

Functional Screening of FxxLF-Like Peptide Motifs Identifies SMARCD1/BAF60a as an Androgen Receptor Cofactor that Modulates TMPRSS2 Expression

Dennis J. van de Wijngaart, Hendrikus J. Dubbink, Michel Molier, Carola de Vos, Jan Trapman, and Guido Jenster

Departments of Urology (D.J.v.d.W., G.J.) and Pathology (D.J.v.d.W., H.J.D., M.M., C.d.V., J.T.), Josephine Nefkens Institute, Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands

Androgen receptor (AR) transcriptional activity is tightly regulated by interacting cofactors and cofactor complexes. The best described cofactor interaction site in the AR is the hormone-induced coactivator binding groove in the ligand-binding domain, which serves as a high-affinity docking site for FxxLF-like motifs. This study aimed at identifying novel AR cofactors by *in silico* selection and functional screening of FxxLF-like peptide motifs. Candidate interacting motifs were selected from a proteome-wide screening and from a supervised screening focusing on components of protein complexes involved in transcriptional regulation. Of the 104 peptides tested, 12 displayed moderate to strong *in vivo* hormone-dependent interactions with AR. For three of these, ZBTB16/PLZF, SMARCA4/BRG1, and SMARCD1/BAF60a, the full-length protein was tested for interaction with AR. Of these, BAF60a, a subunit of the SWI/SNF chromatin remodeling complex, displayed hormone-dependent interactions with AR through its FxxFF motif. Vice versa, recruitment of BAF60a by the AR required an intact coactivator groove. BAF60a depletion by small interfering RNA in LNCaP cells demonstrated differential effects on expression of endogenous AR target genes. AR-driven expression of *TMPRSS2* was almost completely blocked by BAF60a small interfering RNA. In summary, our data demonstrate that BAF60a directly interacts with the coactivator groove in the AR ligand-binding domain via its FxxFF motif, thereby selectively activating specific AR-driven promoters. (*Molecular Endocrinology* 23: 0000–0000, 2009)

The androgen receptor (AR) is a ligand-dependent transcription factor that is essential for normal male sexual development and for maintaining function of male-specific organs (1, 2). AR and other steroid receptors are members of the nuclear receptor superfamily (3). The nuclear receptor family is characterized by a structural and functional organization, that includes a variable N-terminal domain (NTD) containing activation function 1 (AF-1), a highly conserved DNA-binding domain (DBD), and a moderately conserved C-terminal ligand-binding domain (LBD) containing activation function 2 (AF-2) (3, 4).

In eukaryotic cells, gene expression usually is in a repressed state due to a compact chromatin structure (5). A cascade of events is necessary to allow AR-induced transcription of target genes. The initiating step is binding of

testosterone or the more active metabolite dihydrotestosterone (DHT), leading to a conformational change in the AR LBD, dissociation of heat-shock proteins, and translocation of the AR from the cytoplasm to the nucleus. Here, AR forms homodimers and subsequently recognizes androgen response elements located within enhancer and promoter regions of AR target genes, followed by recruitment of cofactors (6–11). Although for the majority of cofactors the mechanism by which they modulate AR activity still needs to be determined, for several, including CARM1, PRMT1, and members of the p160 family of cofactors (SRC1, TIF2, and SRC3), it is known that they possess intrinsic histone-modifying properties or serve as bridging factor for more potent histone acetyltransferases, such as CBP/p300 and p/CAF (12–15).

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Abbreviations: AR, Androgen receptor; BAF, BRG1-associated factor; DBD, DNA-binding domain; DHT, dihydrotestosterone; ER α , estrogen receptor- α ; FCS, fetal calf serum; GR, glucocorticoid receptor; LBD, ligand-binding domain; NTD, N-terminal domain; PR, progesterone receptor; QPCR, quantitative PCR; siRNA, small interfering RNA.

Multi-subunit cofactor complexes recruited by AR include ATP-dependent chromatin remodeling complexes, such as SWI/SNF. The Mediator/TRAP/DRIP complex bridges the receptor to general transcription factors and RNA polymerase II (9, 11, 16–19). Complex interplay between individual cofactors and cofactor complexes at enhancer and promoter regions creates a dynamic chromatin environment allowing tight regulation of AR target gene expression.

For the majority of the more than 130 proteins known to interact with AR, not only does the physiological relevance still need to be determined, but even how they interact with the AR. So far, the coactivator binding groove formed in the LBD after hormone binding is the only well-described protein-interaction surface of AR (20, 21). Whereas in most nuclear receptors this groove serves as a high-affinity docking site for α -helical LxxLL motifs, the coactivator groove in the AR is rather unique because it prefers binding of FxxLF motifs, which drive the interaction of AR cofactors ARA54, ARA70, and hRAD9 (22–30). Another unique feature of the AR is that an FxxLF motif in the NTD is also able to bind strongly to the AR coactivator groove (31, 32). Although the physiological function of this FxxLF-mediated interaction of AR NTD with AR LBD (N/C interaction) is not fully understood, it is believed to decrease the ligand dissociation rate and to selectively affect gene transcription (25, 33–36). Recently, we demonstrated that N/C interaction takes place in the mobile AR fraction. After binding of AR to DNA, N/C interaction is relieved and the coactivator groove becomes accessible for cofactors (37).

Previous direct mutagenesis studies of the AR FxxLF motif and phage display screenings with random and focused sequences using AR LBD or full-length AR as bait yielded several novel motif variants, including FxxFF, FxxMF, and FxxYF, that are able to bind to the AR coactivator groove (21, 38, 39). A survey of known AR cofactors yielded two proteins, gelsolin and PAK6, that were fully dependent on the FxxFF and FxxMF motif, respectively, for interaction with AR (39). The present study aims at identifying novel AR cofactors on the basis of FxxLF-like motifs. Based on an *in silico* proteome-wide screening and a supervised screening that focused on members of protein complexes involved in transcriptional regulation, over 100 FxxLF-like motifs were selected to be assayed for interaction with AR. Our screenings yielded twelve peptides that displayed moderate to strong hormone-dependent interactions with AR. The FxxFF motif of SMARCD1/BAF60a, a subunit of the SWI/SNF chromatin remodeling complex, also interacted with AR if tested in the context of the full-length protein. Interactions between AR and BAF60a were dependent on the presence of

hormone and required an intact AR cofactor groove and the BAF60a FxxFF motif. Small interfering RNA (siRNA) experiments demonstrated that BAF60a functions as a promoter-selective AR cofactor essential for the expression of the *TMPRSS2* gene.

Results

In silico selection of candidate AR binding partners on the basis of FxxLF-like motifs

To identify novel AR cofactors, two different *in silico* screenings were performed to select for FxxLF-like motif-harboring proteins. The first was based on a proteome-wide screening (see supplemental Fig. S1A, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Using the Reference Sequence (RefSeq) protein database from NCBI (release 15), all human proteins containing an FxxFF, FxxMF, or FxxYF motif were selected. Because of the high number of motifs retrieved (>27,000), additional selection criteria were set. Using the Homologene database, those motifs were selected that are conserved between human and both mouse and rat orthologs. Based on Gene Ontology, motifs were selected that are present in proteins that reside in the same cellular compartments as AR, *i.e.* cytoplasm and nucleus, or of which the subcellular localization was unknown. Proteins present in other cellular compartments as well as secreted proteins were excluded. Finally, proteins were selected that are expressed in at least one of the same tissues as AR, including prostate, epididymis, seminal vesicles, and testis, or of which the expression pattern was unknown. Based on these criteria, 33 FxxFF, 27 FxxMF, and 11 FxxYF motifs in these proteins were assayed as peptide for interaction with AR (supplemental Table S1). In addition, based on similar criteria, but using the SwissProt database, 18 FxxLF motifs were selected and tested for AR interaction (supplemental Table S1). These selections also yielded the FxxLF motifs in the known AR cofactors PAK6, gelsolin, and ARA70. However, these motifs were not analyzed further. Our selections did not yield the FxxLF motifs in the AR cofactors ARA54 and hRAD9 due to the absence of a rat ortholog for these proteins.

A second screening was based on a focused selection method (supplemental Fig. S1B). Because the AR is a transcription factor, proteins were selected that are part of two multi-subunit complexes involved in the transcription process: SWI/SNF and Mediator/TRAP/DRIP. SWI/SNF complexes consist of a core ATPase, either SMARCA2/BRM or SMARCA4/BRG1, and 10–12 BRG1-associated factors (BAFs). Mediator/TRAP/DRIP may consist of more than 16 components. Because the number of FxxLF, FxxFF,

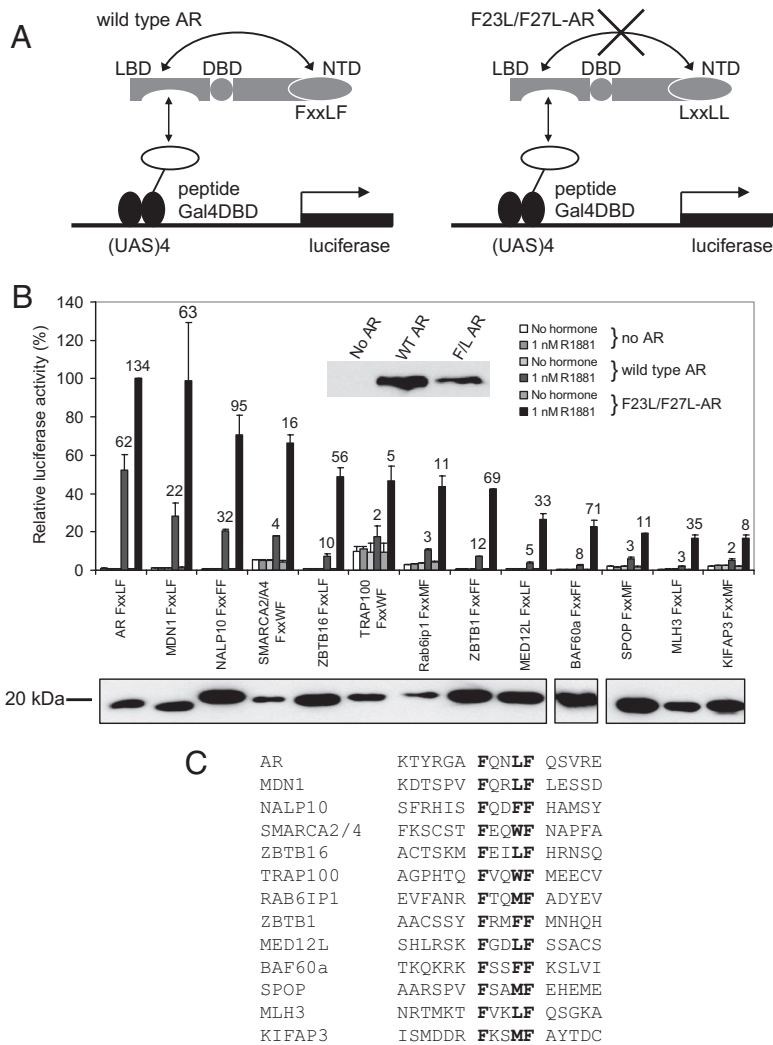


FIG. 1. Mammalian one-hybrid analysis yields 12 AR-interacting peptides. A, Schematic representation of the mammalian one-hybrid assay. A, Peptides were fused to Gal4DBD and served as bait for full-length wild-type AR (*left*) or F23L/F27L-AR in which the competitive FxxLF motif in the NTD has been substituted by LxxLL (*right*). In case of interaction between peptide and AR LBD, the AR NTD serves as transcription activation domain. Interactions were determined in the absence and presence of hormone using Hep3B cells. B, Twelve peptides interact with AR. Only peptides are shown of which the interaction capacity was more than 20% as compared with the interaction of AR FxxLF motif with F23L/F27L-AR or have a more than 5-fold hormone-induced interaction with F23L/F27L-AR. The luciferase activities in the absence or presence of wild-type AR and F23L/F27L-AR are shown. Luciferase activities were determined in the absence or presence of 1 nM R1881. Each bar represents the mean relative luciferase activity of two independent experiments (\pm SD). Interaction of the AR FxxLF motif with F23L/F27L-AR was set to 100% and mean fold inductions are shown *above the bars*. Expression of the Gal4DBD-peptide fusion proteins was visualized in a Western blot using antibodies against Gal4DBD and is shown *below the figure*. *Inset*, Expression of wild-type AR (WT) and F23L/F27L-AR (F/L). C, Amino acid sequences of the peptides interacting with AR (see B).

FxxMF, and FxxYF motifs in these proteins is limited, we extended the selection criteria by inclusion of related motifs, like FxxWF, FxxIF, FxxYL, and FxxLY, which also had to be conserved in mouse and rat orthologs. These criteria yielded 15 motifs (supplemental Table S2). Together with the 89 motifs selected in the first screening, a total of 104 motifs were assayed for AR interaction.

Functional screening yields 12 AR-interacting motifs

The 104 selected motifs were tested for interaction with AR in a mammalian one-hybrid assay as shown schematically in Fig. 1A. Vectors were constructed expressing the motifs linked to Gal4DBD, and these fusion proteins were assayed for interaction with full-length wild-type AR (Fig. 1A, *left*) or a mutant AR in which the competing FxxLF motif in the NTD was substituted by inactive LxxLL (F23L/F27L-AR; Fig. 1A, *right*). If the AR is recruited by Gal4DBD-peptide, the AR will transactivate the (UAS)₄TATA-Luc reporter in the presence of 1 nM R1881 but not in the absence of hormone.

Most selected peptides displayed weak interactions with F23L/F27L-AR (<20% interaction capacity as compared with AR FxxLF motif or less than 5-fold hormone induction) or did not interact at all (data not shown). Twenty peptides displayed elevated luciferase activities even in the absence of hormone (between 5 and 20% luciferase activity as compared with the hormone-dependent interaction of AR FxxLF peptide with F23L/F27L-AR). Eight peptides displayed high basal activities (>20% activity compared with the AR FxxLF interaction with F23L/F27L-AR; supplemental Tables S1 and S2). These elevated and high basal activities were caused by the peptides themselves because similar luciferase values were obtained in the absence of AR (data not shown). In general, peptides that showed elevated or high basal activities were characterized by an overall negative net charge, which corresponds with earlier publications showing that such sequences may have intrinsic activity (40).

Our screenings yielded 12 peptides that showed modest to strong interactions with F23L/F27L-AR (>20% interaction capacity as compared with the AR FxxLF motif and more than 5-fold hormone induction; Fig. 1B). Interactions were reduced when assayed with wild-type AR, demonstrating competition between the peptide and the FxxLF motif in the AR NTD for binding to the coactivator binding groove in the LBD. Eight peptides (MDN1, NALP10, ZBTB16, Rab6IP1, ZBTB1, SPOP, MLH3, and KIFAP3) were derived from the proteome-wide screening. Four peptides (SMARCA2/A4, TRAP100, MED12L, and SMARCD1/BAF60a) were derived from the supervised screening. Of these interacting peptides, four contained

an FxxLF, three an FxxFF, and three an FxxMF motif (Fig. 1C). SMARCA2/A4 and TRAP100 interacted with the AR via a novel FxxWF motif. The elevated luciferase activities of both peptides shown in the absence of hormone were also observed in the absence of AR (Fig. 1B). Western blot analysis demonstrated expression levels of the ARs and the 12 interacting peptides (Fig. 1B).

Interaction of full-length BAF60a with AR is dependent on an intact FxxFF motif

Of the 12 peptide motifs interacting with AR, three were selected for interaction with AR in their respective full-length protein context. Although MDN1 FxxLF and NALP10 FxxFF were the strongest AR-interacting peptides (Fig. 1B), they were not further analyzed yet because of the size of the protein (MDN1, 5596 amino acid residues) or less likely AR-mediated function (NALP10, negative regulator of inflammatory and apoptotic signal transduction), respectively. SMARCA4/BRG1, one of the two core ATPases of the SWI/SNF chromatin remodeling complex, was selected for further analysis because of the strong interaction and its novel FxxWF motif. ZBTB16, ZBTB1, and SPOP are related members of the family of BTB/POZ domain-containing proteins. Of these, ZBTB16, a transcriptional repressor also known as PLZF, was selected for further analysis because it showed strongest interactions with AR (Fig. 1B). Of the remaining motifs, the BAF60a subunit of the SWI/SNF complex was selected for further analysis because it displayed the highest hormone inductions with both wild-type AR and F23L/F27L-AR.

Figure 2A schematically shows the fragments of ZBTB16, SMARCA4, and BAF60a assayed for AR interaction and the relative positions of the motifs in these proteins. Protein fragments were fused to Gal4DBD and evaluated for interaction with AR in a mammalian one-hybrid assay as described above. Although ZBTB16 FxxLF and SMARCA4 FxxWF interacted with AR as peptides, no interactions were observed if both motifs were in a larger protein fragment (Fig. 2B). In contrast, hormone-induced AR interaction was observed with BAF60a protein (Fig. 2B). Interactions of BAF60a with AR were completely dependent on an intact motif, because a mutant BAF60a, in which the FxxFF motif was mutated into FxxAA, did not interact (Fig. 2B). Western blot analysis shows the expression and size of all fusion proteins (Fig. 2B).

To validate the hormone-dependent interaction of AR with BAF60a *in vivo*, we performed co-immunoprecipitation experiments. Using LNCaP cells, we found that BAF60a was co-immunoprecipitated with AR in the presence of R1881 (Fig. 2C). Vice versa, AR was hormone-dependently precipitated with BAF60a, confirming a

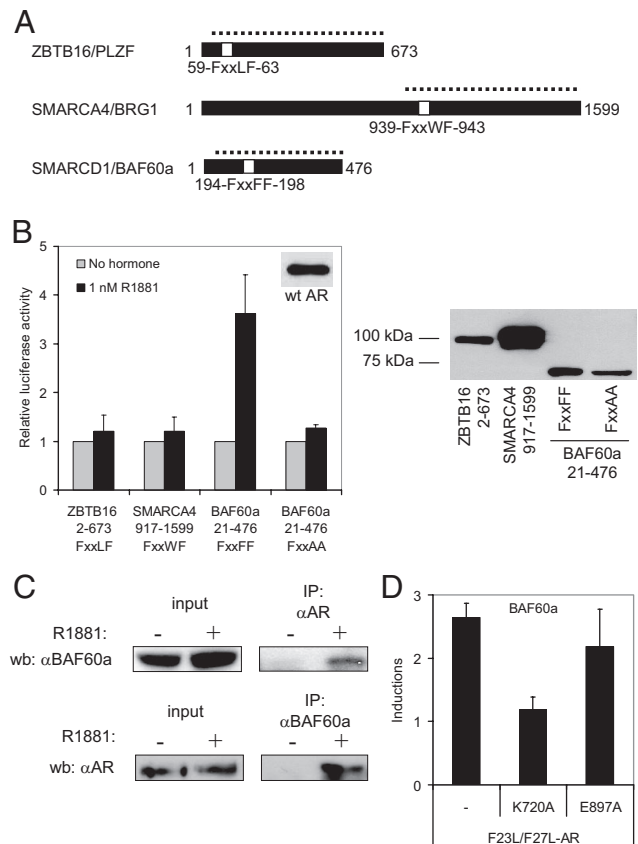


FIG. 2. Full-length BAF60a displays hormone- and motif-dependent interactions with AR. **A**, Schematic representation of proteins tested for interaction with AR. Shown are the relative position of the motif in the protein (*white box*) and the fragment of ZBTB16, SMARCA4, and BAF60a used for interaction with AR (*dotted line*). **B**, Mammalian one-hybrid analysis of proteins tested for interaction with AR. Experimental setup was similar to that described Fig. 1, A and B. Data shown are the relative interactions. Interaction of each protein with AR in absence of hormone was set to 1. *Bars* represent the mean of two independent experiments (\pm SD). Western blot visualizing the expression of Gal4DBD-protein fusions by Gal4DBD antibody staining is shown on the *right*. **C**, AR (*upper panel*) and BAF60a (*lower panel*) were used to pull down BAF60a and AR, respectively, in the absence (–) or presence (+) of 1 nM R1881. Input is about 15% of the lysate used in the pull-down experiment (see *Materials and Methods* for details). **D**, Mammalian one-hybrid assay showing the interaction of BAF60a with AR mutated in the coactivator groove (K720A or E897A). Data show the mean fold R1881-induced interactions of three independent experiments (\pm SEM).

physical interaction between the two proteins. To study the involvement of the AR coactivator groove in recruitment of BAF60a, we mutated the charge clamp residues K720 and E897, located at the extremities of the groove, into alanine residues. Binding of BAF60a to AR was not affected by the E897A mutation (Fig. 2D). However, AR was unable to bind BAF60a if the K720 residue was mutated, demonstrating that the coactivator groove in the AR plays a critical role in binding BAF60a.

BAF60a preferentially interacts with AR

Previously, we demonstrated that several FxxFF motifs selectively bind AR LBD (39). To investigate whether this

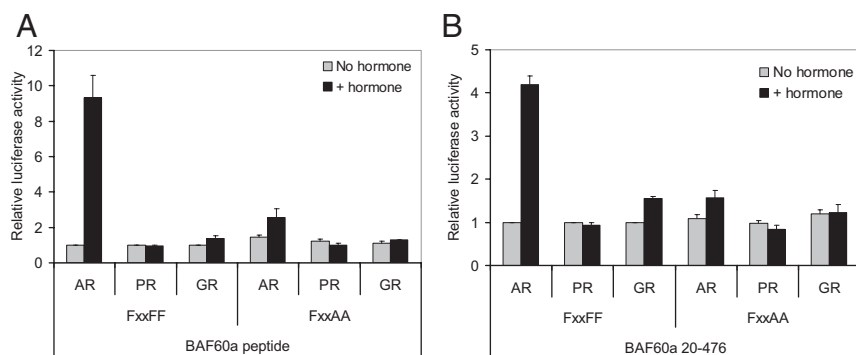


FIG. 3. BAF60a preferentially interacts with AR. Mammalian one-hybrid assay showing the interactions of BAF60a FxxFF and FxxAA peptides (A) or proteins (B) with full-length AR, PR, and GR. Experimental setup is similar to that described in Fig. 2, A and B. Bars represent mean relative luciferase activities of three independent experiments (\pm SEM) in absence (gray bars) or presence (black bars) of hormone. Interaction of the wild-type motif-harboring peptide or protein with a nuclear receptor in the absence of hormone was set to 1. Hormones used were 1 nM R1881 for AR and PR and 10 nM dexamethasone for GR.

is also true for BAF60a peptide and protein, mammalian one-hybrid assays were carried out with progesterone receptor (PR) and glucocorticoid receptor (GR).

The nuclear receptor interaction profiles of BAF60a peptide (Fig. 3A) and protein (Fig. 3B) were highly similar. Both BAF60a peptide and protein displayed strong hormone-dependent interaction with AR but not at all with PR. Weak interactions were observed with GR. A control peptide, D11 LxxLL (41), displayed strong interactions with all three nuclear receptors (supplemental Fig. S2). Mutating the FxxFF motif in the peptide or the protein into FxxAA resulted in strongly reduced interactions of BAF60a with AR. So, the FxxFF motif is essential for AR interaction.

BAF60a is essential for AR-dependent TMPRSS2 expression

To study whether BAF60a plays a functional role in AR target gene expression, siRNA experiments were carried out. BAF60a has two highly homologous family members, SMARCD2/BAF60b and SMARCD3/BAF60c, with conserved FxxFF motifs. Therefore, also the contribution of these two family members on AR-regulated transcription was investigated.

LNCaP cells were transfected with siRNA against each of the three BAF60 members, after which the mRNA levels were measured by quantitative PCR (QPCR). All three BAF60s were expressed in LNCaP cells, and expression of each individual BAF60 member was specifically inhibited by the corresponding siRNA (supplemental Fig. S3). Next, we investigated the role of the different BAF60s in regulating transcription from endogenous androgen-responsive genes in prostate cancer cells. LNCaP cells were transfected with control siRNA (siControl), siRNA against AR (siAR), or siRNA against the three individual BAF60 members, followed by incubation with R1881 or

vehicle for 8 h. Expression of *SGK*, *SARG*, *NDRG1*, *PSA*, *KLK2*, and *TMPRSS2* mRNA was measured by QPCR (Fig. 4). Expression of all target genes was strongly induced in the presence of hormone (siControl) and was dependent on AR because inhibition of AR expression with siRNA (siAR) strongly inhibited target gene expressions. Depletion of BAF60a, BAF60b, and BAF60c with siRNA did not affect or weakly decreased the hormone-dependent expression of *SGK*, *SARG*, and *NDRG1* (Fig. 4, A–C), whereas *PSA* and *KLK2* expression were reduced to about 50% in the presence of each individual siRNA (Fig. 4, D and

E). In contrast, a differential effect was observed on *TM-PRSS2* expression (Fig. 4F). Expression of *TM-PRSS2* was weakly reduced (about 30%) after depletion of BAF60b and BAF60c but was almost completely abolished after BAF60a depletion. These data show that BAF60a is essential for high AR-dependent expression of *TM-PRSS2*.

Discussion

This study aimed at identifying novel AR cofactors that directly interact with AR on the basis of FxxLF-like motifs binding to the ligand-induced coactivator-binding groove in the AR LBD. *In silico* peptide motif selections followed by functional interaction assays yielded SMARCD1/BAF60a, a component of the SWI/SNF chromatin remodeling complex, as a novel AR cofactor that directly and hormone-dependently interacts with the AR coactivator groove via its FxxFF motif. We further demonstrated that BAF60a differentially affected AR target gene expression and that BAF60a was essential for high *TM-PRSS2* expression.

Our search for novel AR cofactors started with a proteome-wide *in silico* screening to select for potentially AR-interacting motifs, based on sequences previously demonstrated to bind strongly AR LBD, such as FxxLF, FxxFF, FxxMF, and FxxYF (21, 38, 39). Selected motifs were then tested as peptides in mammalian one-hybrid assays to determine their interaction capacity with AR. Although this approach is semi-high-throughput, it has several advantages over conventional screening methods, such as yeast two-hybrid and phage display. A major advantage is that selection criteria can be defined, like conservation, tissue of expression, and subcellular localization. In addition, motifs can be selected that otherwise could have been missed by screening of cDNA libraries,

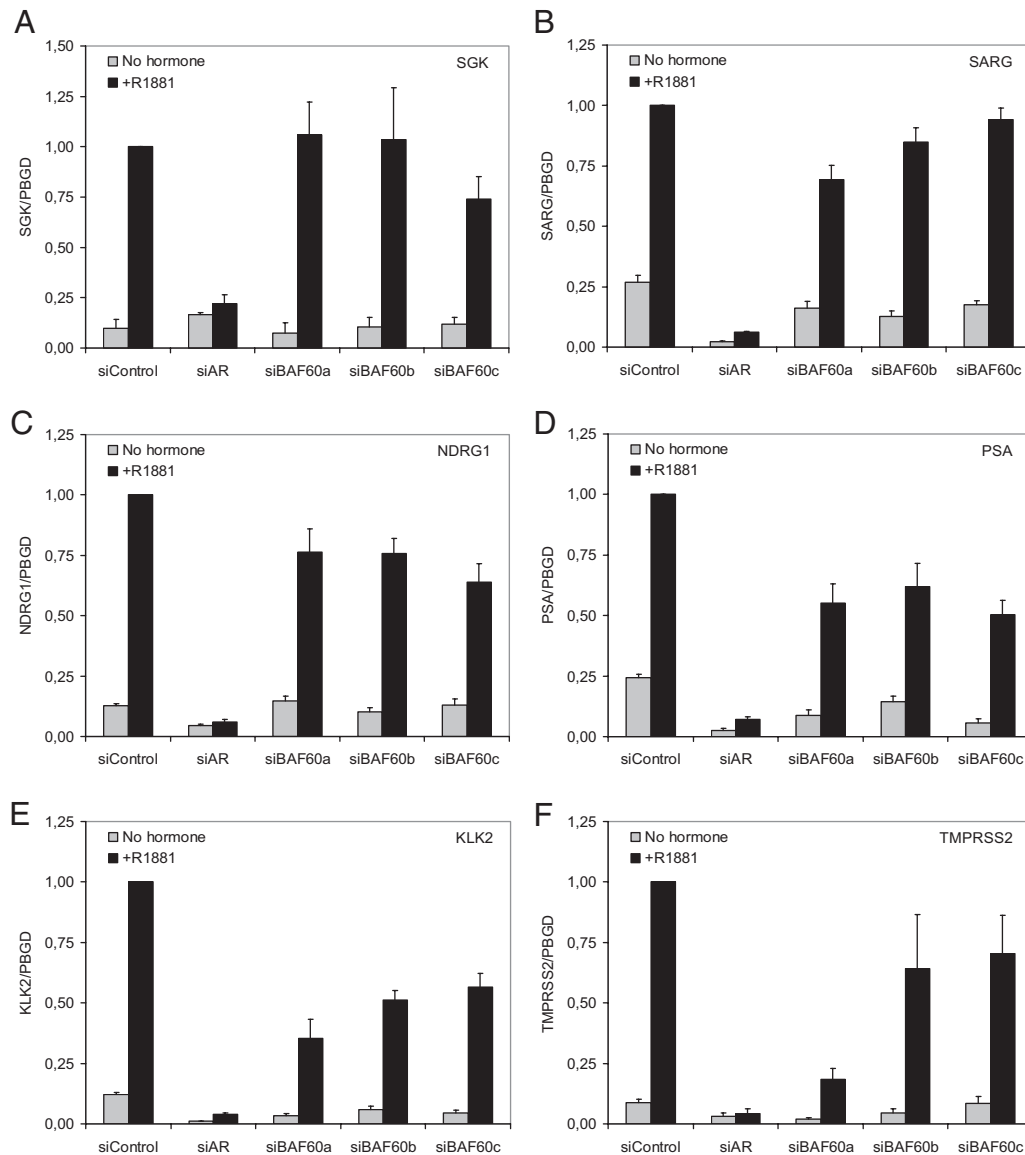


FIG. 4. BAF60a selectively affects the hormone-dependent expression of the AR target gene *TMPRSS2*. LNCaP cells were transfected with nontargeting siRNA (siControl), siRNA directed to AR (siAR), or siRNA against BAF60a, BAF60b, or BAF60c as described in *Materials and Methods*. mRNA levels of AR target genes *SGK* (A), *SARG* (B), *NDRG1* (C), *PSA* (D), *KLK2* (E), and *TMPRSS2* (F) were determined by QPCR in the absence or presence of 1 nM R1881. Results shown are the average of three to six QPCR experiments (\pm SEM) divided over two individual siRNA experiments. Expressions were normalized to *PBGD* values and are relative to the hormone-dependent expression in the presence of siControl.

for example because the motif is located at the very N terminus of a protein or is present in a sequence under-represented in libraries. On the other hand, *in silico* screenings are dependent on database information. Because most databases are incomplete, it can be predicted that potentially interacting motifs were missed, like the FxxLF motifs in ARA54 and hRAD9. We also focused on a limited number of potentially AR-interacting FxxLF-like motifs using stringent selection criteria. Furthermore, it cannot be excluded that as yet unidentified cofactors exist that interact via a different type of FxxLF-like motif not included in our search.

Of the 104 selected FxxLF-like motifs assayed for interaction with AR, 12 displayed moderate to strong inter-

actions. From this low number it is again clear that the three core residues of FxxLF-like motifs are not the sole interaction determinants (24, 32, 42). It may be that the noninteracting peptides do not form an α -helical structure or that residues flanking the FxxLF-like motif prevent stable interactions with the AR LBD surface. Although comparison of the amino acid sequences of the interacting peptides did not reveal a clear consensus in residues flanking the FxxLF-like motifs, all interacting peptides (except for ZBTB1) contained positively charged residues N-terminally to the core motif; a tendency for charged residues at the C terminus was less clear (Fig. 1C). This correlates with previously published data demonstrating the importance of charged residues flanking the

core motif, which form electrostatic interactions with oppositely charged residues on the AR LBD surface (42). However, charged residues are not the only determinant for peptide interaction, because many noninteracting peptides from our screening contain a similar charge distribution.

We identified two peptides, SMARCA2/SMARCA4 and TRAP100, that interacted with AR via a novel type of motif: FxxWF. Previous screenings for novel AR-interacting motifs already demonstrated that W (Trp) residues could be compatible with binding to the AR coactivator binding groove when present at positions +1 and +5 (21, 25, 43). Our previous screening at position +4 of the AR FxxLF motif demonstrated that substitution of L (Leu) for W (Trp) resulted in a peptide weakly interacting with the AR LBD (39). The data obtained in this study show that SMARCA2/A4 and TRAP100 FxxWF motifs do interact with AR, suggesting that in these amino acid contexts, W + 4 is able to obtain a conformation that is favorable for interaction with the AR coactivator groove.

Three motifs were selected for further analysis in full-length proteins. Of the 12 AR-interacting peptide motifs, eight were derived from the proteome-wide screening. With current knowledge, the molecular functions of three of these (NALP10, Rab6IP1, and KIFAP3) could not be linked to AR transcription regulation, and these motifs were therefore not selected. Two peptides (MDN1 and MLH3) were not selected because of protein size (MDN1 consists of almost 5600 amino acid residues) or because of relative weak interaction. However, both proteins remain interesting candidates for AR binding and will be part of future study. Three peptides (ZBTB1, ZBTB16, and SPOP) are present in members of the family of BTB/POZ domain-containing proteins. Because the motifs are located at similar positions within the BTB/POZ domain, ZBTB16 was selected because it showed strongest interactions with AR. The remaining four peptides were derived from the screening focusing on complexes involved in transcriptional processes. Two of these (BAF60a and SMARCA4) were selected for further analysis. Of the other two, TRAP100 was previously shown to interact with AR and to enhance AR-dependent transcription (19). However, whether this interaction is directly through its FxxWF motif remains to be determined. Also the involvement of MED12L in AR-dependent transcription remains to be investigated.

Although SMARCA4 FxxWF and ZBTB16 FxxLF interacted with AR as peptides, no interactions were observed if these motifs were tested in large protein fragments. This suggests that in these fragments, the helical structure of the motif is disturbed or that the motif is hidden in the three-dimensional structure of the protein.

Analyzing crystal structures of the BTB-POZ domain in ZBTB16 revealed that the FxxLF motif is oriented toward the interior of this domain, which may explain why interaction was only found for the peptide. Although BTB/POZ domains are poorly conserved among the different family members, the residues N-terminally to the motif are highly conserved (44). Together with the observation that the FxxLF-like motifs are located at similar positions within the BTB/POZ domains, it seems unlikely that other members of this family do interact with AR via this domain.

Both SMARCA4/BRG1 and BAF60a are components of SWI/SNF chromatin remodeling complexes. SWI/SNF core complexes consist of the ATPase BRM or BRG1 and the subunits BAF47/INI1/SNF5, BAF155, and BAF170 (45). Other subunits commonly found include BAF53, BAF57, BAF60, BAF180, and BAF250 (46–50). Recruitment of SWI/SNF complexes is crucial for transcriptional activity by essentially all nuclear receptors (46, 51–54). More recently, it has been demonstrated that chromatin-bound AR recruits the SWI/SNF core ATPases BRM and BRG1 and requires a functional SWI/SNF complex for its transcriptional activity (16, 17, 55). Although there was a preference for the recruitment of SWI/SNF complexes containing BRM (17), *in vitro* interaction assays failed to detect a direct interaction between BRM and AR (56). Similarly, no direct interactions were observed between BRG1 and GR or estrogen receptor- α (ER α) (57, 58). Our results, which failed to detect an interaction between BRG1 and AR, are consistent with these previous observations and suggest that recruitment of SWI/SNF complexes to nuclear receptors, including AR, is not via the core ATPases but via the other subunits. Recently, it was demonstrated that BAF57 displayed hormone-independent interactions with AR and was essential for hormone-dependent AR transcriptional activity (56). Here we show that BAF60a serves as a good candidate bridging factor for a hormone-dependent interaction of SWI/SNF with AR.

The BAF60 family of proteins consists of BAF60a, BAF60b, and BAF60c, which appear to have ubiquitous expression patterns (49, 59, 60). Highest levels of BAF60b are found in pancreas and lung, whereas BAF60c is preferably expressed in brain and muscle tissue. Depletion in mice demonstrated that BAF60c was essential for development of the heart and for left-right asymmetry (61, 62). In addition, BAF60c was demonstrated to interact with the nuclear receptors ER α , peroxisome proliferator-activated receptor- γ , retinoic acid receptor, retinoid X receptor- α , farnesoid X receptor, and liver receptor homolog 1 (59, 63). Also BAF60a has been found to interact with nuclear receptors, including GR, PR, ER α , farnesoid X receptor, and peroxisome proliferator-activated receptor- γ (58). Although no interactions were observed with

vitamin D receptor and retinoid X receptor- α as individual proteins (58), BAF60a did interact with these proteins when present as heterodimer (64). Glutathione S-transferase pull-down and immunoprecipitation experiments demonstrated that BAF60a and BAF60c interacted with nuclear receptors in the absence of hormone, and interactions were not enhanced by ligand. Furthermore, it was demonstrated that BAF60a harbors two discrete protein interaction surfaces (58). An N-terminal fragment was necessary for the interaction with BRG1, GR DBD, and other nuclear receptors, whereas a C-terminal fragment was essential for interaction with the SWI/SNF core components BAF155 and BAF170. These observations are in contrast with our findings regarding AR. First, we found that interactions between BAF60a and AR were hormone dependent. Second, we found that the interactions were dependent on the BAF60a FxxFF motif and on AR groove residue K720 implying that the interaction of BAF60a with AR occurs via the coactivator groove in the LBD. And third, the FxxFF motif is present in the C-terminal fragment of BAF60a. These observations indicate a unique mode of interaction between BAF60a and AR as compared with other nuclear receptors.

Conflicting data exist on the presence of BAF60 proteins in SWI/SNF complexes. Wang *et al.* (49) showed that at least BAF60a and BAF60b are present in separate SWI/SNF complexes, whereas Lemon *et al.* (46) found BAF60a and BAF60b in the same complex. Our results showed that depletion of the different BAF60 members by siRNA had no or limited effect on AR-dependent expression of *SGK*, *SARG*, and *NDRG1* (Fig. 4, A–C), suggesting that there is redundancy between the different BAF60 members, that other subunits in the same complex determine recruitment by AR, or that expression of these genes is independent of BAF60-containing complexes. Each of the three BAF60 members was essential for optimal expression of *PSA* and *KLK2* (Fig. 4, D and E). In contrast, involvement of BAF60 members in expression of *TMPRSS2* revealed that only BAF60a was essential for expression or *TMPRSS2*, suggesting that there is little redundancy (Fig. 4F). This is the first report in which a functional difference for BAF60 proteins on different target promoters is described. Recently, it was reported that the *TMPRSS2* gene was frequently fused to members of the Ets family of transcription factors in prostate cancers (65–67). Because of the gene fusion, expression of *ERG* and other *Ets* factors is now under control of the AR-responsive *TMPRSS2* promoter, leading to aberrant androgen-regulated overexpression of *Ets* factors. Our data indicate that BAF60a may serve as a target in these types of tumors by affecting AR transcription involving the *TMPRSS2* promoter.

It is currently not known why there is a specific effect of BAF60a on *TMPRSS2* expression. AR regulates expression of genes involved in different cellular processes such as proliferation, differentiation, and cell survival. So, maybe BAF60a is essential for expression of genes involved in specific processes. However, further experiments need to be performed to clarify this.

Our results and previous observations demonstrated that SWI/SNF recruitment to AR involves at least two different BAF subunits: BAF57 and BAF60a. Similar multi-subunit interactions with SWI/SNF have also been observed for GR. Whereas BAF57 and BAF60a interacted hormone independently with GR, BAF250 was found to interact in a hormone-dependent way (47, 58). These results demonstrate that also hormone-dependent interactions are likely to be essential for the recruitment of SWI/SNF to liganded nuclear receptors. Recently, we showed that after DNA binding, AR N/C interaction is relieved and the coactivator binding groove is accessible for FxxLF-like motifs present in cofactors (37). We hypothesize that upon binding to enhancer and/or promoter regions of specific target genes, like *TMPRSS2*, AR recruits the SWI/SNF complex via BAF60a through a direct interaction between its FxxFF motif and the AR coactivator groove, and via BAF57 via a different interaction outside the AR LBD, leading to chromatin remodeling and subsequent target gene expression.

Materials and Methods

Plasmids

Mammalian expression plasmids encoding Gal4DBD-peptide fusion proteins were generated by in-frame insertion of double-stranded synthetic oligonucleotides with 5'-*Bam*HI and 3'-*Eco*RI cohesive ends into the corresponding sites of pM-B/E (25). Peptide expression constructs were sequenced to verify correct reading frames. In addition, proteins encoded by these plasmids were analyzed for size and expression by Western blotting.

pM-B was constructed by cutting pM (Takara Bio, Otsu, Shiga, Japan) with *Eco*RI after which the cohesive ends were filled up with Klenow enzyme and religated. pM-SMARCA4/BRG1 was generated by subcloning a *Hind*III fragment encoding amino acids 917–1599 of SMARCA4/BRG1 from pBJ-BRG1 (kindly provided by Dr. Gerald Crabtree, Stanford University Medical School, Stanford, CA) into the *Hind*III site of pM-B. pM-B/E-ZBTB16 was obtained by subcloning a *Bam*HI/*Eco*RI-digested PCR fragment encoding amino acid residues 2–673 of ZBTB16 into pM-B/E. PCR was performed using primer pair 5'-GATCGGATCCTC-GATCTGACAAAAATGGGCATG-3' and 5'-GATCGAAT-TCTCACACATAGCACAGGTAGAGG-3' (*Bam*HI and *Eco*RI sites in *bold*) on pSPORT6-ZBTB16 (RZPD, Berlin, Germany). pM-BAF60a was obtained by subcloning a *Bam*HI/*Xho*I fragment encoding amino acid residues 20–476 of BAF60a from pcDNA-BAF60a [kindly provided by Dr. Nick Koszewski, University of Kentucky (Louisville, KY) and described previously

(64)] into the *Bam*HI/*Sa*II sites of pM. FxxAA variant of pM-BAF60a was generated by QuikChange (Stratagene, La Jolla, Ca) using primer pair 5'-GGAAGTTCTCTTCCGCTGCTA-AGTCCTTGG-3' and 5'-CCAAGGACTTAGCAGCGGAA-GAGAACTTCC-3' (base substitutions in *bold*) according to the manufacturer's instructions. All constructs generated by PCR and QuikChange mutagenesis were verified by sequence analysis, whereas protein size and expression were determined by Western blotting.

Mammalian constructs expressing wild-type AR (pCMVAR0); the AR mutants F23L/F27L-mutated AR (pCMVF23L/F27L-AR), F23L/F27L-AR K720A (pCMVF23L/F27L-ARK720A), and F23L/F27L-AR E897A (pCMVF23L/F27L-ARE897A); rat GR; and Gal4DBD-D11 LxxLL have been described previously (24, 25, 41, 68). The expression construct for progesterone PR and the (UAS)₄TATA-Luc reporter construct were kindly provided by Dr. Leen Blok and Magda Meester (Erasmus Medical College, Rotterdam, The Netherlands), respectively.

Mammalian cell culture

Hep3B cells were cultured in α -MEM (Bio-Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) and antibiotics, whereas LNCaP cells were maintained in DMEM (Bio-Whittaker) supplemented with 5% FCS and antibiotics.

Mammalian one-hybrid assay

Hep3B cells were plated at a density of 5×10^4 cells per well of a 24-well plate and were allowed to grow for 24 h. Four hours before transfection, the medium was replaced by 250 μ l α -MEM supplemented with 5% charcoal-stripped FCS, antibiotics, and hormone or vehicle. Transfections were performed in 25 μ l α -MEM containing 1 μ l Fugene 6 (Roche Diagnostics, Mannheim, Germany); 50 ng Gal4DBD-peptide or Gal4DBD-protein expression construct (pM); 50 ng AR, PR, or GR expression construct; and 150 ng (UAS)₄TATA-Luc reporter per well. Luciferase activities were measured 24 h after transfection as described previously (25).

Western blot analysis

Hep3B cells were transfected with 250 ng Gal4DBD-peptide or Gal4DBD-protein expression construct as described above. Twenty-four hours after transfection, cells were lysed in 100 μ l Laemmli buffer (50 mM Tris, 10 mM dithiothreitol, 10% glycerol, 2% sodium dodecyl sulfate, and 0.001% bromophenol blue). Lysates were boiled and subjected to 10% SDS-PAGE gel, after which proteins were transferred to a nitrocellulose membrane. Blots were incubated with monoclonal antibodies directed against Gal4DBD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and subsequently with horseradish peroxidase-conjugated goat antimouse antibody (Dako, Glostrup, Denmark). Proteins were visualized using SuperSignal West Pico chemiluminescent blotting substrate from Pierce (Rockford, IL), followed by exposure to x-ray film.

Coimmunoprecipitation

Immunoprecipitation was performed essentially as described previously (32). In short, hormone-depleted LNCaP cells were transiently transfected with expression vectors encoding BAF60a and AR using the calcium phosphate method (69). After incubation in 1 nM R1881 or vehicle for 24 h, the cells were lysed and rotated overnight at 4 C in the presence of

antibody against AR (F39.4) or BAF60a (BD Transduction Laboratories, Lexington, KY). Next, goat antimouse agarose was added followed by rotation for 5 h. Subsequently, the beads were washed and the precipitated protein complexes were boiled in Laemmli sample buffer and subjected to 10% SDS-PAGE. Western blotting was performed using antibodies directed against AR or BAF60a as described above.

siRNA transfection

LNCaP cells were seeded in 25-cm² culture flasks in DMEM supplemented with 5% FCS. After 72 h, the medium was replaced with DMEM supplemented with 5% charcoal-stripped FCS. Four hours later, siRNAs were transfected according to the manufacturer's protocol using Dharmafect 3 (Dharmacon, Lafayette, CO) and allowed to grow for another 48 h. Cells were then treated with 1 nM R1881 or vehicle for 8 h, after which they were trypsinized and harvested. Cell pellets were stored at -80 C until RNA isolation. BAF60a, BAF60b, and BAF60c siRNAs as well as control nontargeting siRNA were obtained as pre-designed siRNA pools from Dharmacon. siRNA against AR (5'-GCAGUAUCCGAAGGCAGCA-3') was ordered as annealed double-stranded siRNA from QIAGEN (Valencia, CA).

RNA isolation, cDNA preparation, and QPCR

Analysis of AR target gene mRNA expression in absence or presence of siRNA was performed by QPCR using the SYBR Green method (Applied Biosystems, Foster City, CA). Total RNA was isolated using the RNeasy kit (QIAGEN). Synthesis of cDNA and performance of the QPCR have been described previously (65). Amounts of specific mRNAs for each sample were determined relative to porphobilinogen deaminase (*PBGD*) by the standard curve method. Primer combinations used are indicated in supplemental Table S3.

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Address all correspondence and requests for reprints to: Guido Jenster, Ph.D., Department of Urology, Josephine Nefkens Institute, Erasmus Medical College, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. E-mail: g.jenster@erasmusmc.nl.

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