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Amino acids 3–13 and amino acids in and flanking the ^{23}FxxLF^{27} motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor

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The N-terminal domain (NTD) and the ligand-binding domain (LBD) of the androgen receptor (AR) exhibit a ligand–dependent interaction (N/C interaction). Amino acids 3–36 in the NTD ($AR_{3–36}$) play a dominant role in this interaction. Previously, it has been shown that a ΦxxΦΦ motif in $AR_{3–36}$, ^{23}FxxLF^{27}, is essential for LBD interaction. We demonstrate in the current study that $AR_{3–36}$ can be subdivided into two functionally distinct fragments: $AR_{3–13}$ and $AR_{16–36}$. $AR_{3–13}$ does not directly interact with the AR LBD, but rather contributes to the transactivation function of the AR.NTD-AR.LBD complex. $AR_{16–36}$, encompassing the ^{23}FxxLF^{27} motif, is predicted to fold into a long amphipathic α-helix. A second ΦxxΦΦ candidate protein interaction motif within the helical structure, ^{30}VREVI^{34}, shows no affinity to the LBD. Within $AR_{16–36}$, amino acid residues in and flanking the ^{23}FxxLF^{27} motif are demonstrated to modulate N/C interaction. Substitution of Q24 and N25 by alanine residues enhances N/C interaction. Substitution of amino acids flanking the ^{23}FxxLF^{27} motif by alanines are inhibitory to LBD interaction.

Keywords: androgen receptor; transcription activation domain; ligand-binding domain; amphipathic α-helix; FxxLF.

The androgen receptor (AR) is a member of the steroid receptor subgroup of the nuclear receptor family of transcription factors. Nuclear receptors have a modular structure, composed of a moderately conserved carboxy-terminal ligand-binding domain (LBD) folded in 12 α-helices, a highly conserved central DNA-binding domain (DBD) and a nonconserved N-terminal domain (NTD). Most nuclear receptors contain two transactivation functions: AF-1 in the NTD, and AF-2 in the LBD. Ligand-activated nuclear receptors bind as homo- or heterodimers to hormone-response elements in the regulatory regions of their target genes. Together with coactivators, general transcription factors and RNA polymerase II, they form a stable transcription initiation complex [1–4].

Upon ligand binding, the LBD acquires a conformation that facilitates the interaction with coactivators. Best studied in this regard are the interactions with the p160 coactivators SRC1, TIF2/GRIP1 and ACTR/RAC3. The nuclear receptor interaction domains of p160 coactivators contain LxxLL motifs (NR boxes) which bind to a hydrophobic cleft in the agonist-activated LBD. Antagonists induce a different LBD conformation which inhibits the interaction with coactivators and enables the binding of corepressors [3,5].

P160 coactivators not only bind to the LBD, but also to the NTD [6,7]. This interaction is independent of the NR boxes. As shown for the estrogen receptor α (ERα), simultaneous NTD and LBD binding by one coactivator can confer synergism of AF-1 and AF-2 activities, which might be necessary for optimal functioning [8].

Like shown for other nuclear receptors, p160 coactivators can bind the AR LBD by their LxxLL motifs, and they interact with the AR NTD, independent of these motifs [9–11]. In contrast to AR AF1, which is strong, AF-2 needs overexpression of a p160 coactivator to become manifest [9,10,12–15]. Many other proteins with known or unknown functions have been found to interact with the AR. An overview of AR-interacting proteins is presented in the AR mutations database (http://www.mcgill.ca/androgendb) [16].

Previously, a ligand-dependent functional interaction between the AR subdomains NTD and LBD, has been described [17–19]. This N/C interaction might be intra- or intermolecular [15,17–19]. *In vitro* pull-down experiments indicated that the AR N/C interaction is direct [11]. The AF-2 core domain in helix 12 of the AR LBD was shown to be involved in this interaction [11,15]. In the AR NTD, two regions are involved in the functional interaction with the AR LBD: $AR_{3–36}$, including the ^{23}FxxLF^{27} motif, and $AR_{370-494}$, which encompasses a transactivation function

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Abbreviations: AF, transactivation function; AR, androgen receptor; DBD, DNA-binding domain; DHT, dihydrotestosterone; E_2, estradiol; ERα, estrogen receptor α; GalAD, Gal4 transactivating domain; GAlDBD, Gal4 DNA-binding domain; LBD, ligand-binding domain; N/C interaction, interaction between NTD and LBD; NR, nuclear receptor; NTD, N-terminal domain; PR, progesterone receptor; R1881, methyltrienolone.

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and a presumed supplementary protein interaction domain [15,20]. In the present study, $AR_{3–36}$ is subdivided into two fragments: $AR_{3–13}$ and $AR_{16–36}$, which are further characterized.

EXPERIMENTAL PROCEDURES

Materials and plasmid construction

Dihydrotestosterone (DHT) was purchased from Steraloids (Wilton, NH, USA), R1881 (methyltrienolone) was from NEN (Boston, MA, USA).

Standard procedures were utilized for PCR and molecular cloning [21]. PCR products were inserted in pGEM-T Easy (Promega, Madison, WI, USA). All plasmids were sequenced to verify their correct construction. Primer sequences are shown in Table 1. AR numbering corresponds to a length of 919 amino acids, as employed by The Androgen Receptor Gene Mutations Database (http://www.mcgill.ca/androgendb).

Yeast expression constructs

pGalAD-AR.NTDwt ($AR_{3–503}$), originally derived from the yeast expression vector pACT2 (Clontech, Palo Alto, CA, USA), and pGalDBD-AR.LBD ($AR_{661-919}$), originally derived from the yeast expression vector pGBT9 (Clontech), were previously described as AR.N8 (high) and pGAL4(DBD)AR(LBD), respectively [15,18]. pGalAD-AR.NTDΔ1–13 was obtained by exchange of a 75-bp *Sma*I fragment of pGalAD-AR.NTDwt with a corresponding fragment derived from a PCR product synthesized with primers pr14 and pr1B, utilizing $pSVAR_0$ [22] as template. pGalAD-AR.NTDΔ3–36 was obtained by excision of a 117-bp *Sma*I fragment from pGalAD-AR.NTDwt. For generation of pGalAD-AR.NTD23/27RR, pGalAD-AR.NTD30/33RR, pGalAD-AR.NTD24/25AA and pGalAD-AR.NTD26/27AA, a 117-bp *Sma*I fragment of pGalAD-AR.NTDwt was exchanged with corresponding fragments containing the indicated mutations, which were obtained by PCR on the template pGalAD-AR.NTDwt utilizing primer G4AD1 (Clontech) in combination with one of the following oligonucleotides: pr23/27RR, pr30/33RR, pr24/25AA, and pr26/27AA (mutated codons are underlined in Table 1).

The AR peptide construct pGalAD-$AR_{2–36}$ was obtained by insertion of a 117-bp *Bam*HI/*Eco*RI fragment, which was synthesized by PCR on the template $pSVAR_3$ [23], utilizing primers pr2–36sense and pr2–36antisense, into the corresponding sites of pACT2 (Clontech). All other pGalAD-ARpeptide constructs were generated by *Bam*HI/*Eco*RI in frame insertion of double-stranded oligonucleotides into the corresponding sites of $pACT_2$ (Clontech), yielding pGalAD-$AR_{1–14}$, pGalAD-$AR_{16–36}$, pGalAD-$AR_{17–32}$, pGalAD-$AR_{24–39}$, pGalAD-$AR_{17–32}$ (18/19AA), pGalAD-$AR_{17–32}$(20/21AA), pGalAD-$AR_{17–32}$(23 A), pGalAD-$AR_{17–32}$(24/25AA), pGalAD-$AR_{17–32}$(26/27AA), pGalAD-$AR_{17–32}$(28/29AA) and pGalAD-$AR_{17–32}$(30/31AA). Oligonucleotides for these AR peptide expression constructs were: pr1–14sense, pr1–14antisense, pr16–36sense, pr16–36antisense, pr17–32sense, pr17–32antisense, pr24–39sense, and pr24–39antisense. Primers pr18/19AA, pr20/21AA, pr22A, pr24/25AA, pr26/27AA, pr28/29AA, and pr30/31AA sense and antisense oligonucleotides were modified pr17–32 sense and antisense oligonucleotides, containing GCTGCA (sense) and TGCAGC (antisense) as two adjacent alanine codons at the indicated positions.

Mammalian cell expression constructs

pMMTV-LUC, pSVAR.NTDwt ($AR_{1–503}$) [originally described as pSVAR($TAD_{1–494}$)] and pSVAR.DBD.LBD ($AR_{537–919}$) (originally described as pSVAR-104) were previously published [18,23,24]. Insertion of a 1.9-kb *Hind*III fragment from $pSVAR_3$ in *Hind*III digested $pGAD_{424}$ (Clontech) yielded $pGAD_3$. $pGAD_3$.NTDΔ3–13

Table 1. Primers for construction of plasmids.

Primer name	Primer sequence
pr14	5′-TCTAGATTCCCGGGTCCGCCGTCCAAGACCTACCGAGG-3′
pr1B	5′-CAGCAGCAGCAAACTGGC-3′
pr23/27RR	5′-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGCGCAGATTCTGGCGAGCTCCT-3′
pr30/33RR	5′-CTGGGGCCCGGGTTCTGGATCCGTTCGCGGCGGCTCTGGAACAGATTCTGGAA-3′
pr24/25AA	5′-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGAACAGAGCCGCGAAAGCTCC-3′
pr26/27AA	5′-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGGCCGCATTCTGGAAAGCTCC-3′
pr2–36sense	5′-AATTGGGGATCCGAGAAGTGCAGTTAGGGCTGGGAAGG-3′
pr2–36antisense	5′-GATCGAATTCGTTCTGGATCACTTCGCGCACGCTC-3′
pr1–14sense	5′-GATCGAAGTGCAGTTAGGGCTGGGAAGGGTCTACCCTCGGCCGG-3′
pr1–14antisense	5′-AATTCCGGCCGAGGGTAGACCCTTCCCAGCCCTAACTGCACTTC-3′
pr16–36sense	5′-GATCTCCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATCCAGAACG-3′
pr16–36antisense	5′-AATTCGTTCTGGATCACTTCGCGCACGCTCTGGAACAGATTCTGGAAAGCTCCTCGGTAGGTCTTGGA-3′
pr17–32sense	5′-GATCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCG-3′
pr17–32antisense	5′-AATTCGCGCACGCTCTGGAACAGATTCTGGAAAGCTCCTCGGTAGGTCTT-3′
pr24–39sense	5′-GATCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATCCAGAACCCGGGCCCCG-3′
pr24–39antisense	5′-AATTCGGGGCCCGGGTTCTGGATCACTTCGCGCACGCTCTGGAACAGATTCTG-3′
pr172B	5′-CGGAGCAGCTGCTTAAGCCGGGG-3′
pr-242	5′-AAGCTTCTGCAGGTCGACTCTAGG-3′
PDsense	5′-GATCCATATCGATAAGCTTAGATCTGAATTCA-3′
PDantisense	5′-AATTCAGATCTAAGCTTATCGATATG-3′

was obtained by insertion of a 75-bp *Sma*I fragment synthesized by PCR on the $pSVAR_0$ template, utilizing primers pr14 and pr172B, into the *Xba*I(Klenow-filled)/*Sma*I sites of $pGAD_3$. Exchange of a 1.5-kb *Hin*dIII/*Bst*EII fragment of pSVAR.NTDwt with the corresponding fragment of $pGAD_3$.NTDΔ3–13 yielded pSVAR.NTDΔ3–13. $pGAD_3$Δ3–37 was obtained by excision of a 108-bp fragment from $pGAD_3$ by *Xba*I(Klenow-filled)/*Sma*I digestion. $pSVAR_8$ was obtained by exchange of a 1.8-kb *Hin*dIII fragment of $pSVAR_3$ with the corresponding fragment of $pGAD_3$Δ3–37. For construction of pSVAR.NTDΔ3-37, a 1.7-kb *Hin*dIII/Asp718 fragment of pSVAR.NTDwt was exchanged with the corresponding fragment of $pSVAR_8$. pSVAR.NTD23/27RR, pSVAR.NTD30/33RR, pSVAR.NTD24/25AA and pSVAR.NTD26/27AA were obtained by exchange of a 348-bp *Hin*dIII/*Sma*I fragment of pSVAR.NTDwt with corresponding fragments synthesized by PCR on the $pSVAR_0$ template, utilizing primer pr-242 and one of the mutant primers pr23/27RR, pr30/33RR, pr24/25AA or pr26/27AA.

Pull-down constructs

For pSVAR.NTDwt and pSVAR.NTDmutant see Mammalian cell expression constructs. pCMV-GST-AR.LBD ($AR_{664–919}$) was generated as follows: pGEX-2TK-CHB was obtained by *Bam*HI/*Eco*RI in frame insertion of a double-stranded oligonucleotide in the corresponding sites of pGEX-2TK (Amersham Biosciences, Uppsala, Sweden). Oligonucleotides were PDsense and PDantisense. Insertion of the AR.LBD *Cla*I/*Bgl*II fragment from pAR_{34} [23] into the corresponding sites of pGEX-2TK-CHB yielded pGST-AR.LBD. Insertion of the AR LBD *Bam*HI/*Sal*I fragment of pGST-AR.LBD into the corresponding sites of pCMV-GST [25] yielded pCMV-GST-AR.LBD.

Yeast growth, transformation and β-galactosidase assay

Yeast strain Y190 (Clontech), containing an integrated Gal4 driven UAS_{GAL1}-lacZ reporter gene, was utilized for two-hybrid experiments. Yeast cells were grown in the appropriate selective medium (0.67% w/v yeast nitrogen base without amino acids, 2% w/v glucose, pH 5.8), supplemented with the required amino acids. Yeast transformation was carried out according to the lithium acetate method [26]. A yeast liquid β-galactosidase assay was performed to quantify the interaction of GalAD-AR.NTDwt, GalAD-AR.NTDmutant and GalAD-ARpeptide proteins with GalDBD-AR.LBD. In short, stationary phase cultures of Y190 yeast transformants grown in selective medium were diluted in the same medium supplemented with 1 μM DHT or without hormone, and grown until an OD_{600} between 0.7 and 1.2. Next, β-galactosidase activity was determined as described previously [18].

Mammalian cell culture, transfection, and luciferase assay

Chinese hamster ovary (CHO) cells were maintained in DMEM/F12 culture medium, supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (Life Technologies, Gaithersburg, MD, USA). Cells were plated in 24-well plates at a density of 2×10^4 cells per well, in a total volume of 0.5 mL. Cells were transfected with MMTV-LUC reporter plasmid (50 ng·well^{-1}) and pSVAR.DBD.LBD (10 ng·well^{-1}) together with increasing amounts of pSVAR.NTDwt or pSVAR.NTDmutant (10, 30, 100, 300 ng·well^{-1}), supplemented with pTZ19 as carrier DNA to a total amount of 300 ng·well^{-1}, utilizing 0.5 μL FuGENE transfection reagent (Roche Inc., Mannheim, Germany) per well. After overnight incubation with or without 1 nM R1881, cells were harvested and luciferase measurement was performed as described previously [27].

Protein extraction and Western blot analysis

Yeast protein extracts were obtained by direct lysis of yeast cells in 2 × SDS gel-loading buffer by a freeze/thawing cycle and boiling, according to Sambrook and Russell (2001) [21]. Western blot analysis for detection of GalAD fusion proteins was performed as previously described, utilizing a GAL4AD monoclonal antibody (Clontech) [18].

CHO cells were plated at a density of 1.5×10^6 cells per 80 cm^2 flask and the next day were transfected with 1 μg pSVAR.NTDwt or pSVAR.NTDmutant, utilizing 12 μL FuGENE transfection reagent. After overnight incubation, cells were harvested by scraping in 1 mL NaCl/P_i and centrifugation (5 min, 800 ***g***). Protein extracts were obtained by lysis of the pelleted cells in 60 μL lysis buffer A (20 mM Tris, 1 mM EDTA, 0.1% Nonidet P40, 25% glycerol, 20 mM Na-molybdate, pH 6.8), with addition of 0.3 M NaCl, followed by three cycles of freeze/thawing and centrifugation (10 min at 400 000 ***g***). Western blot analysis for detection of AR.NTD proteins was performed as previously described, utilizing AR antibody SP061 [18,28].

Pull-down assay

CHO cell plating, transfection, harvesting, and protein extraction were carried out as described in the previous section, except that 3 μg pCMV-GST-AR.LBD and 1 μg pSVAR.NTDwt or pSVAR.NTDmutant were utilized, and that transfection and cell lysis were in the absence or presence of 100 nM R1881. Protein lysate (5 μL) was directly applied on a 10% SDS/PAGE gel (10% input). Lysate (50 μL) was mixed with 150 μL buffer A, with or without 100 nM R1881, and rotated for 5 h at 4 °C with 25 μL glutathione–agarose beads (Sigma-Aldrich, Deisenhofen, Germany). Next, agarose beads were washed five times with buffer A supplemented with 0.1 M NaCl with or without 100 nM R1881, boiled in 30 μL Laemmli sample buffer and 25 μL supernatant was separated over a 10% SDS/PAGE gel. After Western blotting, visualization of input and precipitated AR.NTD proteins was carried out as described above.

RESULTS

Systems for detection of androgen receptor N/C interaction

The ligand-dependent interaction between AR NTD and AR LBD, N/C interaction, was studied in yeast and mammalian *in vivo* protein interaction systems, and in

pull-down assays. In the yeast two-hybrid system, vectors encoding the Gal4 transactivating domain (GalAD) fused to AR NTDwt, AR NTDmutant or ARpeptides derived from AR NTD, were transfected to a yeast strain, which expressed the Gal4 DNA-binding domain (GalDBD) linked to AR.LBD (Fig. 1A). Upon incubation with DHT, N/C interaction mediated the expression of an integrated UAS_{GAL1}-lacZ reporter gene, which was assessed in a β-galactosidase assay. Note that in this assay the transactivating function is provided by both AR NTD and GalAD.

In the mammalian protein interaction system, vectors encoding wild type or mutated AR NTD, and AR DBD-LBD were cotransfected to CHO cells (Fig. 1B). R1881-induced activity of a transiently transfected androgen-inducible MMTV promoter was assessed in a luciferase assay. Note that in this assay the transactivating function is solely contributed by AR NTD.

In pull-down assays the fusion protein GST-AR.LBD and wild type or mutated AR.NTD proteins were transiently expressed in CHO cells.

$AR_{3–13}$ modulates the androgen receptor N/C interaction

As assayed in the yeast protein interaction system, deletion of $AR_{3–36}$ (GalAD-AR.NTDΔ3–36) completely abolished the ligand-dependent functional N/C interaction (Fig. 2A). Deletion of the N-terminal 13 amino acids (GalAD-AR.NTDΔ1-13) resulted in a slightly diminished (approximately 20%) N/C interaction. Because GalAD-AR.NTDΔ1-13 was expressed at a higher level than GalAD-AR.NTDwt (Fig. 2C), the decrease of AR N/C interaction caused by $AR_{1–13}$ deletion might actually be more than observed.

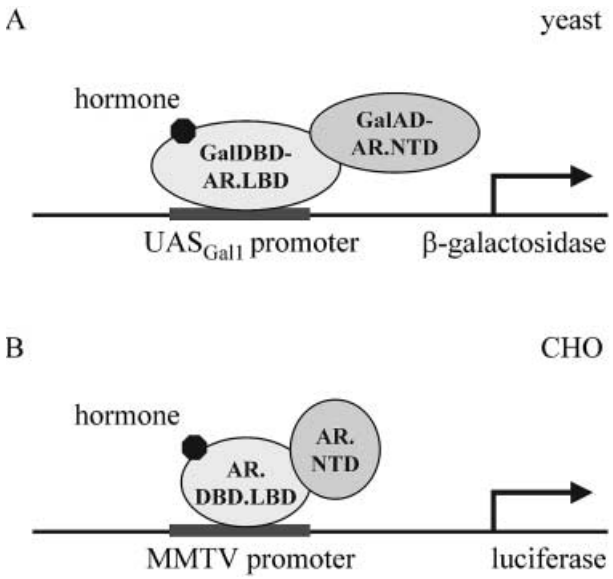

Fig. 1. Schematic representation of *in vivo* protein interaction systems utilized in this study. (A) Yeast protein interaction (two-hybrid) system. DHT-dependent interaction between GalAD-AR.NTD and GalDBD-AR.LBD induces expression of the UASGAL1 regulated lacZ reporter gene. Cotransfection of pGBT9 and pACT2, which encode GalDBD and GalAD, respectively, does not induce reporter gene expression (data not shown). Similarly, individually expressed GalDBD-AR.LBD and GalAD-AR.NTD are not active in this assay. (B) Mammalian (CHO cells) protein interaction system. R1881-dependent interaction between AR.NTD and AR.DBD.LBD induces MMTV-promoter driven luciferase expression. Separately expressed AR.DBD.LBD and AR.NTD are unable to activate the MMTV promoter (data not shown).

Similar to the yeast assay, in the mammalian protein interaction assay, deletion of AR_{3-37} completely prevented N/C interaction (Fig. 2B). A much more pronounced effect of $AR_{3–13}$ deletion on N/C interaction was observed as compared to the yeast assay. The approximately 90% drop in activity is indicative of an important role of $AR_{3–13}$ in N/C interaction. The diminished interaction was not due to a lower expression level of AR.NTDΔ3–13. In fact, AR.NTDΔ3–13 expression was higher than AR.NTDwt expression (Fig. 2C).

To investigate whether $AR_{3–13}$ directly binds to AR LBD, pull-down experiments were carried out. The results are presented in Fig. 3. In the absence of ligand, none of the AR NTD proteins showed LBD interaction. However, in the presence of ligand, both AR.NTDwt and AR.NTDΔ3–13 bound to AR LBD with similar affinity (Fig. 3). In contrast, AR.NTDΔ3–37 did not interact.

$AR_{2–14}$ cannot autonomously interact with the androgen receptor LBD

To substantiate the modulating role of $AR_{2–14}$ in N/C interaction, as suggested by the experiments described above, the individual peptides $AR_{2–36}$, $AR_{2–14}$ and $AR_{16–36}$ coupled to GalAD (Fig. 4A) were assayed in the yeast protein interaction system (Fig. 4B). No substantial interaction with AR.LBD was found for GalAD-$AR_{2–14}$. Activity was retained for approximately 60% in the GalAD-$AR_{16–36}$/AR.LBD complex. Because the GalAD-$AR_{2–36}$ expression level was lower than that of GalAD-$AR_{16–36}$ (Fig. 4C), the actual difference in activity between GalAD-$AR_{2–36}$ and GalAD-$AR_{16–36}$, might be larger.

Analysis of ^{30}VREVI^{34} in androgen receptor N/C interaction

Prediction programs of protein secondary structures (see http://npsa-pbil.ibcp.fr) indicated a long α-helical structure for $AR_{20–34}$. A helical wheel drawing of this region predicted an amphipathic character of this helical structure (Fig. 5A) [29]. At positions 15 and 37, the putative α-helix is flanked by proline residues. Within the helix, two candidate ΦxxΦΦ protein interaction motifs (Φ is any hydrophobic amino acid residue and x is any amino acid residue) are present: ^{30}VREVI^{34} and the previously identified ^{23}FQNLF^{27} motif (Fig. 5B) [20,30,31]. To investigate whether like ^{23}FQNLF^{27}, ^{30}VREVI^{34} could contribute to N/C interaction, two constructs were generated, expressing either the complete ^{30}VREVI^{34} or the complete ^{23}FQNLF^{27} motif linked to GalAD (Fig. 5B). As expected, in the yeast protein interaction system, ligand-dependent interaction with AR LBD could easily be detected for GalAD-$AR_{17–32}$. However, the interaction was weak for GalAD-$AR_{24–39}$ (Fig. 5C). Low activity was not due to decreased protein expression (Fig. 5D).

In a complementary yeast protein interaction experiment, the ^{30}VREVI^{34} motif in GalAD-AR.NTDwt was modified

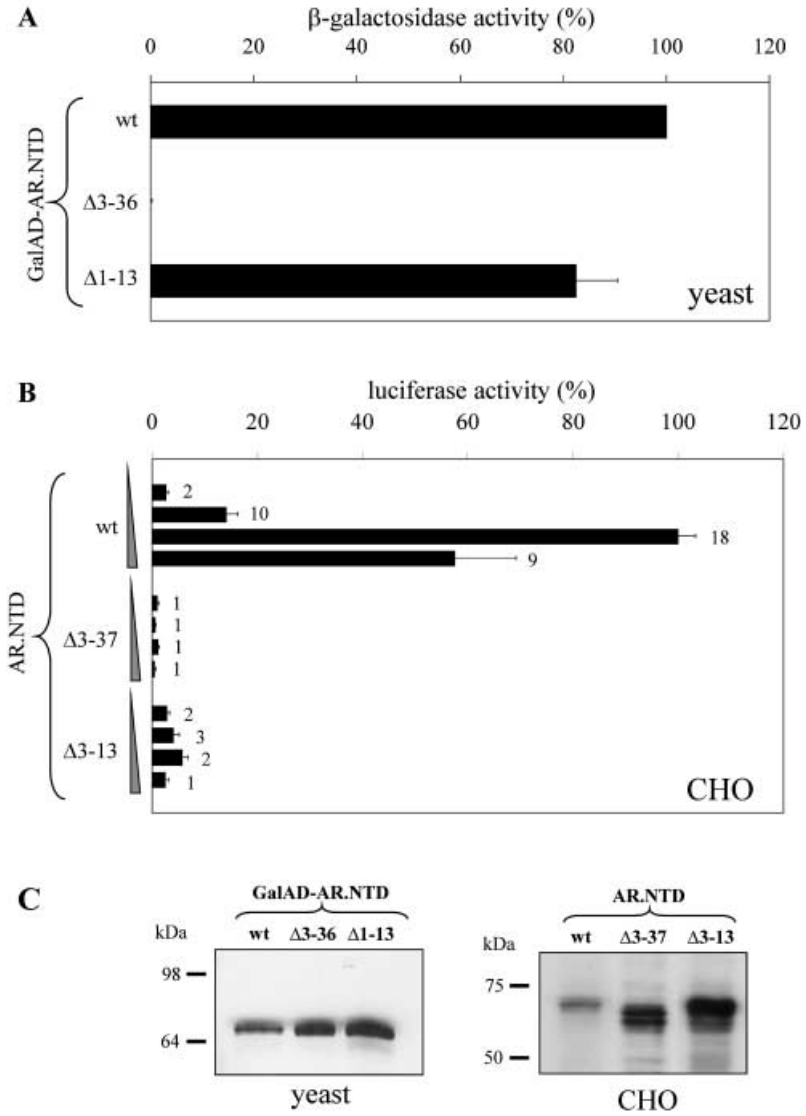

Fig. 2. AR3–13 modulates androgen receptor N/C interaction. (A) Interaction of AR.NTDwt and N-terminal deletion mutants with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment the activity of GalAD-AR.NTDwt was set at 100%. Each bar represents the mean (± SEM) β-galactosidase activity of three independent experiments. (B) Interaction of AR.NTDwt and deletion mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or mutant (see Experimental procedures). In each experiment, carried out in triplicate, the mean of the highest AR.NTDwt value was set at 100%. Each bar represents the mean (± SEM) luciferase activity of three independent experiments. Fold induction is shown to the right of each bar and represents the ratio of activities determined in the presence and absence of R1881. (C) Western analysis of indicated GalAD-AR.NTD proteins in the yeast protein interaction system (left panel) and of indicated AR.NTD proteins in the mammalian protein interaction system (right panel). See Experimental procedures for details.

by substitution of two hydrophobic amino acids by arginine residues, resulting in GalAD-AR.NTD30/33RR. These substitutions might cause steric hindrance in the interaction with the AR LBD surface, change the charge and disrupt the proposed amphipathic α-helical structure of $AR_{16–36}$. GalAD-AR.NTD23/27RR was utilized as control. Substitution of V30 and V33 partially reduced the interaction, whereas the F23R,F27R mutation completely abolished the interaction (Fig. 6A). Expression levels of GalAD-AR.NTDwt and GalAD-AR.NTD30/33RR were similar (Fig. 6C).

Results obtained in the mammalian protein interaction system, utilizing the AR.NTD30/33RR mutant and AR.NTD23/27RR, were essentially identical to the observations made in the yeast system (Fig. 6B). A partial inhibition of AR N/C interaction was observed for AR.NTD30/33RR, and an almost complete inhibition for AR.NTD23/27RR.

Pull-down experiments confirmed and extended the *in vivo* protein interaction experiments (Fig. 6D). AR N/C interaction was diminished due to 30/33RR substitutions, and completely abolished by 23/27RR substitutions.

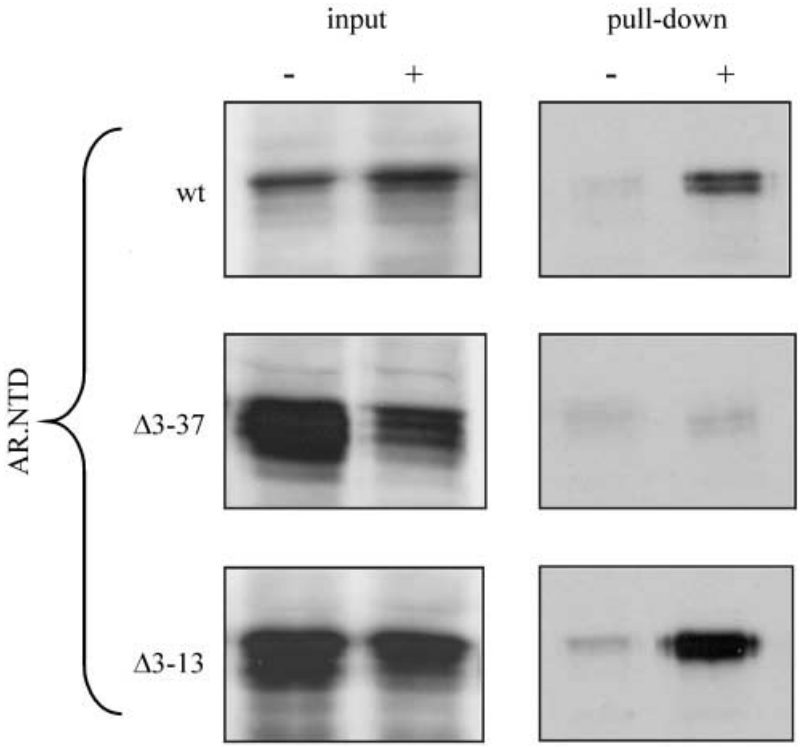

Fig. 3. AR3–13 is not involved in direct binding of AR NTD to AR LBD. Interaction of AR.NTDwt and N-terminal deletion mutants with GST-AR.LBD as studied by pull-down assays. Proteins were produced in CHO cells by cotransfection of pCMVAR.LBD and pSVAR.NTDwt or indicated deletion constructs. CHO cells were cultured in the absence (–) or presence (+) of 100 nM R1881. Input is 1/10th of the lysate utilized in a pull-down experiment. See Experimental procedures for details.

Amino acid residues flanking F23, L26 and F27 modulate androgen receptor N/C interaction

To study in more detail the role of 24/25QN in the ^{23}FQNLF^{27} motif in AR N/C interaction, these amino acids were substituted by 24/25AA. In both the yeast and mammalian protein interaction assay, GalAD-AR.NTD24/25AA and AR.NTD24/25AA formed even more active complexes with AR LBD than with wild-type AR NTD (Fig. 7A,B) (note the low expression levels of the 24/25AA mutants in both systems; Fig. 7C). As expected, AR.NTD26/27AA was incapable to interact with AR.LBD.

To extend these findings, an alanine scan was carried out for peptide GalAD-AR$_{17–32}$ (Fig. 8A). Results of the yeast protein interaction assay are shown in Fig. 8(B). Substitution of amino acids 23, 26 and 27 completely abolished interaction with GalDBD-AR.LBD and alanines at positions 24 and 25 increased the interaction capacity. All alanine substitutions of amino acids flanking ^{23}FQNLF^{27} reduced the binding to AR LBD. Most prominent inhibitory effects were found for amino acid residues directly flanking ^{23}FQNLF^{27}. Note that expression levels of the peptide constructs were similar (Fig. 8C).

DISCUSSION

Previously, we and others demonstrated a ligand–dependent functional interaction between AR NTD and AR LBD. Amino acids 3–36 in the NTD (AR$_{3–36}$), including the ^{23}FxxLF^{27} motif, play a pivotal role in N/C interaction [15,20]. Here we studied the function of the AR$_{3–36}$ subdomain AR$_{3–13}$ in N/C interaction and the role of individual amino acid residues in and flanking the ^{23}FQNLF^{27}motif in AR$_{16–36}$ in N/C interaction.

Yeast protein interaction assays indicated that AR$_{3–13}$ contributed to the ligand-induced transactivation function of the AR.NTD/AR.LBD complex (Figs 2 and 4). Pull-down experiments provided evidence that AR$_{3–13}$ does not directly interact with AR LBD (Fig. 3). On first sight, conflicting results were obtained in the yeast and mammalian protein interaction assays (Fig. 2). In the yeast assay, reporter gene activity, which monitored the N/C interaction, was partly reduced by AR$_{3–13}$ deletion, whereas in the mammalian assay almost all reporter gene activity was lost. The most obvious difference between both assays is the coupling of AR.NTD to GalAD in the yeast assay, and the absence of a second transactivation domain linked to AR NTD in the mammalian assay. The latter assay completely depends on the intrinsic transactivating function of AR NTD and thus does not allow discrimination between loss of AR.NTD-AR.LBD binding and loss of AR.NTD transactivating function. In the yeast assay, loss of transactivation function of AR NTD mutants, which retain AR LBD interacting capacity, like AR.NTDΔ3–13, will be masked by the GalAD transactivating function. So, AR$_{3–13}$ is not essential but rather modulates N/C interaction, most probably by affecting the transactivation function of AR.NTD. Alternative explanations might be induction of a more favorable NTD conformation or stabilization of the *in vivo* N/C interaction, which are not reflected in the pull-down assays and peptide interaction experiments. Unfortunately, the

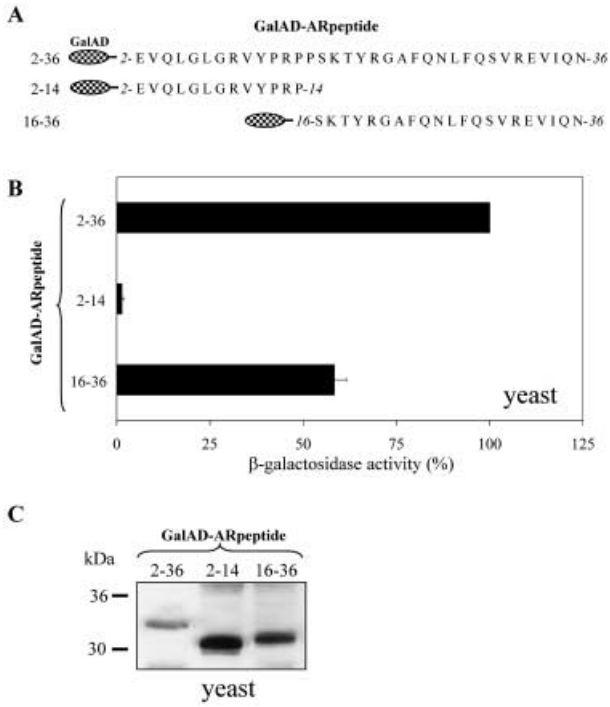

Fig. 4. AR2-14 cannot autonomously interact with AR LBD. (A) AR peptides utilized in GalAD-ARpeptide fusion proteins in the yeast protein interaction system. (B) Interaction of indicated GalAD-ARpeptides with GalDBD-AR.LBD in yeast in the presence of 1 μM DHT. In each experiment the activity of GalAD-AR2-36 was set at 100% (see also legend to Fig. 2A). (C) Western analysis of indicated GalAD-ARpeptide proteins in yeast. For details, see Experimental procedures.

primary structure and the predicted secondary structure of AR_{3-13} do not give a clue to a more precise description of its function (data not shown). However, the fact that, between species, AR_{3-13} is one of the most conserved regions of AR NTD, underscores a presumed important role in AR function [32].

The second domain that was studied, AR_{16-36}, is essential in N/C interaction. The predicted structure indicated that AR_{16-36} can fold in a remarkably long amphipathic α-helical structure, suggesting an important protein interaction interface [29]. AR_{16-36} contains two ΦxxΦΦ putative protein interaction motifs: ^{23}FxxLF^{27}, which was found to be pivotal for direct N/C interaction [20, this study], and ^{30}VxxVI^{34} (Figs 5 and 6). The latter sequence modulates N/C interaction. Amino acid residues in this sequence might contribute to the stability of the predicted α-helix. Alternatively, they might make additional contacts to the LBD surface. This is also true for other amino acid residues flanking the ^{23}FxxLF^{27} motif (Fig. 8). Remarkably, substitution of Q24 and N25 by alanines increased N/C interaction (Figs 7 and 8).

The AR FxxLF motif shows similarities to LxxLL motifs [5,33,34] present in nuclear receptor interaction domains (NR boxes) of p160 coactivators. LxxLL motifs are essential in the interaction with LBDs [33]. They bind to a hydrophobic cleft in nuclear receptor LBDs, which is marked by a charged clamp composed of a highly conserved lysine and glutamate residue in helix 3 and

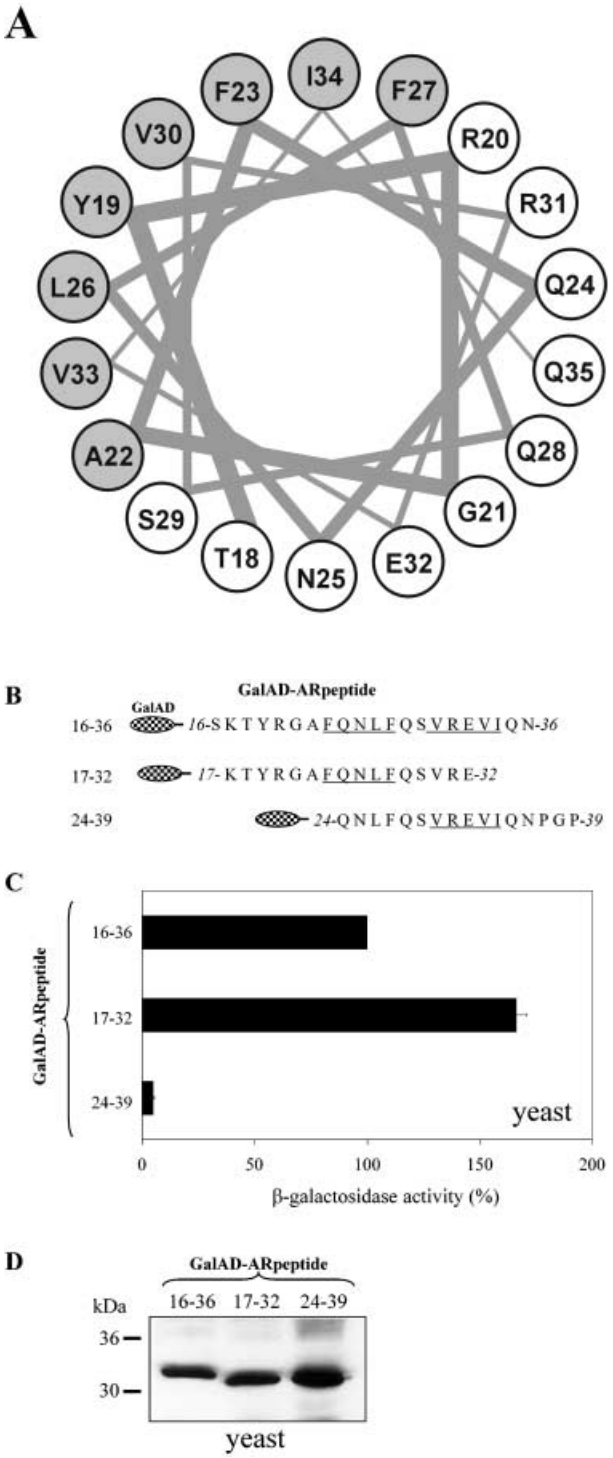

Fig. 5. Analysis of a predicted long amphipathic α-helix of AR18–35 in AR N/C interaction. (A) A helical wheel drawing of AR18–35 predicts a long amphipathic α-helical structure. Gray circles represent hydrophobic amino acids. (B) GalAD-ARpeptide fusion proteins utilized in the yeast protein interaction system. The ΦxxΦΦ motifs 23FQNLF27 and 30VREVI34 are underlined. (C) Interaction of GalAD-ARpeptides with GalDBD-AR.LBD in yeast in the presence of 1 μM DHT. In each experiment the activity of GalAD-AR16–36 was set at 100% (see also legend to Fig. 2A). (D) Western analysis of indicated GalAD-ARpeptide proteins in the yeast system. For details, see Experimental procedures.

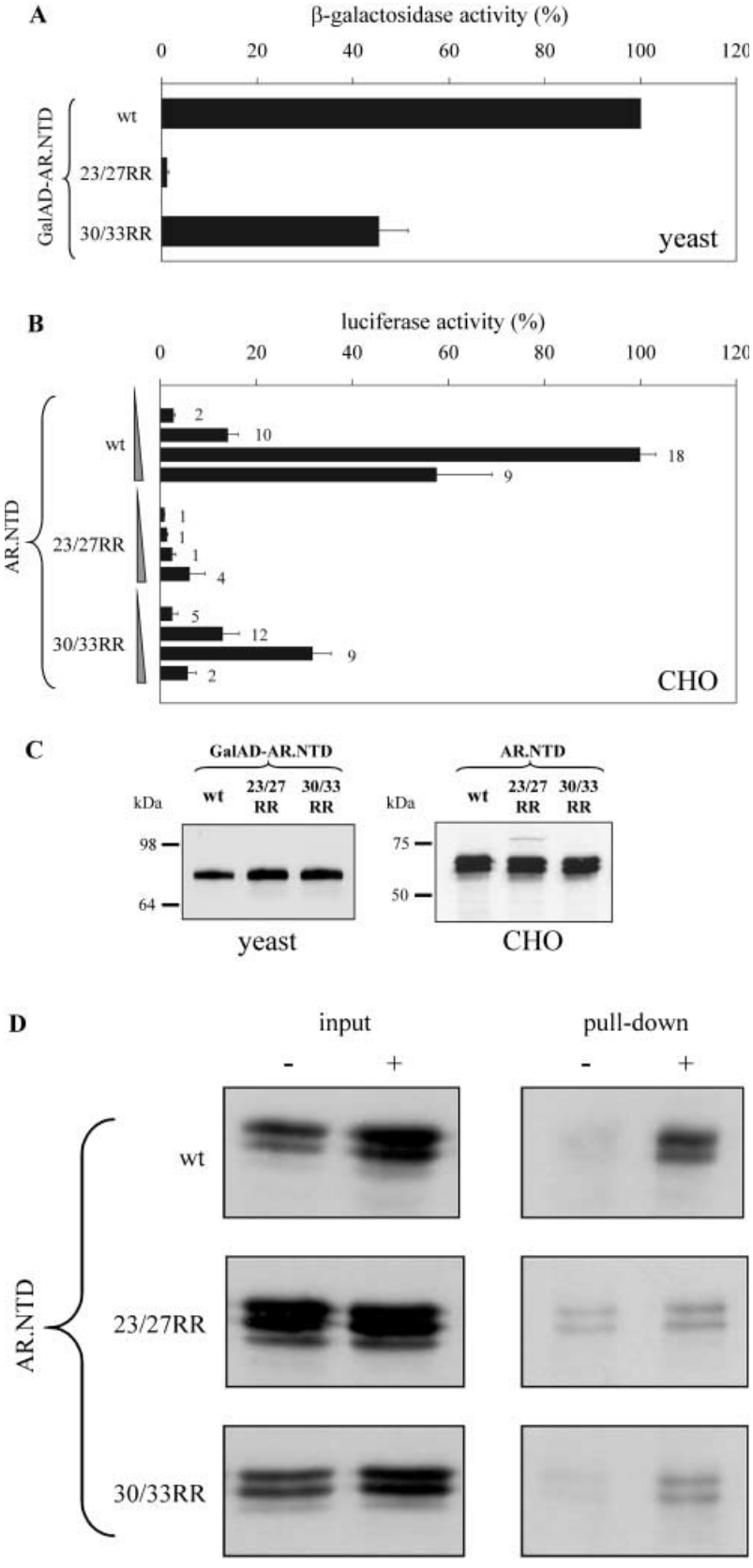

Fig. 6. 30VREVI34 is not essential for AR N/C interaction. (A) Interaction of GalAD-AR.NTDwt and mutants with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment GalAD-AR.NTDwt activity was set at 100% (see legend to Fig. 2A). (B) Interaction of AR.NTDwt and mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or indicated mutants (see Experimental procedures and legend to Fig. 2B). (C) Western analysis of indicated GalAD-AR.NTD proteins in the yeast system (left panel) and indicated AR.NTD proteins in the mammalian system (right panel) (see also Experimental procedures). (D) Pull-down assays showing interaction of AR.NTDwt and mutants with GST-AR.LBD (see also legend to Fig. 3).

helix 12 of the LBD, respectively (K720 and E897 in AR) [35–37]. AR K720 and E897 are both involved in the ligand–dependent interaction between AR LBD and the coactivator TIF2 [9,11,15]. However, in the FxxLF-mediated AR N/C interaction, E897 is essential, but K720 can be replaced by many other amino acids, without

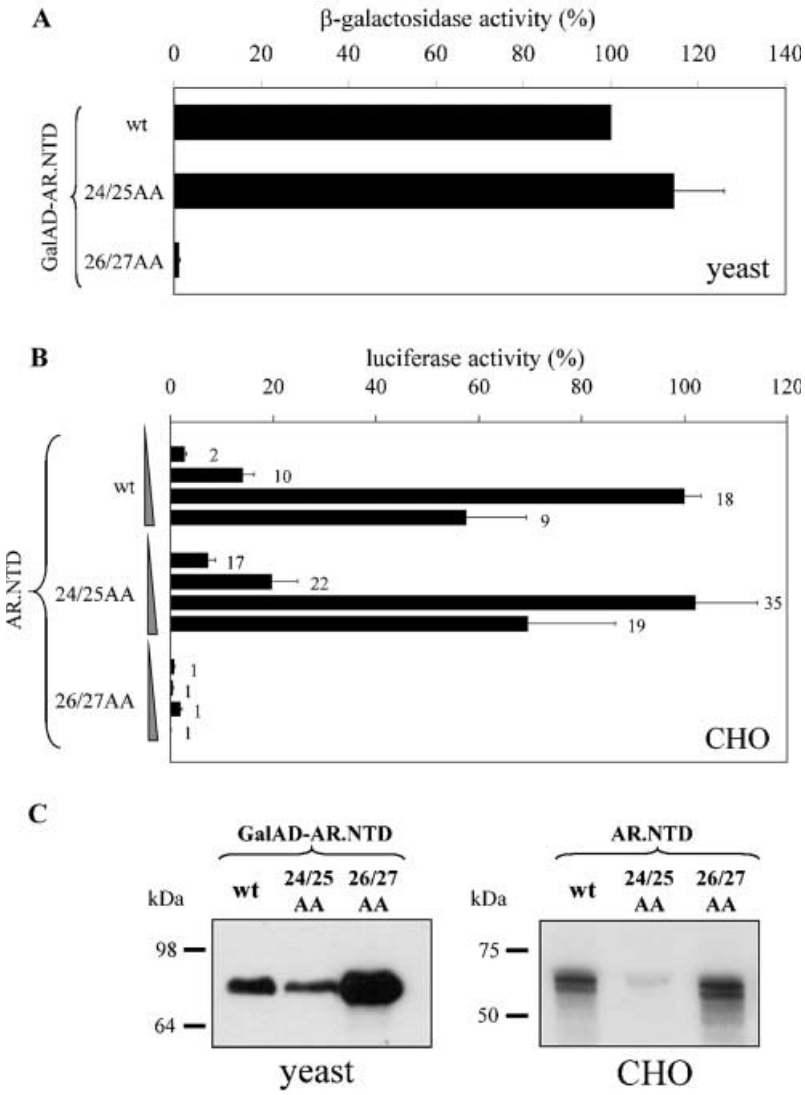

Fig. 7. Alanine substitutions of Q24 and N25 stimulate AR N/C interaction. (A) Interaction of GalAD-AR.NTDwt and mutants with GalDBD-AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment, GalAD-AR.NTDwt activity was set at 100%. See also legend to Fig. 2A. (B) Interaction of AR.NTDwt and mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or mutants (see Experimental procedures and legend to Fig. 2B). (C) Western analysis of indicated GalAD-AR.NTD proteins in the yeast protein system (left panel) and indicated AR.NTD proteins in the mammalian system (right panel). For details, see Experimental procedures.

affecting N/C interaction [9,11,15,38]. So, the AR N/C interaction is similar, but not identical, to LxxLL-mediated coactivator–LBD interaction.

The 3D structures of agonist bound LBD/LxxLL peptide complexes of several nuclear receptors have been elucidated, and interactions of the peptide backbone and its amino acid side chains with the LBD surface have been identified [5,36,37,39]. It is presumed that upon binding to the LBD surface, the LxxLL motif adapts a short α-helical structure, which is stabilized by interaction with the charged clamp [5,36,37]. The first and last leucine residue in the LxxLL motif enter the hydrophobic cleft in the LBD, and directly contact amino acid residues within the cleft. The variable amino acids (xx) in the LxxLL motif point away from the cleft and seem not to interact directly with the LBD surface. Structural data for AR.LBD/LxxLL peptides are not available but, because AR.LBD/coactivator interaction also depends on K720 and E897, it might be predicted that they will be similar to LBD/LxxLL peptide complexes studied so far [9,11,15]. Because K720 is not essential for $AR^{23}FxxLF^{27}$/AR.LBD interaction, the structure of this complex might be different. A different complex would also explain the stimulation of $AR^{23}FxxLF^{27}$/AR.LBD interaction by substitution of Q24 and N25 by alanine residues. Structural analyses of AR.LBD/$AR_{16–36}$ complexes have to reveal the function of amino acid residues flanking F23, L26 and F27 and answer the question as to whether or not the entire long amphipathic $AR_{16–36}$ α-helix is required for a stable AR NTD/LBD complex.

The LxxLL-like motifs LxxIL, FxxLL, and L/IxxI/VI, have been found in LBD binding coactivators or corepressors [40–43]. FxxLF motifs that are able to contact AR LBD, have only been found in AR NTD and most recently in the AR coactivators ARA54 and ARA70, suggesting a specific role of these motifs in AR function [44–47]. The increasing number of proteins found to interact with the AR LBD raises the question of the physiological relevance of the many interactions. It remains to be established whether all interactions take place in living cells under physiological conditions, whether interactions with different proteins are simultaneous or consecutive events, and which interactions are most stable and most specific. Recently, a start has been made to identify factors, including the AR, present in the transcription initiation complex of the prostate specific antigen enhancer/promoter, using chromatin immunoprecipitation (ChIP) [48].

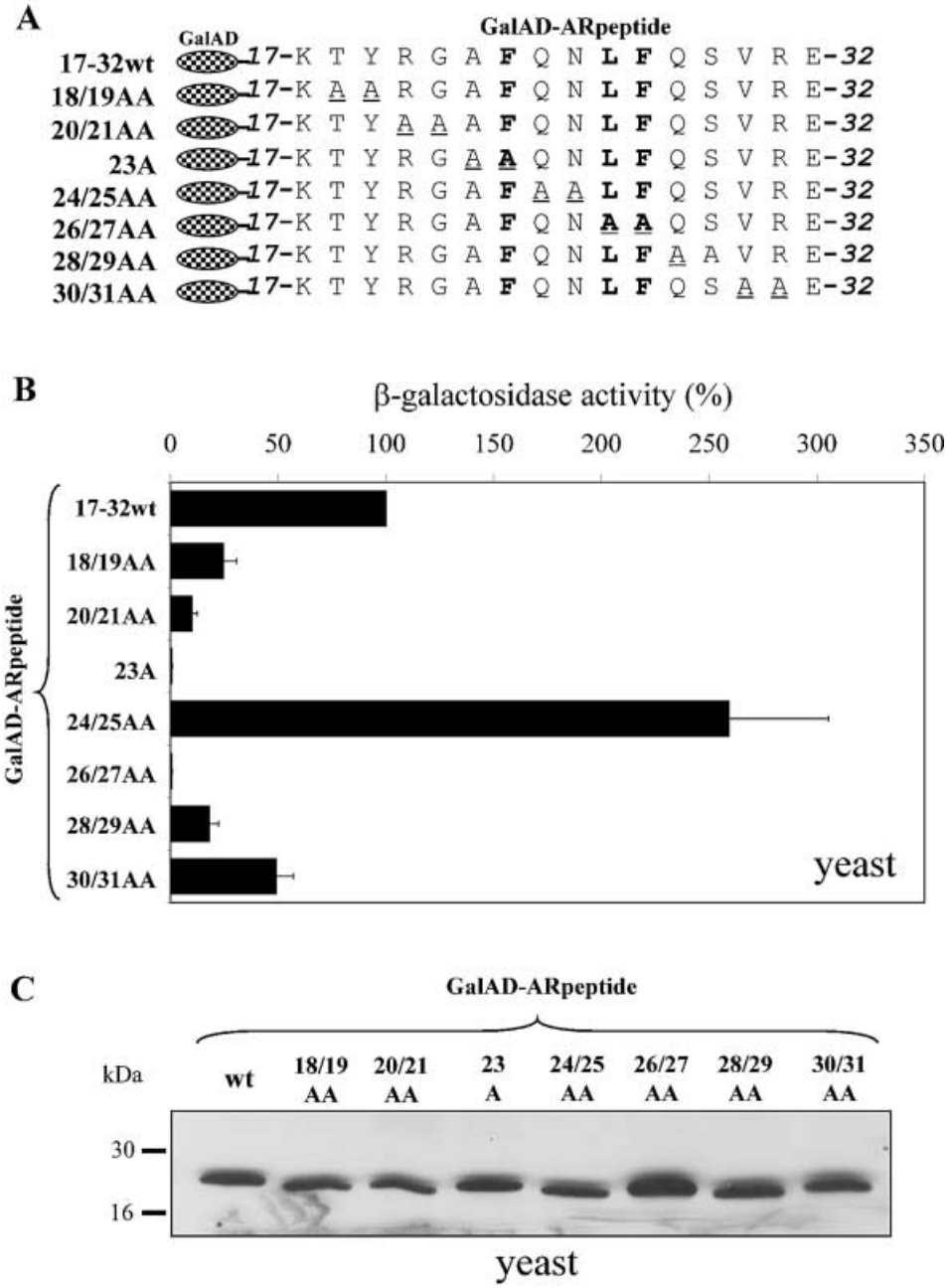

Fig. 8. Alanine scanning of AR17–32: amino acids flanking F23, L26 and F27 modulate AR N/C interaction. (A) GalAD-ARpeptide fusion proteins in the yeast protein interaction system. (B) Interaction of GalAD-ARpeptides with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment the activity of GalAD-AR17–32 was set at 100%. See also legend to Fig. 2A. (C) Western analysis of indicated GalAD-ARpeptide proteins in the yeast assay. For details, see Experimental procedures.

Another question concerns the interaction of $AR_{16–36}$ with other proteins. One candidate might be the TFIID TATA box-binding protein associated factor 31, $TAF_{II}31$, which has been found to interact with FxxΦΦ motifs in acidic transcription activation domains of p65 (nuclear factor-kappa B), VP16, p53 and related proteins [31,49–51].

AR NTD has previously been proposed to accommodate more than one AR LBD interacting domain [9,15,20]. A candidate second domain is ^{433}WHTLF^{437}, which was found to modulate ^{23}FxxLF^{27} function [20]. Another candidate motif is ^{179}LxxIL^{183} [9]. However, peptides containing these motifs were unable to interact with AR LBD in the yeast protein interaction assay, excluding their role as a second autonomous interaction motif in AR NTD (data not shown).

N/C interaction is not unique for the AR, but has also been described for other nuclear receptors. ERα ligand-dependent direct N/C interaction has been demonstrated, which was disrupted by amino acid substitutions that affect receptor function [52,53]. The ERα N/C interaction could be induced by the agonist estradiol (E_2), but not by the antagonist ICI164 384 [53]. Recently, it was found that the ERα N/C

interaction was required for SRC-1-mediated synergism between AF-1 and AF-2 function [8,53]. The progesterone receptor (PR) showed direct N/C interaction in the presence of agonist R5020, but not in the presence of antagonist RU486 [54]. LxxLL motifs in the PR-B form were most probably not involved, because the shorter PR-A form, lacking these motifs, also showed N/C interaction [55].

The role of the N/C interaction in full-length AR function is not well understood. Ligand-dependent AR N/C interaction affects ligand dissociation [11,20,56]. Whether this is a direct or an indirect effect is unknown. Disruption of the N/C interaction by mutation of the ^{23}FxxLF^{27} motif has a limited effect on full length AR transactivation function [20, Steketee, unpublished observation]. However, several AR LBD mutants with reduced or completely abolished N/C interaction have been found in androgen insensitivity patients [11,56,57]. Additionally, both N/C interaction and the transactivating function of the AR prostate cancer mutant T877A can be induced by natural low affinity ligands like progesterone or E_2 or the AR antagonist cyproterone acetate [18].

In conclusion, we propose that AR_{3-36} is involved in a dynamic sequence of protein interaction events, including N/C interaction, in regulation of AR function. Detailed knowledge on the role of the AR N/C interaction would require the elucidation of its function under more physiological conditions, including the study of mouse models carrying AR mutants defective in N/C interaction.

ACKNOWLEDGMENT

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