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Use of a modified yeast one-hybrid screen to identify BAF60a interactions with the Vitamin D receptor heterodimer

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Abstract

A modified yeast one-hybrid screen was used to isolate proteins capable of interacting with the Vitamin D receptor (VDR) heterodimer complex while driving expression from a repressor Vitamin D response element (VDRE). Four of nine independent colonies recovered in the screen coded for full-length BAF60a, a component of the mammalian SWI/SNF complex. Deletion studies in yeast were unable to localize a unique region of BAF60a responsible for interaction with the heterodimer complex, as only the full-length protein would support reporter gene expression. Pull-down analyses revealed that BAF60a displayed strong interactions with either the unliganded or liganded heterodimer complex, but neither individual receptor component alone. Transient transfection analysis in opossum kidney (OK) cells indicated that BAF60a decreased basal transcriptional activity from the negative VDRE, but had no effect on hormone-induced repression. Transcriptional activity from an enhancer VDRE also exhibited decreased basal transcriptional activity, but also augmented hormone-dependent enhancer activity, resulting in an overall increased sensitivity to hormone. In summary, BAF60a has been identified as a factor that specifically interacts with the VDR heterodimer complex using a modified yeast one-hybrid selection strategy. This suggests that BAF60a may be a link between mammalian SWI/SNF-like chromatin remodeling complexes and the VDR heterodimer.

Keywords: Transcription; Vitamin D

1. Introduction

Great strides have been made in understanding the interaction of nuclear receptors with co-modulator proteins and their role in chromatin remodeling as a means of activating transcription of target genes [1]. A simple model has emerged for type II nuclear receptors involving the association of co-repressor molecules with the unliganded receptors to repress basal transcription. Following addition of ligand, a conformational change occurs in the nuclear receptor leading to the dissociation of co-repressor molecules and the recruitment of a variety of co-activator proteins, many of which are directly or indirectly involved in the remodeling of chromatin and leading to the activation of gene transcription. For the VDR (NR1I1, [2]), co-activators include members of the p160 family of proteins such as SRC-1, GRIP-1 and RAC-3 as well as a multi-protein complex referred to as the DRIP complex ([3] and references therein). NcoA-62 appears to be a co-modulator unique to the VDR complex and also acts as an enhancer of Vitamin D-regulated gene transcription [4]. The multi-protein PBAF complex, which is highly related to the human SWI/SNF-B complex, has been shown to be associated with the VDR heterodimer in a ligand-dependent manner to promote transactivation [5]. More recently, the chromatin remodeling multi-protein complex WINAC was shown to contain components of SWI/SNF and interact with the VDR through the Williams syndrome transcription factor in a ligand-independent manner [6]. This complex is thought to target the unliganded VDR to promoters whereupon ligand-binding results in the recruitment of requisite co-modulators.

While the above model is useful for explaining events surrounding transcriptional activation of target genes, far less

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is currently known as to how these same nuclear receptors may actively repress transcription in response to hormone in target cells. However, several recent studies highlighting Vitamin D's capacity to repress gene transcription through protein–protein interactions between the VDR complex and co-modulatory factors have been reported. Repression of the atrial-specific slow myosin heavy chain 3 gene (*slow* MyHC3) is mediated by a negative VDRE in the promoter that binds the heterodimer complex [7]. *Irx4*, a homeobox gene, is expressed in ventricular cells and mediates repression of *slow* MyHC3 in those cells by interacting with the RXR component of the heterodimer complex as it is bound to the DNA response element. Conversely, the promyelocytic leukemia zinc finger (PLZF) protein has been shown to associate with the VDR, can be found in immunoprecipitates and DNA-binding complexes formed by the receptor and when over-expressed in the U937 monocytic cell line it significantly impaired Vitamin D-dependent activation from the p21 promoter construct [8]. Using a yeast two-hybrid approach it was demonstrated that the unliganded VDR could interact with a variant of N-CoR, RIP13Δ1 [9], and addition of ligand disrupted the interaction. Another member of the N-Cor family, Alien, was also shown to interact with the unliganded VDR, and hormone addition again significantly reduced this interaction [10].

The VDR interacts as a heterodimer complex with a VDRE in the promoter region of the cPTH gene to repress transcription in just such a hormone-dependent manner [11]. Mutation of two base-pairs in the 3′ half of this element was sufficient to alter the transcriptional potential from being a repressor DNA element to a hormone-dependent enhancer [12]. Thus, it is apparent that the interface of the nuclear receptor complex and DNA response element represents a key determinant in eliciting either repression or enhancement of the transcriptional response. In the present report a modified yeast one-hybrid screen to evaluate proteins capable of interacting with the heterodimer complex as it is bound to the cPTH VDRE is described. As a result of this strategy, it was found that BAF60a, a member of the SWI/SNF complex [13,14], specifically interacted with the heterodimer complex, but not with either of the individual receptor components alone. BAF60a also had a strong influence on Vitamin D-mediated transcriptional activity from an enhancer VDRE. The data suggest that BAF60a may be a link between SWI/SNF chromatin remodeling complexes and the VDR heterodimer.

2. Materials and methods

2.1. General

All enzymes were purchased from New England BioLabs (Beverly, MA) unless otherwise specified. The human HeLa cell cDNA-hybrid library was purchased from BD Biosciences (Palo Alto, CA). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The thymidine kinase/luciferase (tk/Luc) reporter vector was a generous gift from Dr. D. Kaetzel, University of Kentucky, Lexington, KY. Synthesis of cPTH- and hOC-tk/Luc reporters was accomplished by insertion of double-stranded oligonucleotides into the *Bam*HI/*Xho*I site of the tk/Luc reporter vector and confirmed by sequence analysis. The DNA sequence of the cPTH VDRE oligonucleotide (top strand) was 5′-TCGACATGAGGGTCAGGAGGGTGTGCTGA; the hOC VDRE oligonucleotide (top strand) was 5′-TCGACCACCGGGTGAACGGGGGCATTGCA; the chicken vitellogenin II (cVitII) estrogen response element (ERE) was 5′-TCGACTTCCTGGTCAGCGTGACCGGAGCA. CMV-driven human VDR (hVDR) expression vector was a generous gift from Dr. L. Freedman, Memorial Sloan-Kettering, New York, NY. CMV human estrogen receptor α (hERα) was a generous gift from Dr. Ok-Kyong Park-Sarge, University of Kentucky, Lexington, KY. The human RXRα (hRXRα) expression plasmid was prepared from previously described vectors [15], and subcloned into the pcDNA3.1 expression vector (Invitrogen Life Technologies, Carlsbad, CA). Vitamin D hormone was kindly provided by Dr. Ron Horst, National Animal Disease Center, Ames, IA. 9-cis Retinoic acid was purchased from Sigma (St. Louis, MO). The purified anti-VDR 4707 antibody used in the Western blotting assay has been described previously [16], while the anti-RXR polyclonal antibody (Δ197) was purchased from Santa Cruz Biotech (Santa Cruz, CA). DNA sequencing of recovered pACT2 plasmids from the yeast screen was performed at the University of Kentucky Molecular Structural Analysis Facility.

The copper-inducible yeast expression vector YEphRXRα (Trp) has been described previously [17]. In similar fashion, the yeast expression vector YEphVDR (His) was generated by digestion of CMV-hVDR with the combination of *Nde*I/*Bam*HI to excise the hVDR coding region and treated with T4 DNA polymerase to generate blunt-ends. The copper-inducible yeast expression vector YEpBEP (His) [18] was digested with BamHI, made blunt-ended with T4 DNA polymerase and the ligated with the hVDR coding region fragment. The YRpcPTH and YRphOC reporter vectors (Ura) were generated by cloning single copies of oligonucleotides for the cPTH and hOC VDREs into the *Xho*I/*Bgl*II sites of YRpC3 [19]. Sequences were confirmed by dideoxynucleotide sequencing chemistry.

The yeast cell line BJ5409 (Matα, *his*3Δ200, *leu*Δ2, *trp*1, *ura*3-52, *gal*) was maintained in 1× yeast nitrogen base (YNB) without amino acids and ammonium sulfate media, supplemented with 50 g/l ammonium sulfate, 2% glucose, 20 mg/l adenine sulfate and the required amino acids. The yeast cells were transformed sequentially with reporter vector (either YRpcPTH or YRphOC), YEphVDR and YEphRXRα using lithium acetate. Expression of hVDR and hRXRα proteins was confirmed by Western blotting.

2.2. Yeast one-hybrid screen

Screening of the YRpcPTH/YEphVDR/YEphRXRα transformed BJ5409 yeast strain using the HeLa cDNA/pACT2/Gal4-AD library (Leu) was performed using lithium acetate. Transformants were spread on 150 mm plates containing 2% bacto agar, 1× YNB, 2% glucose, 20 mg/ml adenine sulfate, 60 μg/ml X-gal, 10 μM copper sulfate and 250 nM $1,25(OH)_2D_3$ (Solid Media A). Over 4–7 days colonies producing varying degrees of blue color (total of 99) were picked and grown on two different plates: Solid Media A and an analogous solid media except for the substitution of copper sulfate with 50 μM EDTA to limit expression of hVDR and hRXRα proteins from their copper-inducible promoters (Solid Media B). Those transformant colonies that exhibited copper-inducibility for β-galactosidase activity (total of 15 colonies) were grown at 30 °C in the appropriate drop-out (-His, -Leu, -Trp, -Ura) culture media that was 1× YNB, 2% glucose and 20 mg/ml adenine sulfate. The plasmid DNA was recovered from each culture and then used to individually back-transform the parental YRpcPTH/YEphVDR/YEphRXRα/BJ5409 cells using lithium acetate. The transformed yeast were grown on selective media (-His, -Leu, -Trp, -Ura) lacking copper sulfate and Vitamin D_3. Three individual colonies from each of the transformant plates were picked and assessed for β-galactosidase activity using the yeast transcription assay (see below). Based on these latter results, a total of nine individual plasmids were then sequenced.

2.3. Yeast transcription assay

Yeast transcription assays in a 96-well format were performed essentially as previously described [18]. Briefly, prototrophic yeast transformants were grown under selection to an A_{260} of 0.5–1.0. Cells were then diluted to an A_{260} of ca. 0.05 in selective media containing copper sulfate (10 μM), and with or without $1,25(OH)_2D_3$ (250 nM) in a total volume of 100 μl and incubated at 30 °C. After 24 h, A_{260} readings were taken, the cells were lysed, ONPG substrate was added, the plates incubated at 37 °C for 30 min, stop buffer added and the plates were read at A_{415}. Normalized β-galactosidase values were obtained from triplicate samples as a measure of A_{415}/A_{600}.

2.4. Deletion analysis

The 3′ and 5′ deletion mutants were obtained by PCR amplification using the recovered BAF60a/pACT2 plasmid as template. All of the 3′ deletion mutants were anchored at the 5′ end by the oligonucleotide: 5′-CTATTCGATGATGAAGATACCCCACCAAACCC. The following oligonucleotides were used to obtain the indicated 3′ deletion mutants: 1–109, 5′-GGACTCGAGAGGCCTCTTGGATATCTAGCCGT; 1–225, 5′-GGACTCGAGACATCAGTAGGACAGTACACCGT; 1–283, 5′-GGACTCGAGACTGCTGCAGGTACTTGTCACAG; 1–341, 5′-GGACTCGAGACTGGGTCTTCAAGGTGTCATCC. All of the 5′ deletion mutants were anchored at the 3′ end by the oligonucleotide: 5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT. The following oligonucleotides were used to obtain the indicated 5′ deletion mutants: 335–453, 5′-CAAGGATCCGTGACACCTTGAAGACCCAGA; 276–453, 5′-CAAGGATCCGTGACAAGTACCTGCAGCAGA; 218–453; 5′-CAAGGATCCGACGGTGTACTGTCCTACTGA; 105–453, 5′-CAAGGATCCGAGATATCCAAGAGGCCTTGA. Samples were amplified using Advantage™ Genomic Polymerase (BD Biosciences, Palo Alto, CA) and two-step cycling using an Ericomp PowerBlock I thermal cycler in 0.2 ml tubes: 94 °C/15 s, 68 °C/2 min for 30 cycles. All PCR fragments were digested with the combination of *Bam*HI/*Xho*I, gel purified, subcloned into the same sites of pAct2/Gal4-AD (BD Biosciences, Palo Alto, CA) yeast expression plasmid and sequenced. The individual deletion mutant clones were then used to transform the YRpcPTH/YEphVDR/YEphRXRα BJ5409 yeast strain using lithium acetate. Yeast were grown on solid media containing 2% bacto agar, 1× YNB, 2% glucose and 20 mg/ml adenine sulfate (-His, -Leu, -Trp, -Ura). Individual colonies were picked and grown in selective media containing 1× YNB, 2% glucose and 20 mg/ml adenine sulfate (-His, -Leu, -Trp, -Ura) and assessed for mutant protein expression by Western blotting (data not shown) with an anti-HA antibody (Zymed Laboratories, South San Francisco, CA). The deletion mutants were then analyzed for β-galactosidase activity in the yeast transcription assay.

2.5. GST pull-down analysis

The BAF60a/pAct2 clone was cut with *Xma*I to release a ca. 2.2 kb fragment. The fragment was cloned into the same corresponding site in the baculovirus pAcGHLT-A vector (BD Biosciences, Palo Alto, CA) and orientation determined by restriction digestion with *Kpn*I. Sf9 cells were co-transfected with BAF60a/pAcGHLT-A and linearized Baculogold vector (BD Biosciences, Palo Alto, CA) using Tfx20 (Promega Corp., Madison, WI). After three rounds of virus amplification, Sf9 cells were infected 1:10 with recombinant viral media and after 48 h cells were harvested and whole-cell extracts prepared as previously described [20]. Expression of GST/BAF60a was confirmed by Western blotting with an anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and compared with Sf9 cells producing GST alone (data not shown). Sf9 whole-cell extracts expressing either GST or GST/BAF60a were mixed with 0.1 volumes of a 10% Triton X-100 solution and then diluted 1:3 in PBS. This dilution was added to glutathione agarose and mixed for 30 min at 4 °C. Samples were pelleted at 2000 × *g* and washed 4× PBS followed by 1× KTEDG-150/0.1% NP-40 (150 mM KCl, 20 mM Tris, pH 7.4, 1.5 mM EDTA, 2 mM DTT, 5% glycerol). Samples were then resuspended in KTEDG-150/0.1% NP-40 containing 0.5 mM leupeptin/0.05% sodium azide and stored at

4 °C. Aliquots were removed and assessed for concentration and purity by SDS-PAGE in conjunction with Coomassie blue staining.

Recombinant hVDR- and hRXRα-containing extracts were prepared from Sf9 cells as previously described [20]. Cytosols were diluted into ice-cold buffer containing a final concentration of 80 mM NaCl, 20 mM KCl, 20 mM Tris (pH 7.5), 1.5 mM EDTA, 2 mM DTT, 0.5% CHAPS, 0.1 mM sodium vanadate, 10 mM sodium fluoride, 0.5 mM leupeptin, 5% glycerol in the presence or absence of 250 nM $1,25(OH)_2D_3$. Following a 30 min incubation on ice, aliquots for control samples were removed and denatured in SDS buffer. The remaining sample was divided and applied to glutathione-agarose matrix bound with either GST-BAF60a or GST. Samples were subsequently incubated at room temperature for 60 min. The matrices were then washed 7 ml × 1 ml with buffer (100 mM NaCl, 20 mM Tris (pH 7.5), 1.5 mM EDTA, 2 mM DTT, 0.05% Triton X-100). Samples were then re-suspended in SDS denaturing buffer and heated at 95 °C for 5 min.

2.6. Western blotting

The protein samples were separated on 10–20% gradient gels (BioWhittaker Molecular Application, Rockland, ME), transferred onto polyvinylidenefluoride membranes and blocked for 30 min at 4 °C with 1% nonfat dry milk in PBS/0.05% Tween 20. Incubation with the appropriate dilution of the primary antibody (1:3000 for VDR; 1:10,000 for RXR blots) was continued in the same buffer overnight with gentle agitation. The blots were then washed 3 × 10 min with PBS/Tween and then incubated with horseradish peroxidase-linked secondary antibodies (1:10,000 dilution). Following three washes as described above, the blotted proteins were revealed by chemiluminescent detection (Pierce Biotechnology, Rockford, IL).

2.7. Transient transfection

Opossum kidney (OK) cells (American Type Culture Collection, CRL-1840) were maintained in Dulbecco's modified Eagle's media/F-12 (1:1) with 10% charcoal-stripped fetal bovine serum containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C. Cells were plated in 24-well plates and transfected with the appropriate VDRE/tk-Luc reporter construct (100 ng), CMV-β-galactosidase expression vector (20 ng), CMV-hVDR expression vector (0.5 ng), CMV-hRXRα expression vector (0.5 ng) and the indicated variable amounts of CMV-BAF60a expression vector made up to 500 ng total DNA per well with pTZ19R carrier plasmid DNA. For comparison with estrogen-regulated gene transcription, transfection was similarly carried out with the cVitII ERE/tk-Luc construct (100 ng), CMV-β-galactosidase expression vector (20 ng), CMV-ERα expression vector (1.0 ng) and the indicated variable amounts of CMV-BAF60a expression vector and pTZ19R in media lacking phenol red. The pcDNA3.1 mammalian expression vector was included in all transfections as needed to keep total amounts of transfected expression vectors the same. Cells were transfected in triplicate using lipofectamine (1.5 μl per well) and Plus reagent (1.5 μl per well) for 3 h in media lacking serum, followed by supplementation to 1% serum and treatment with or without 50 nM hormone for 42 h. Cell lysates were prepared by washing the cells with phosphate-buffered saline solution (3×), followed by overlaying with lysis buffer (Promega Corp., Madison, WI) and three rounds of freeze-thawing. Luciferase activity was determined and normalized with respect to values for β-galactosidase enzymatic activity. Average values were calculated from triplicate treatments ± S.E.M. and are reflective of two to three independent experiments.

3. Results

A means of screening cDNA libraries for interacting proteins that utilized full-length nuclear receptors to drive expression from enhancer-linked reporters in yeast has been described [17,21]. Because of the relative importance associated with the sequence of the cPTH VDRE, it seemed that a modification might be adapted to screen for proteins capable of interacting with the VDR complex bound to a repressor DNA element. It was reasoned that expressing full-length hVDR and its heterodimer partner, hRXRα, would necessarily result in either repression or an inability to enhance transcription of a β-galactosidase reporter driven by the cPTH VDRE in yeast (Fig. 1a). Indeed, no activity above baseline was observed when the cPTH/β-galactosidase reporter was expressed in BJ5409 yeast cells expressing full-length hVDR/hRXRα proteins, while a significant increase in enzyme activity was observed when the analogous β-galactosidase reporter linked to the human osteocalcin enhancer VDRE was evaluated in combination with the expression of both receptors (Fig. 1b). Based on this result, it seemed plausible that a hybrid cDNA library of proteins fused to the Gal4 activation domain (Gal4AD) would provide a means of screening for interacting proteins that would be capable of eliciting an enhancement of β-galactosidase gene expression from the cPTH VDRE. In this scenario, hybrid proteins interacting with the hVDR/hRXRα complex bound to the cPTH VDRE might overcome the necessarily repressive potential of the complex and activate transcription of the β-galactosidase gene (Fig. 1). It was anticipated that there would be low background in this colorimetric selection screen because of the inherent repressive potential of the heterodimer complex-binding to the cPTH VDRE.

This modified one-hybrid strategy was then utilized to screen a cDNA-Gal4AD library derived from HeLa cells with the hVDR/hRXRα complex driving β-galactosidase gene expression from the cPTH VDRE in BJ5409 yeast cells.

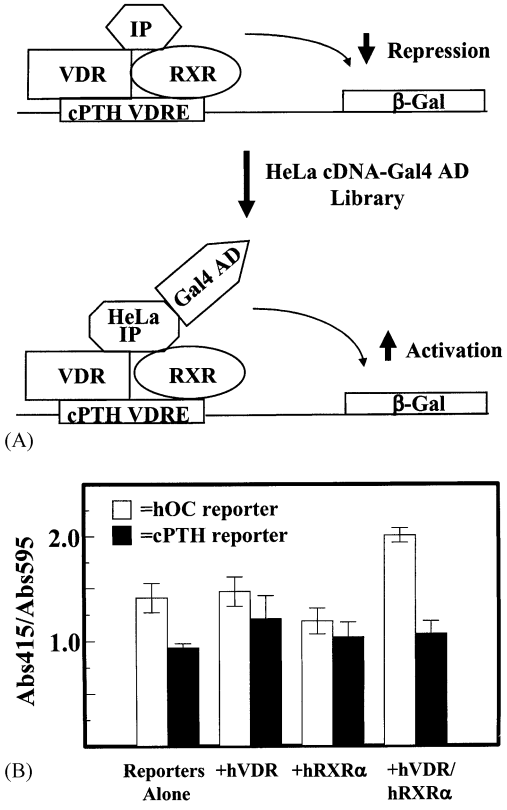

Fig. 1. (A) Schematic showing the strategy employed to screen proteins capable of interacting with the hVDR/hRXRα complex bound to the cPTH VDRE. (B) Assessment of the transcriptional potential of the hOC and cPTH VDREs in yeast cells. BJ5409 yeast were transformed with the indicated reporter plasmids alone or in the presence of the indicated receptor expression plasmids. Individual clones were grown in the presence of calcitriol and assessed for the ability to produce β-galactosidase activity.

Over 180,000 transformed yeast clones were obtained from the initial screen, of which 99 colonies of varying degrees of blue color were observed and picked for further analysis. Secondary screening on the same selection plates quickly reduced the number of potential clones to less than twenty. Plasmid DNA harboring the hybrid proteins was recovered by nutritional selection and used to retransform the parental hVDR/hRXRα/cPTH/BJ5409 reporter yeast strain. Three individual colonies from each transformation were randomly selected for growth in liquid culture and β-galactosidase activity quantitated by assessing the conversion of ONPG substrate (Fig. 2). Following this analysis a total of nine individual clones remained that were capable of conferring increased β-galactosidase enzymatic activity compared to the parental strain. These plasmids were subjected to sequencing analysis and a BLAST search indicated that four of the clones coded for the same protein, BAF60a or SMARCD1 [13,14]. Based on PCR analysis and restriction enzyme digestion, three of these clones coded for a full-length 453 amino acid isoform of BAF60a (GeneBank accession AF109733), while the fourth BAF60a clone coded for a smaller splice variant of this protein (GeneBank accession NM_003076). The particular full-length BAF60a fusion

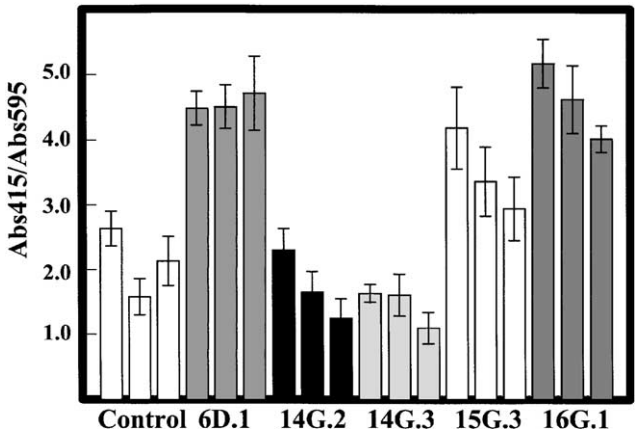

Fig. 2. Quantitative assessment of β-galactosidase activity for recovered plasmids from the HeLa cell cDNA hybrid library screen following retransformation of parental BJ5409 yeast expressing hVDR/hRXRα/cPTH VDRE reporter. Three individual colonies were picked from each retransformation and β-galactosidase activity assessed as described in Materials and Methods in the presence of calcitriol. Five representative retransformed plasmids are shown relative to the parental strain control. The sample labeled '15G.3' was one of the four independent recovered plasmids that coded for full-length BAF60a.

clone used in the following experiments in yeast included an additional 4 amino acids (PRPG) at the amino terminus resulting from in-frame translation of 12 bp of 5′ untranslated mRNA, while the splice variant form was not utilized.

To determine the hormone-dependence of the interaction of the VDR complex with the BAF60a fusion protein, a yeast transcription assay was utilized [18]. Three independent colonies of the yeast expressing BAF60a/hVDR/hRXRα/cPTH-β-galactosidase reporter were randomly selected and grown in liquid culture treated with or without hormone for 24 h. As seen in Fig. 3, there

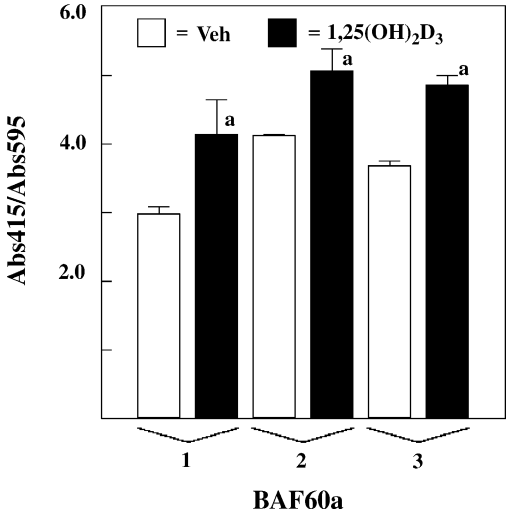

Fig. 3. Hormone dependence of the BAF60a interaction with the parental BJ5409 yeast expressing hVDR/hRXRα/cPTH VDRE reporter. Three individual colonies were picked and grown in the presence and absence of calcitriol and assessed for the ability to produce β-galactosidase activity. [a]Significantly different than vehicle, $P < 0.05$.

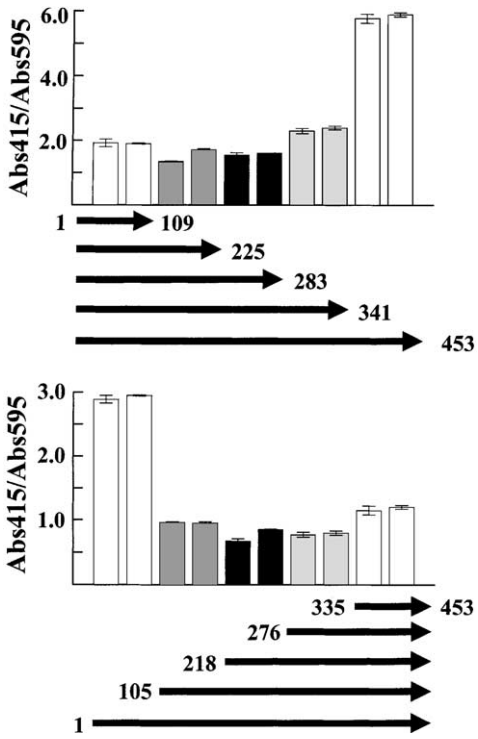

Fig. 4. Deletion analysis of BAF60a interaction with parental yeast expressing hVDR/hRXRα/cPTH VDRE reporter plasmids. PCR was used to amplify coding regions of BAF60a truncated from the C-terminal (top panel) or N-terminal (bottom panel) portion of the molecule. Following PCR the DNA fragments were subcloned in-frame into pACT2 yeast expression vector and transformed into the parental yeast strain. Two individual colonies from each transformation were picked and assessed for β-galactosidase activity in the presence of calcitriol.

was a small increase in β-galactosidase activity in the presence of hormone for each of the tested colonies, approaching a maximum of 40% in the case of sample 1. Thus, both the unliganded and liganded VDR complex appeared capable of interacting with BAF60a using the cPTH-β-galactosidase reporter in yeast, with hormone providing a modest increase in measurable enzyme activity.

An inspection of the protein sequence of BAF60a revealed several potential protein–protein interaction motifs, including two LXXLL motifs in the central portion of the molecule. To determine which regions of BAF60a might be involved in the interactions with the hVDR/hRXRα complex, a series of DNA fragments were generated by PCR that spanned various regions of the 453 amino acid protein using primers anchored from either the N- or C-termini. These PCR fragments were cloned in-frame into the pACT2/Gal4-AD vector to create analogous Gal4-AD fusion products and their expression in yeast verified by Western blotting with an anti-HA antiserum (data not shown). β-Galactosidase enzyme activity was then assessed from two independent yeast clones randomly selected from each of the transformations (Fig. 4). Interestingly, none of the truncated BAF60a proteins anchored from either the N- or C-terminal regions exhibited β-galactosidase enzymatic activities comparable to the results obtained with the full-length protein. Based on this result, it would appear that the intact BAF60a molecule is required to form an interaction with the heterodimer complex and no truncated region of the protein by itself is capable of reconstituting the wild-type activity.

The BAF60a cDNA was then excised and cloned in-frame into a baculovirus-derived GST-expression vector, the protein expressed in Sf9 insect cells and purified by affinity chromatography with glutathione-agarose. This material was then used in pull-down experiments with recombinant hVDR- and hRXRα-containing extracts also prepared from Sf9 insect cells [15,20]. Unexpectedly, no specific interaction was observed when either hVDR- or hRXRα-containing extracts alone were used in separate experiments (Fig. 5). Instead, strong interactions with BAF60a were only observed when combined hVDR or hRXRα extracts were used in the pull-down assay. The results also indicated that this interaction of the combined extracts with BAF60a was independent of the presence or absence of the hormonal form of Vitamin D.

The effects of BAF60a expression on Vitamin D-regulated transcription in mammalian cells were then examined. OK cells have previously been used in the laboratory to examine the effects on Vitamin D-dependent transcription for both repressor and enhancer DNA elements [11,22]. Initially, the effects of BAF60a expression were evaluated using the cPTH-tk/Luc reporter in OK cells. As observed in Fig. 6A, increased expression of BAF60a caused a dose-dependent decrease in basal expression from the reporter gene, but had essentially no effect on the actual hormone-dependent repressive response itself. Thus, because the overall basal activity declined with increasing BAF60a expression, it follows that the degree of hormone-dependent repression, that is the difference between the basal and hormone-dependent activity observed in these cells, also became less pronounced.

The effects on gene activation were also assessed using an analogous reporter construct driven by the human osteocalcin VDRE (hOC-tk/Luc), a well-characterized Vitamin D-dependent enhancer of transcriptional activity [23,24]. As seen in Fig. 6B, titration of BAF60a expression resulted in both a modest decrease in basal expression of luciferase activity while at the same time producing an nominal increase in hormone-dependent activity. The net effect on the Vitamin D-dependent enhancer element was to increase the hormone-dependent sensitivity of the response from two-fold in the absence of BAF60a to ca. three-fold at the highest level of BAF60a expression.

As a further comparison, analogous experiments were performed utilizing an estrogen response element enhancer linked reporter, together with an expression vector for the hERα (Fig. 6C). Treatment with estrogen resulted in a ca. two-fold increase in transcriptional activity in the OK cells. Increasing co-transfection with the BAF60a expression vector resulted in a gradual decline in basal activity; however, unlike the situation with the Vitamin D enhancer there was

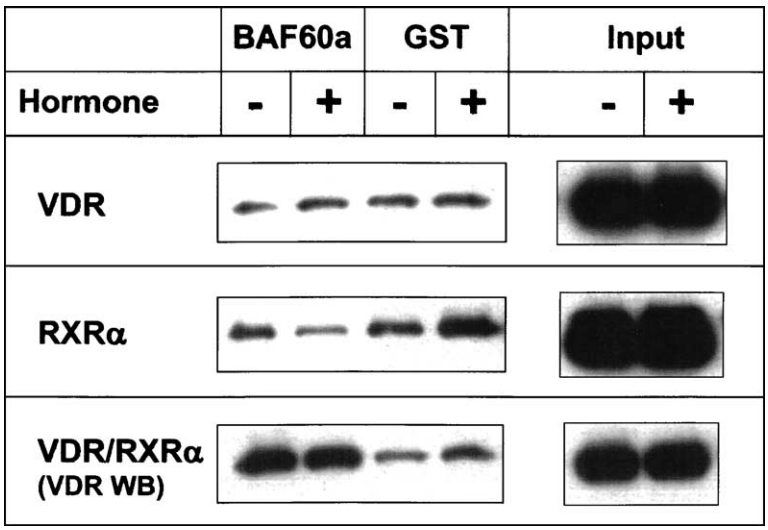

Fig. 5. GST pull-down analysis of BAF60a interactions with hVDR and hRXRα. GST-BAF60a was purified on glutathione agarose and incubated with the indicated recombinant receptor extracts obtained from baculovirus infection of Sf9 insect cells. Experiments were performed either in the presence or absence of hormone; calcitriol for the VDR-containing incubations or 9-cis retinoic acid for RXRα alone. Western blots were analyzed for either VDR or RXRα proteins (ca. 33% of the recovered pull-down material) relative to control blots corresponding to ca. 5% of initial input for the pull-down experiment.

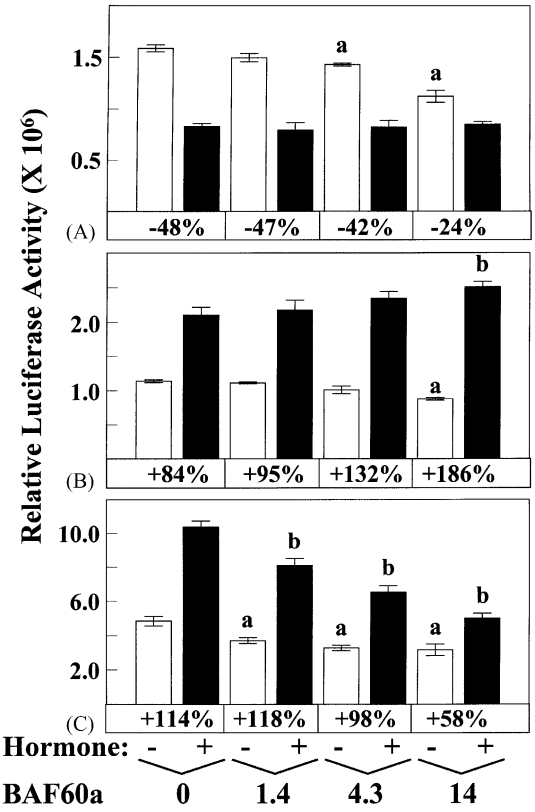

Fig. 6. Transient transfection analysis in OK cells. OK cells were transfected with a fixed amount of hVDR/hRXRα expression vectors together with either cPTH-tk/Luc (A) or hOC-tk/Luc (B) reporters and the indicated amounts of BAF60a expression vector (ng per well). In panel (C), a fixed amount of ERα expression vector was transfected with an ERE-tk/Luc reporter and the indicated amounts of BAF60a expression vector. Percentages reflect the hormone-dependent change from corresponding vehicle treatment. [a]Significantly different from vehicle treatment in the absence of BAF60a expression vector, $P < 0.05$; [b]significantly different from hormone treatment in the absence of BAF60a expression vector, $P < 0.05$.

a roughly parallel decline in hormone-dependent transcription up until the highest amount of co-transfected BAF60a. As such, the estrogen-dependent enhancer activity generally declined throughout the BAF60a co-transfection experiment and an overall decrease in sensitivity to hormone was observed, quite opposite to the results seen with the Vitamin D enhancer in the above experiments. This implies that BAF60a exerts some level of specificity in the interaction with different nuclear receptor complexes under these conditions.

4. Discussion

The mammalian SWI/SNF nuclear complex is composed of multiple subunits that are intimately involved in regulating gene transcriptional events. Distinct SWI/SNF complexes have been noted [13], with certain subunits present in both types of complexes. Nuclear receptors have been shown to interact with components of the SWI/SNF complex [25,26]. More recently, PBAF, a multi-protein complex that is highly related to the human SWI/SNF-B complex, was shown to be associated with the VDR heterodimer complex [5]. Another chromatin remodeling complex, WINAC, that contains elements of SWI/SNF was also shown to interact with the VDR [6]. Although the SWI/SNF complex has largely been linked with chromatin remodeling leading to the activation of gene transcription, recent reports have also described its role in repressing gene activity in yeast [27].

BAF60 is a component of the mammalian SWI/SNF complex and three different isoforms have been identified, BAF60a, BAF60b and BAF60c [14]. BAF60a is thought to be ubiquitously expressed, while the other two forms

exhibit more restricted tissue expression patterns. BAF60a, or its yeast homologue Swp73p, have been implicated in interactions with the glucocorticoid receptor [13,28] and AP-1 [29]. Its presence was noted in the PBAF complex interacting with the VDR heterodimer [5], and it was also observed in the WINAC complex that interacts with the VDR [6]. In this latter case, the association of the VDR with WINAC was determined to be ligand-independent, similar to the present observations with BAF60a and the VDR heterodimer. Thus, the present data appears to support the previous findings and extend them to identify BAF60a as a possible link between various chromatin remodeling complexes and the VDR heterodimer.

Unlike the situation described for other co-modulator proteins in which a peptide region can be identified as playing a prominent role in protein–protein contacts, BAF60a interactions with the hVDR/hRXRα heterodimer would appear to require an intact molecule. This suggests a more complex role than a singular protein–protein interaction interface, and might be explained by a complex structural interplay of BAF60a with the heterodimer complex to activate gene transcription in the yeast screen. Previous work suggests that Rsc6, a yeast homologue of Swp73p, is part of the functional core of the SWI/SNF-related complexes [30]. The present data suggest a similar role for BAF60a, in which the three-dimensional structure of BAF60a could be critical to the overall integrity of the interaction between the VDR heterodimer and the core of such multi-protein complexes as PBAF or WINAC.

Direct interactions between BAF60a and the heterodimer complex were observed in the pull-down experiments, in contrast to the lack of binding observed between BAF60a and the individual members of the heterodimer when they were assessed in the analogous experiments. The BAF60a-VDR heterodimer interaction also appeared to be largely independent of hormone, both in yeast where only a modest increase in β-galactosidase activity was observed, as well as in the pull-down experiments. Increasing amounts of transfected BAF60a had its most profound effect on basal activity from the cPTH VDRE reporter construct, with no changes in the absolute repressor activity observed in response to hormone. Collectively, this would be consistent with the aforementioned role of BAF60a as a part of some core complex, that when over-expressed can promote association with the unliganded VDR complex leading to a reduction in basal activity. However, it is unclear if this observation represents a specific interaction because there was a general decline in basal activity across all the various reporters evaluated in the transfection analysis.

The most significant effect on transcriptional activity occurred when BAF60a was included in transient transfections utilizing an enhancer hOC VDRE reporter construct. This was observed both on the basal activity in the absence of hormone, whereby levels fell in response to increased levels of BAF60a, while at the same time there was an increased response in hormone-dependent enhancer activity. The net effect was to improve the overall sensitivity of the response to hormone from the hOC VDRE. The PBAF complex was shown to promote ligand-dependent gene transactivation by the VDR heterodimer complex [5], thus the ability of increasing amounts of transfected BAF60a to enhance transcription from the hOC VDRE reporter construct would be consistent with the prior report. The lack of strong hormone dependence for the interaction of BAF60a with the VDR heterodimer in the present study, however, would suggest that other components of PBAF might constitute the ligand-dependent trigger leading to transactivation.

It was previously noted that the polarity of the VDR heterodimer complex binding to the cPTH VDRE, with VDR occupying the 5′ half-site and RXR the 3′ position [12], was opposite relative to the alignment of the heterodimer bound to the hOC enhancer element [31]. Therefore, the question arises as to how BAF60a can recognize the VDR complex bound to either the repressor or enhancer elements when they are oriented differently with regards to the polarity of binding by the respective receptors. However, it should be stressed that the cross-linking studies used to assess the polarity of binding to the cPTH VDRE were not absolute in assigning orientation of the respective receptors. The experiments indicated that a fraction of the cross-linked heterodimer complex was aligned similarly to the hOC VDRE, that is, with RXR occupying the 5′ half-site and VDR the 3′ half-site. Thus, the recovery of BAF60a in the modified yeast one-hybrid screen may be reflective of that portion of the heterodimer complex binding to the cPTH VDRE in the altered polarity, i.e. as if bound to an enhancer element. This would also be consistent with the empirical observation of a relatively prolonged time for color development when individual yeast colonies containing BAF60a with the hVDR/hRXRα/cPTH-β-galactosidase vectors were grown on solid media. In the meantime, the remaining five clones recovered from the yeast screen are currently being assessed for their impact on both Vitamin D-dependent transcriptional repression and enhancement activities.

In summary, the present work outlines another approach to identifying protein co-factors that are involved in nuclear receptor transcription. It has also added BAF60a to the growing list of proteins capable of interacting with the VDR, and suggests that it may be a link between SWI/SNF-like complexes such as PBAF and WINAC, which have been previously shown to be capable of mediating Vitamin D-dependent transactivation, and the VDR heterodimer.

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References

[1] A. Aranda, A. Pascual, Nuclear hormone receptors and gene expression, Physiol. Rev. 81 (2001) 1269–1304.

[2] N.R.N. Committee, A unified nomenclature system for the nuclear receptor superfamily, Cell 97 (1999) 161–163 (letter).

[3] C. Rachez, L.P. Freedman, Mechanisms of gene regulation by Vitamin D(3) receptor: a network of coactivator interactions, Gene 246 (2000) 9–21.

[4] T.A. Baudino, D.M. Kraichely, S.C. Jefcoat Jr., S.K. Winchester, N.C. Partridge, P.N. MacDonald, Isolation and characterization of a novel coactivator protein, NCoA-62, involved in Vitamin D-mediated transcription, J. Biol. Chem. 273 (1998) 16434–16441.

[5] B. Lemon, C. Inouye, D.S. King, R. Tjian, Selectivity of chromatin-remodelling cofactors for ligand-activated transcription, Nature 414 (2001) 924–928.

[6] H. Kitagawa, R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, S. Kato, The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome, Cell 113 (2003) 905–917.

[7] G.F. Wang, W. Nikovits Jr., Z.Z. Bao, F.E. Stockdale, Irx4 forms an inhibitory complex with the Vitamin D and retinoic X receptors to regulate cardiac chamber-specific slow MyHC3 expression, J. Biol. Chem. 276 (2001) 28835–28841.

[8] J.O. Ward, M.J. McConnell, G.W. Carlile, P.P. Pandolfi, J.D. Licht, L.P. Freedman, The acute promyelocytic leukemia-associated protein, promyelocytic leukemia zinc finger, regulates 1,25-dihydroxyVitamin D(3)-induced monocytic differentiation of U937 cells through a physical interaction with Vitamin D(3) receptor, Blood 98 (2001) 3290–3300.

[9] P.P. Dwivedi, G.E. Muscat, P.J. Bailey, J.L. Omdahl, B.K. May, Repression of basal transcription by Vitamin D receptor: evidence for interaction of unliganded Vitamin D receptor with two receptor interaction domains in RIP13delta1, J. Mol. Endocrinol. 20 (1998) 327–335.

[10] P. Polly, M. Herdick, U. Moehren, A. Baniahmad, T. Heinzel, C. Carlberg, VDR-Alien: a novel, DNA-selective Vitamin D(3) receptor-corepressor partnership, FASEB J. 14 (2000) 1455–1463.

[11] S.M. Liu, N. Koszewski, M. Lupez, H.H. Malluche, A. Olivera, J. Russell, Characterization of a response element in the 5′-flanking region of the avian (chicken) PTH gene that mediates negative regulation of gene transcription by 1,25-dihydroxyVitamin D3 and binds the Vitamin D3 receptor, Mol. Endocrinol. 10 (1996) 206–215.

[12] N.J. Koszewski, S. Ashok, J. Russell, Turning a negative into a positive: Vitamin D receptor interactions with the avian parathyroid hormone response element, Mol. Endocrinol. 13 (1999) 455–465.

[13] W. Wang, Y. Xue, S. Zhou, A. Kuo, B.R. Cairns, G.R. Crabtree, Diversity and specialization of mammalian SWI/SNF complexes, Genes Dev. 10 (1996) 2117–2130.

[14] H.Z. Ring, V. Vameghi-Meyers, W. Wang, G.R. Crabtree, U. Francke, Five SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARC) genes are dispersed in the human genome, Genomics 51 (1998) 140–143.

[15] R.L. Horst, T.A. Reinhardt, J.P. Goff, N.J. Koszewski, J.L. Napoli, 9,13-Di-cis-retinoic acid is the major circulationg geometric isomer of retinoic acid in the periparturient period, Arch. Biochem. Biophys. 322 (1995) 235–239.

[16] M.C. Langub, T.A. Reinhardt, R.L. Horst, H.H. Malluche, N.J. Koszewski, Characterization of Vitamin D receptor immunoreactivity in human bone cells, Bone 27 (2000) 383–387.

[17] K.W. Henry, X. Yuan, N.J. Koszewski, H. Onda, D.J. Kwiatkowski, D.J. Noonan, Tuberous sclerosis gene 2 product modulates transcription mediated by steroid hormone receptor family members, J. Biol. Chem. 273 (1998) 20535–20539.

[18] K. Henry, M.L. O'Brien, W. Clevenger, L. Jow, D.J. Noonan, Peroxisome proliferator-activated receptor response specificities as defined in yeast and mammalian cell transcription assays, Toxicol. Appl. Pharmacol. 132 (1995) 317–324.

[19] T.A. Pham, Y.P. Hwung, D.P. McDonnell, B.W. O'Malley, Transactivation functions facilitate the disruption of chromatin structure by estrogen receptor derivatives in vivo, J. Biol. Chem. 266 (1991) 18179–18187.

[20] N.J. Koszewski, T.A. Reinhardt, R.L. Horst, Vitamin D receptor interactions with the murine osteopontin response element, J. Steroid Biochem. Mol. Biol. 59 (1996) 377–388.

[21] H. Chen, R.J. Lin, R.L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M.L. Privalsky, Y. Nakatani, R.M. Evans, Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300, Cell 90 (1997) 569–580.

[22] N.J. Koszewski, S. Kiessling, H.H. Malluche, Isolation of genomic DNA sequences that bind Vitamin D receptor complexes, Biochem. Biophys. Res. Commun. 283 (2001) 188–194.

[23] N.J. Koszewski, H.H. Malluche, J. Russell, Vitamin D receptor interactions with positive and negative DNA response elements: an interference footprint comparison, J. Steroid Biochem. Mol. Biol. 72 (2000) 125–132.

[24] K. Ozono, J. Liao, S.A. Kerner, R.A. Scott, J.W. Pike, The Vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer, J. Biol. Chem. 265 (1990) 21881–21888.

[25] H. Inoue, T. Furukawa, S. Giannakopoulos, S. Zhou, D.S. King, N. Tanese, Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors, J. Biol. Chem. 277 (2002) 41674–41685.

[26] B. Belandia, R.L. Orford, H.C. Hurst, M.G. Parker, Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes, EMBO J. 21 (2002) 4094–4103.

[27] J.A. Martens, F. Winston, Evidence that Swi/Snf directly represses transcription in *S. cerevisiae*, Genes Dev. 16 (2002) 2231–2236.

[28] B.R. Cairns, R.S. Levinson, K.R. Yamamoto, R.D. Kornberg, Essential role of Swp73p in the function of yeast Swi/Snf complex, Genes Dev. 10 (1996) 2131–2144.

[29] T. Ito, M. Yamauchi, M. Nishina, N. Yamamichi, T. Mizutani, M. Ui, M. Murakami, H. Iba, Identification of SWI.SNF complex subunit BAF60a as a determinant of the transactivation potential of Fos/Jun dimers, J. Biol. Chem. 276 (2001) 2852–2857.

[30] I. Treich, L. Ho, M. Carlson, Direct interaction between Rsc6 and Rsc8/Swh3, two proteins that are conserved in SWI/SNF-related complexes, Nucleic Acids Res. 26 (1998) 3739–3745.

[31] C.H. Jin, J.W. Pike, Human Vitamin D receptor-dependent transactivation in *Saccharomyces cerevisiae* requires retinoid X receptor, Mol. Endocrinol. 10 (1996) 196–205.