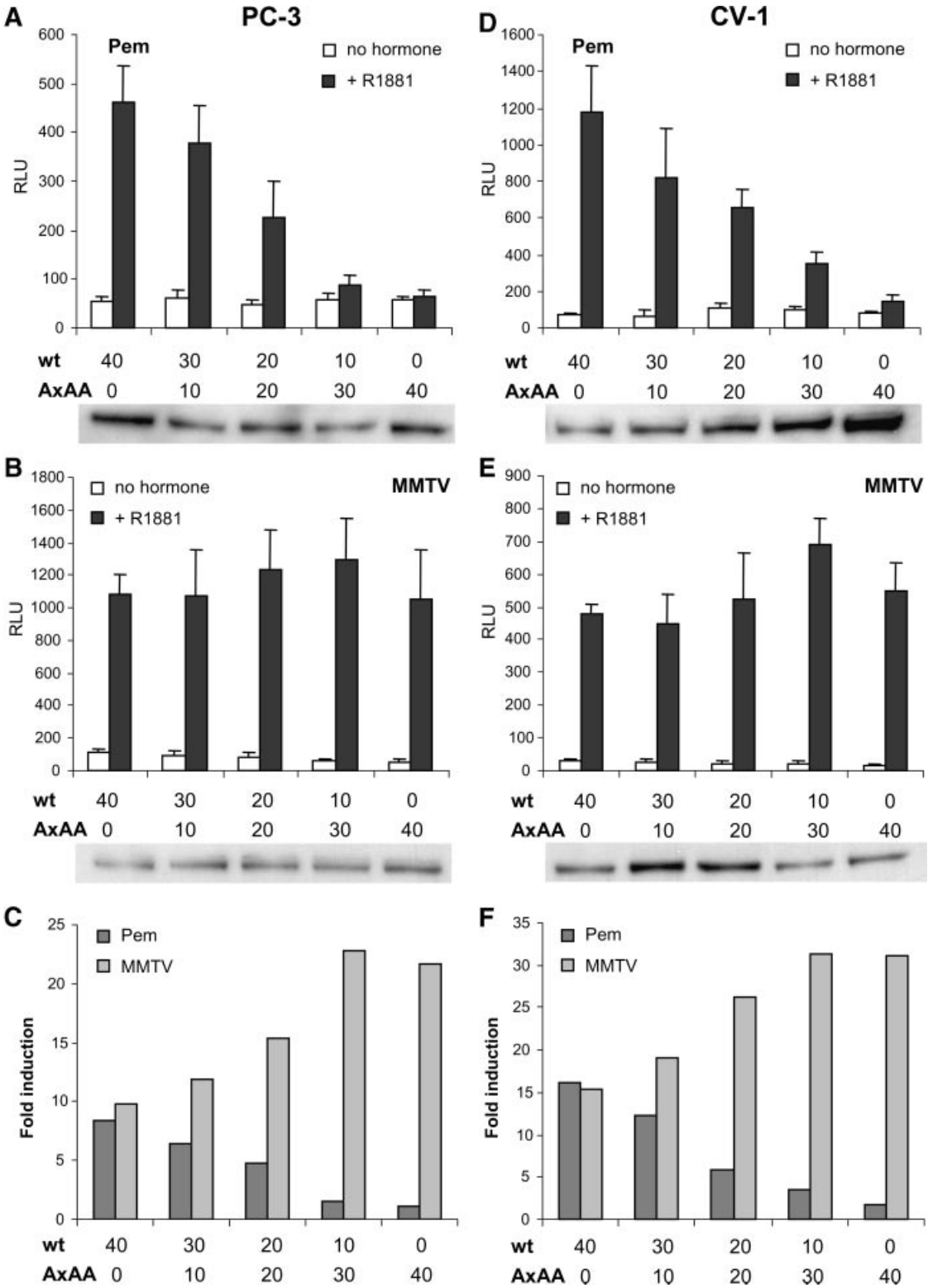


Fig. 4. Concentration-dependent effects of AR acetylation site mutants. Cells were transfected with the indicated amounts of expression vector for wt AR or for the triple mutant form, while keeping the total DNA amount constant, and with different reporter constructs. Treatment was with 1 nM R1881 (black bars) or with vehicle (white bars). The reporter activity measured is given in relative light units (RLU) in panels **A**,**B** and **D**,**E** and in fold induction in panels **C**,**F**. The results are a representative of three separate experiments and the bars are the mean ± SD of sextuplicate values. Western blot analysis of AR levels is shown for each experiment. **A**: Transfection of PC-3 cells with the mouse Pem promoter reporter. **B**: Transfection of PC-3 cells with the MMTV promoter reporter. **C**: Comparison of inductions seen for the Pem (dark gray) and MMTV (light gray) promoters in PC-3 cells. **D**: Transfection of CV-1 cells with the mouse Pem promoter reporter. **E**: Transfection of CV-1 cells with the MMTV promoter reporter. **F**: Comparison of inductions seen for the Pem (dark gray) and MMTV (light gray) promoters in CV-1 cells.

mutations in this region have been shown to reduce transport to the nucleus [Zhou et al., 1994; Thomas et al., 2004]. In order to determine the effect of the acetylation site mutations on subcellular localization, we fractionated PC-3 cells expressing wt AR or the AxAA mutant and performed Western blot analysis (Fig. 5A). In the absence of hormone both AR forms were mostly located in the cytoplasm, as expected. Following R1881 treatment, a sizeable fraction of the wt AR pool was translocated into the nucleus. When looking at the AxAA mutant we found that comparatively more remained in the cytoplasm after hormone treatment. A significant amount was however also present in the nucleus. RNA polymerase II and GAPDH levels were furthermore determined to ascertain that the nuclear and cytoplasmic fractions had been properly separated (Fig. 5A).

These fractionation experiments indicate that elimination of all AR acetylation sites still allows nuclear translocation after androgen treatment.

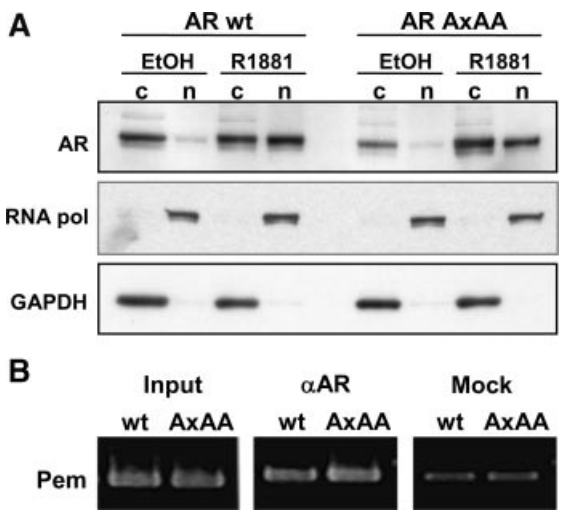


Fig. 5. Subcellular localization and binding of the AR triple mutant to the Pem promoter. **A**: PC-3 cells transfected with an expression vector for wt AR or the AxAA form were treated or not with 1 nM R1881. Cytoplasmic (c) and nuclear (n) fractions were prepared. Western blot analysis was performed with antibodies specific for AR, RNA polymerase II or GAPDH. **B**: PC-3 cells transfected with an expression vector for wt AR or the triple mutant form and with the Pem reporter construct were treated with 1 nM R1881 for 24 h. The cells were cross-linked with formaldehyde, extracts were prepared and the AR was immunoprecipitated. After de-crosslinking, a region of the Pem promoter was amplified by PCR using specific primers (αAR, **middle panel**). Cell extracts prior to immunoprecipitation (Input, **left panel**) served as positive control and extracts incubated without antibody (Mock, **right panel**) as negative control.

Plasmid Immunoprecipitation Shows That Wild-Type AR and the Acetylation-Deficient Form Possess Comparable DNA-Binding Properties

In order to further understand the reason for the non-responsiveness of the Pem promoter to the AR triple mutant, we performed plasmid immunoprecipitation analysis. PC-3 cells transfected with expression vectors for wt AR or the AxAA form, and with the Pem promoter construct were crosslinked by formaldehyde and sonicated. The complex was precipitated by a specific anti-AR antibody and analyzed by PCR using primers recognizing the Pem promoter region. A representative experiment is shown in Figure 5B. Signals of comparable intensities were generated from complexes purified from the PC-3 cells expressing wt AR or the mutated form. The mock-treated cells showed a very weak background signal.

These data show that the AxAA form binds to the Pem promoter as well as the wt form does, suggesting that impaired activity is not linked to reduced promoter recognition.

AR Acetylation and N/C Interaction Are Probably Not Linked

An important role of the interaction between the N-terminal domain (NTD) which contains the transactivation function 1 and the C-terminal ligand-binding domain (LBD) for full activity of the AR has been reported [Ikonen et al., 1997]. This N/C interaction is mainly mediated by the FxxLF motif located at positions 23–27 and less so by the WxxLF motif located at positions 433–437 (Fig. 6A) [He et al., 2000].

As the AR hinge region in which the acetylation sites are located may play a role in modulating the interdomain communication, we compared the activities of AR forms mutated in regions involved in the N/C interaction. We changed FxxLF to FxxAA and WxxLF to WxxAA in the AR sequence (Fig. 6A). Transactivation experiments were performed with these constructs in PC-3 cells (Fig. 6B). When assaying the Pem promoter we found the FxxAA form to give similar induction levels than wt AR whereas the WxxAA form was more than twice as active. Concerning the MMTV promoter, all expression constructs gave similar inductions, with possibly a stronger effect of the WxxAA mutant.

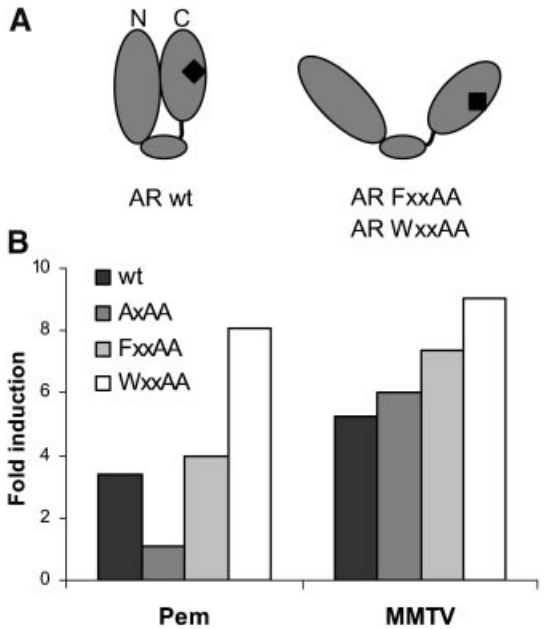


Fig. 6. Mutations of acetylation motif and N/C interaction regions differentially affect AR activity. **A**: Schematic representation of N/C interaction. **B**: PC-3 cells were transfected with 50 ng of expression vector for wt AR (black bars), acetylation-defective mutant (dark gray bars) or N/C interaction mutants (light gray and white bars), and with Pem or MMTV promoter reporter constructs, as indicated. Treatment was with 1 nM R1881. The reporter activity measured is given as fold inductions. The results are a representative of three separate experiments.

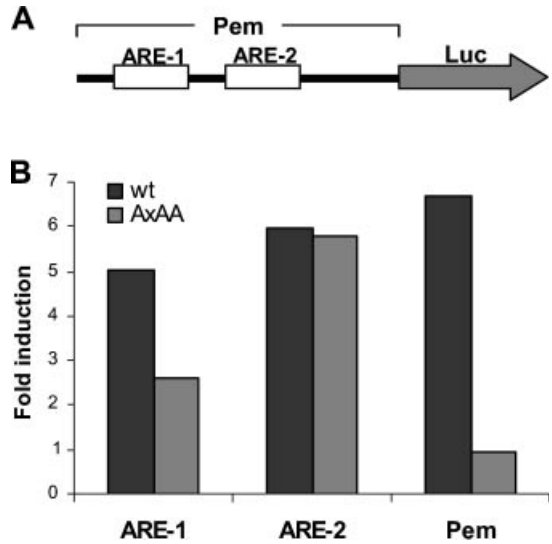


Fig. 7. AR acetylation site mutations have different effects on Pem ARE-1 and ARE-2. **A**: Schematic representation of the mouse Pem promoter. **B**: PC-3 cells were transfected with an expression vector for wt AR (black bars) or triple mutant form (dark gray bars) and with Pem promoter or minimal reporter constructs, as indicated. Treatment was with 1 nM R1881. The reporter activity measured is given as fold inductions. The results are a representative of three separate experiments.

The fact that the AR mutants with impaired N/C interaction behave differently from the acetylation defective form suggests that there is no strong link between both processes.

Acetylation-Deficient AR Mutant Differentially Stimulates Pem ARE-1 and ARE-2

The molecular basis for the differential response of the Pem promoter to the acetylation-defective AR was further analyzed. We had previously shown [Barbulescu et al., 2001] that the androgen response of the Pem promoter is mainly mediated by two potent DNA response elements named ARE-1 and ARE-2 (Fig. 7A). We analyzed the role of these elements in trans-activation assays using reporter constructs containing four copies of each (Fig. 7B). The activity of Pem ARE-1 in presence of the AxAA mutant was half of that seen with wt AR. In contrast, the response of Pem ARE-2 was the same in presence of wt AR and AxAA mutant. As before, the Pem promoter was not stimulated by the AxAA form.

The data establish that two related DNA elements exhibiting selectivity for the AR have strikingly different responses to the AR AxAA mutant. They furthermore suggest that Pem ARE-1, but not ARE-2, plays a role in the non-responsiveness of the Pem promoter to the AxAA form.

DISCUSSION

AR modification by acetyl groups has been directly demonstrated by in vitro acetylation assays and by immunoprecipitation using antibodies specific for acetylated lysines [Fu et al., 2002, 2004a]. The precise sites were mapped by mass spectrometry leading to the identification of three modified lysines belonging to the KLKK sequence located at positions 630–633 in the hinge region. This motif had previously been described in other proteins subject to this post-translational modification [Glozak et al., 2005; Faus and Haendler, 2006].

Several reports relate that preventing AR acetylation is followed by a dramatic loss of activity. Cell-based transactivation experiments using mutants defective at different acetylation sites showed that in DU145, HEK, and COS cells, the androgen-dependent AR activity was much reduced when using MMTV-Luc, PSA-Luc or ARE-Luc as reporter vectors [Fu et al., 2000, 2002]. Conversely, the

QxKK and TxKK mutants which mimic acetylation exhibit stronger activity on the PSA-Luc and MMTV-Luc promoters [Fu et al., 2003a]. In line with this, the HAT Tip60, which acetylates the AR, stimulates its activity, whereas HDAC1 has opposite effects [Gaughan et al., 2002]. Another study shows that the increase in AR acetylation which follows bombesin treatment and p300 stimulation leads to higher ARE-Luc reporter gene activity in PC-3 cells and to increased PSA expression in LNCaP cells [Gong et al., 2006].

In contrast with this, other groups found that deletion of a region comprising the KLKK motif was still compatible with strong hormone-stimulated AR activity. The AR-Δ629–633 mutant is conducive to higher stimulation of the $(ARE)_2$-TATA-Luc reporter in COS and HeLa cells, in comparison to wt AR [Poukka et al., 1999]. When testing a probasin promoter-based reporter plasmid, similar effects are elicited by this deletion mutant and by the wt AR [Karvonen et al., 2006]. Deletion of the larger 629–636 or 628–648 regions also leads to increased AR transactivation potential, as seen in presence of different minimal androgen-responsive promoters in HeLa cells [Wang et al., 2001b; Haelens et al., 2007]. Finally, removal of the complete hinge region (amino acids 628–669) results in an AR form that is three times more active on different androgen-controlled promoters, when tested in COS and HeLa cells [Tanner et al., 2004].

In view of the apparently conflicting data between the point mutants and the hinge deletion mutants we performed a detailed analysis of the response of different androgen-regulated promoters to AR acetylation site modifications. We found that elimination of one or two acetylation sites could either reduce or increase AR activity, depending on the reporter system. These mutations also had an influence on the EC_{50} value of androgen action. We furthermore discovered that the triple acetylation mutant form behaved quite differently from the single or double mutant. Strong activity was seen in presence of the PSA and MMTV promoters but not with the Pem promoter. This implied that the role of K630, K632, and K633 was not equivalent and suggested a complex mechanism in which single, double, and triple acetylation elicited different effects.

The strong discriminatory effects seen for the AR triple acetylation mutant on different promoters were not due to changes in protein levels, in ligand recognition, in nuclear translocation or in promoter binding. An attractive possibility is the altered recognition by co-factors. Indeed, several cofactors that bind to the AR hinge region, such as Ubc9, silencing mediator for retinoic and thyroid hormone receptor, small nuclear ring finger protein, activating signal cointegrator-1, filamin A, androgen receptor corepressor-19, Pod-1 and glycogen synthase kinase-3β, have been described [Poukka et al., 1999, 2000; Lee et al., 2002; Liao et al., 2003; Loy et al., 2003; Jeong et al., 2004; Salas et al., 2004; Hong et al., 2005]. As acetylation of lysines neutralizes their positive charge, this might regulate the interaction of the hinge region with a distinct subset of proteins. This is exemplified by the K630Q and K630T mutations which are better recognized by p300 and by the K630R modification which is preferentially bound by the N-CoR complex [Fu et al., 2002]. A crosstalk between acetylation and other post-translational modifications may also happen. This could occur by competition for the same lysine residue, as has been observed for ERα in which K266 and K268 can either be acetylated or sumoylated [Sentis et al., 2005; Kim et al., 2006], and K302 and K303 either be acetylated or monoubiquitylated [Wang et al., 2001a; Eakin et al., 2007]. Whether this also takes place in the AR KLKK region remains to be determined. Lysine acetylation may also influence other post-translational modifications. This is the case for the AR phosphorylation events that come about in response to activating signaling pathways. Here AR acetylation is essential for activation by the AKT, PKA, and JNK but not by the MAPK pathways [Fu et al., 2004a]. Finally, interplay between acetylation/deacetylation by Tip60/HDAC1 and ubiquitylation by Mdm2 for control of AR stability has been reported [Gaughan et al., 2002].

Promoter-specific effects have been reported for AR mutants defective in their N/C interaction [He et al., 2002; Callewaert et al., 2003]. Mutation of the FxxLF motif which is essential for this interaction has no effect on the activation of the MMTV and Slp promoters but only permits partial response of the PSA and probasin promoters. The deletion of this motif does not influence the transcriptional activation via selective AREs, that is, those that are only stimulated by the AR, but reduces that

mediated by promiscuous response elements, which are stimulated by the AR, GR, PR, and MR [Callewaert et al., 2003]. It was therefore pertinent to compare mutants deficient in N/C interaction and acetylation mutants. Little difference was observed in presence of the MMTV promoter, as expected. In contrast, the N/C interaction mutant was fully active on the Pem promoter. This extends the above-mentioned findings that selective AREs, as found in the Pem promoter, are not dependent on N/C interaction for their stimulation by the AR. In sharp contrast, the acetylation-deficient AR did not activate the Pem promoter. This strongly suggests that preventing the N/C communication does not hinder acetylation at the KxKK motif. A link between N/C interaction and enzymes involved in AR acetylation has however previously been reported. Opposite roles of CBP and SIRT1 in increasing and decreasing N/C interaction respectively, have been documented [Fu et al., 2006]. However, even though these enzymes modify the acetylation status of the AR, it is not clear whether this was the direct cause for the changes in the N/C interaction.

Promoter-selective effects of post-translational modifications have already been found in the case of sumoylation. A differential impact of the sumoylation E2 and E3 enzymes PIASxα and Ubc9 on AR function has been reported [Geserick et al., 2003]. Overexpression of either of them reduces AR activity on minimal promoters containing selective AREs but not on those with promiscuous response elements. Altogether this shows that a subset of androgen target genes, mostly those harboring selective AREs, respond differently to hormone stimulation due to regulatory mechanisms involving the hinge region and N/C interaction. Interestingly, the genes controlled by selective AREs are mainly implicated in reproductive functions, as evidenced by the generation of a transgenic mouse model expressing a mutated AR that cannot activate ARE-dependent genes [Schauwaers et al., 2007]. The ARE-driven Pem gene (recently renamed Rhox5) also plays a role in reproduction, as shown by the corresponding knock-out mice which have reduced sperm motility and fertility [Maclean et al., 2005].

The implications of our findings on the in vivo modulation of androgen action by the AR acetylation status are presently unclear, due to the limitations inherent to cell-based transactivation assays. This aspect could be addressed by generating transgenic mice bearing mutations in the KxKK motif and examining the impact of these modifications on androgen target tissues. In addition, crossing such animals with mice models for prostate cancer (reviewed in Kasper and Smith [2004]) might give insight into a possible role of AR acetylation in this disease. Interestingly, several mutations affecting the hinge region have been described in prostate cancer patients [Buchanan et al., 2001; Shi et al., 2002; Thompson et al., 2003]. Also, a mutation in the KxKK motif that confers growth advantage and resistance against apoptosis in a xenograft model has been identified [Fu et al., 2003a]. Further studies are now needed to understand how the balance between AR acetylation and deacetylation controls gene transcription and to find out whether this post-translational modification plays a role in pathophysiological processes.

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