Journal of Cellular Biochemistry 69:1–12 (1998)

ARTICLES

L7 Protein Is a Coregulator of Vitamin D Receptor-Retinoid X Receptor-Mediated Transactivation

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Abstract The vitamin D receptor (VDR) heterodimerizes with the retinoid X receptor (RXR) and requires additional protein-protein interactions to regulate the expression of target genes. Using the yeast two-hybrid system, we identified the previously described protein L7, that specifically interacted with the VDR in the presence of vitamin D. Deletion analysis indicated, that the N-terminus of L7, which harbours a basic region leucine zipper like domain, mediated interaction with the VDR. Binding assays with purified GST-L7 demonstrated, that L7 specifically pulled down the VDR, that was either expressed in yeast or endogenously contained in the cell line U937. Interestingly, L7 inhibited ligand-dependent VDR-RXR heterodimerization, when constitutively expressed in yeast. We also demonstrate that L7 repressed binding of VDR-RXR heterodimers to a vitamin D response element. Surprisingly, L7 recruited RXR to the same response element in the presence of 9-cis retinoic acid. Ligand-dependent protein-protein interaction in the yeast two-hybrid system confirmed, that binding of L7 also was targeted at the RXR. Our data suggest, that protein L7 is a coregulator of VDR-RXR mediated transactivation of genes, that modulates transcriptional activity by interfering with binding of the receptors to genomic enhancer elements. J. Cell. Biochem. 69:1–12, 1998.

Key words: two-hybrid system; vitamin D receptor; retinoid X receptor; vitamin D; protein L7; basic region leucine zipper domain; coregulation

The vitamin D receptor (VDR) belongs to the group of ligand inducible steroid hormone receptors, that bind as homodimers or as heterodimers to specific DNA elements and activate transcription of various target genes involved in cell proliferation, differentiation, and homeostasis [Beato et al., 1995; Mangelsdorf et al., 1995]. Binding of ligand(s) nevertheless is a prerequisite for the formation of active dimers and transactivation and involves basically an allosteric change in the carboxyterminal activation function AF-2 of nuclear factors [Danielian et al., 1992; Barettino et al., 1994; Bourguet et al., 1995]. Additionally, coactivators, which associate with the nuclear receptors only in the presence of ligand(s), serve as bridging molecules between the receptors and the basal transcription maschinery and thus are involved in transactivation [Le Douarin et al., 1995; Onate et al., 1995; Voegel et al., 1996; Hong et al., 1997; Torchia et al., 1997]. In the absence of ligand(s) cytoplasmic proteins [Pratt, 1993] or nuclear factors which bind preferentially to the hinge region between DNA binding domain and activation domain of several steroid hormone receptors mediate the silencing of basal gene transcription [Chen and Evans, 1995; Hörlein et al., 1995; Perlmann and Vennström, 1995; Zamir et al., 1997]. Upon ligand binding dissociation of these co-repres-

Abbreviations: AD, activating domain; bZip, basic region leucine zipper; DBD, DNA-binding domain; dIdC, polydeoxyinosinic-deoxycytidylic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]); IPTG, isopropyl β-D-thiogalactopyranoside; LBD, ligand-binding domain; MBP, maltose binding protein; ONPG, *o*-nitrophenyl-β-D-galactoside; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; vitamin D, 1,25-dihydroxyvitamin D3; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D galactoside.

Contract grant sponsors: Schering AG, Deutsches Bundesministerium für Bildung, Wissenschaft Forschung und Technologie; Contract grant number: FKZ 0310781.

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Received 15 September 1997; Accepted 20 October 1997

sors and recruitment of co-activators will be induced. Recently, a ligand independent transcriptional repressor was described, which inhibits transactivation by the thyroid and retinoic acid receptors (TR and RAR) by interfering with their DNA binding activity [Burris et al., 1995]. In contrast to the other co-repressors, it is not only located in the nucleus, but also contributes to the ribosomal structure in the cytoplasmic compartment. These findings demonstrate, that transcriptional activation of genes is a complex process, which not only involves the differential control of protein-protein interactions between general transcription factors of the RNA-polymerase II holoenzyme and nuclear receptors by specific nuclear cofactors [Ing et al., 1992; Baniahmad et al., 1993; Blanco et al., 1995]. Moreover, it is obvious that the activity of nuclear receptors is determined by a yet to defined interaction with a new class of receptor binding proteins. In this study, we present a novel VDR binding cofactor, which binds to the receptor in the presence of the natural ligand vitamin D (1,25-dihydroxyvitamin D3). It is the previously described protein L7, which has been shown to play a role in translational regulation as a component of 80S ribosomes [Seshadri et al., 1993; Hemmerich et al., 1993]. In contrast to ligand-dependent co-activators, L7 represses VDR mediated transactivation in yeast and interferes with binding of VDR-RXR heterodimers to DNA. Furthermore we provide evidence for a general influence of the L7 cofactor on receptor controlled transcriptional regulation.

MATERIALS AND METHODS

Materials

Vitamin D (1,25 Dihydroxyvitamin D3) was obtained from Solvay-Duphar (Da Weesp, The Netherlands).The ligand 9-*cis*-retinoic acid was obtained from Biomol (Hamburg, Germany). The anti-vitamin D receptor antibody (9A7) was from Dianova (Hamburg, Germany). The anti-GST-antibody was purchased from Pharmacia (Freiburg, Germany). The isotop [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml) was obtained from Amersham Life Science (Braunschweig, Germany). Primers were purchased from MWG Biotech (Ebersberg, Germany). The human skeletal muscle cDNA library was from Clontech Laboratories (Heidelberg, Germany).

Construction of Plasmids

The expression plasmid pGBT9-RXRα (Gal4-DNA-binding domain-hRXRα 1-462) was constructed by inserting an *Eco*RI/*Xho*I fragment of the entire cDNA of RXRα into *Eco*RI/*Sal*I digested pGBT9 (Clontech Laboratories, Heidelberg, Germany). The RXRα-cDNA [Mangelsdorf et al., 1990] was amplified by PCR using the following primer pair: 5'-TAGTCGAATTCATG-GACACCAAACATT-3' and 5'-GGACACTC-GAGAGGCTGAGAAGAACAG-3'. The plasmids pGBT9-VDR, pGBT9-VDR-DBD(124), pGAD424-RXRβ, and pGBT9-VDR-LBD(298) have been previously described [Berghöfer-Hochheimer et al., 1997]. The expression vector Yepubstu-VDR containing a copper responsive yeast metallothionein promoter was constructed by inserting the entire cDNA of the VDR [Baker et al., 1988] into *Stu*I/*Kpn*I digested Yepubstu (a gift from R. Kölling). The VDR-cDNA was amplified by PCR using the following primer pair: 5'-TATGGCG-GCCAGCACTTCCCTG-3' and 5'-GGCCAGG-TACCCACCAAGGCTCTTG-3'. To construct the maltose binding protein-VDR fusion MBP-VDR, the *Eco*RI/*Bam*HI VDR-insert of pGBT9-VDR was inserted into *Eco*RI/*Bam*HI digested pMAL-c2 (Biolabs, Schwalbach/ Taunus, Germany). To construct pGAD10-L7, pGAD10-L7 derived from the cDNA library was digested with *Pst*I/*Xho*I, the restriction sites filled-in with Klenow-fragment and religated. To obtain pTM175-L7, L7 cDNA was amplified with the following primers: 5'-CGGCCGAATTCATG-GAGGGTGTAGAAG-3' and 5'-ATAATCATC-GATC ACACCTTAGTTCATTC-3' and the PCR product inserted into *Eco*RI/*Cla*I digested pTM175. In pTM175 [Shnyreva and Munder, 1996] transcription was driven by the constitutive yeast glyceraldehyde 3-phosphate dehydrogenase promoter. To obtain a fusion between the glutathione-S-transferase (GST) and the RXRα, GST-RXR, the *Eco*RI/*Xho*I fragment of the RXR shown above was inserted into *Eco*RI/*Xho*I digested pGEX-5X1 (Pharmacia, Freiburg, Germany). To obtain GST-L7bZip (321 bp), L7 cDNA was amplified with the following primers: 5'-GCGGCCGGATCCCCATGGAGGGTG-TAGAAG-3' and 5'-GCTCACTCGAGTGATACC-TCTGATTC-3'. The PCR product was digested with *Bam*HI/*Xho*I and inserted into the likewise digested pGEX-5X-1. Restriction sites in primers are underlined. All cDNAs were amplified by PCR as follows: Template DNA was denaturated at

94°C for 5 min prior to amplification (30 cycles). Cycle conditions were: Denaturation at 94°C for 30 s, annealing for 1 min according to the melting points of the primers (between 55°C and 65°C), polymerisation at 72°C for 2 min. The standard $MgCl_2$ concentration was used for amplification with *Pwo*-polymerase (Boehringer-Mannheim, Germany). The plasmids pGAD-GH-S18 and pTM175–S18 were kindly provided by M. Shnyreva. Sequences of all PCR products were confirmed by dideoxy-sequencing with sequenase 2.0 (Amersham, Braunschweig, Germany).

Strains

For cloning strategies *Escherichia coli* strains DH1 (a gift from J.P. Jost) and JM101 (Kan^s; a gift from K. Weber) were used. For expression of the MBP-VDR fusion protein or the GST fusion proteins *E. coli* strains JM109 (from K. Weber) or BL21 (a gift from A. Hochheimer) were used, respectively. For recovery of library plasmids from the two-hybrid system strain HB101 (Promega, Heidelberg, Germany) was used. The yeast two-hybrid system was propagated in *Saccharomyces cerevisiae* strain SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538,URA3::GAL1-lacZ*), Clontech Laboratories, Heidelberg, Germany). Strain BJ3505 (*MATa, pep4::HIS3, prb1-Δ1.6R, HIS3, lys2-208, trp1-101, ura3-52, gal2, can1)*, a gift from M. Husemann, was used for propagation of Yepubstu-VDR.

Yeast Assays and Two-Hybrid System

Yeast transformations were performed as previously described [Klebe et al., 1983]. Transformants were selected on SD medium (2% glucose, 0,67% yeast nitrogen base without amino acids), supplemented with the appropriate nutrients. Yeast strain SFY526 containing pGBT9-VDR was transformed with a human skeletal muscle cDNA library in pGAD10 (Clontech Laboratories, Heidelberg, Germany) and the cotransformants were tested for β-galactosidase activity in the presence of 10^{-6} M vitamin D in the colony-lift filter assay [Berghöfer-Hochheimer et al., 1997]. To recover library plasmids, total yeast DNA was prepared and transformed in *E. coli* strain HB101. Transformants were selected on M9-minimal medium lacking leucine and containing ampicillin. To ensure identification of the correct cDNAs, isolated plasmids were retransformed into strain SFY526 containing pGBT9-VDR and the cotransformants were again tested for β-galactosidase activity. Quantification of β-galactosidase of yeast transformants was performed in 96-well dishes by using ONPG as previously described [Berghöfer-Hochheimer et al., 1997].

Preparation of Crude Extracts

Yeast strain BJ3505 was transformed with the expression plasmids Yepubstu or Yepubstu-VDR. The transformants were grown overnight in 400 ml SD medium in an orbital shaker (200 rev/min at 30°C) to an $A_{600\ nm}$ of 1.5. Cells were then induced with 0.1 mM $CuSO_4$ for 5 h in the presence of 10^{-6} M vitamin D and harvested by centrifugation. Preparation of the crude extracts has been previously described [Sone et al., 1990]. Expression of the VDR was detected by Western Blot analysis on nitrocellulose (Amersham, Braunschweig, Germany) using the monoclonal 9A7 antibody (1 µg/µl, diluted 1: 400) and peroxidase conjugated goat anti-rat secondary antibody (Dianova, Hamburg, Germany), which was diluted 1:12,000. The myelomonocytic cells of U937 (kindly provided by V. Güntzschel) at a density of 5×10^5 cells/ml were incubated with 10^{-7} M vitamin D 5 h prior to centrifugation of the cells. The nuclear extract was prepared as previously described [Schreiber et al., 1989] and the presence of endogenous VDR was shown by Western Blot analysis.

Interaction of the VDR With GST Fusion Proteins

The pGEX-expression plasmids were transformed into *E. coli* strain BL21 and the transformants were grown in LB medium containing ampicillin and 2% glucose in an orbital shaker (200 rev/min at 37°C). At a density of $A_{600\ nm}$ 0.3 cells were induced with 0.05 mM IPTG and incubated another 3 h. After the cells were harvested by centrifugation and washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM 2-mercaptoethanol, they were lysed by sonication (3 min with intervals of 15 s). According to the manufacturer's instructions (Pharmacia, Heidelberg, Germany) the GST fusion proteins were purified from the crude cell extracts by affinity chromatography with glutathione

sepharose and stored in 10% glycerol, 10 mM 2-mercaptoethanol at -70°C. The proteins were quantified using bovine serum albumin as a standard with the method of Bradford [1976]. For testing interactions individual GST fusion proteins or GST were incubated with yeast or U937 crude extracts containing the VDR in the absence or in the presence of 10^{-6} M vitamin D. Purification of the protein-protein interactions by binding to glutathione sepharose and analysis for VDR content were performed according to MacDonald et al. [1995]. As a size marker for proteins in SDS-polyacrylamide gelelectrophoresis wide range protein marker from Sigma (Munich, Germany) was used.

Gel Shift Analysis

Complementary oligonucleotides derived from the mouse osteopontin promoter [Noda et al., 1990] (5'-TCGAGAAGGTTCACGAGGTTCAC-GTC-3') were annealed and labeled with α-^{32}P-dCTP by Klenow fragment (Boehringer, Mannheim, Germany). The labeled DNA was purified by ultrafiltration (Amicon, Witten, Germany). Equimolar amounts of fusion proteins were incubated with the DNA (30,000 cpm/reaction) in 20 mM HEPES, pH 7,9, 50 mM KCl or 150 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 6 µg poly dI-dC for 25 min at room temperature. If more than one protein was investigated for binding of DNA, the proteins were preincubated for 15 min at room temperature. Vitamin D (10^{-6} M) and/or 9-*cis* retinoic acid (10^{-6} M) were incubated with the receptors prior to the addition of DNA. Concentration of the antibodies was 50 ng/µl. The protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel, run in 0.5 x TBE-buffer (89 mM Tris base, 89 mM boric acid, 4 mM EDTA, pH 7.4) at 25 mA and visualized by autoradiography.

RESULTS

Protein L7 Interacts With the VDR in a Ligand Dependent Manner in the Yeast-Two Hybrid System

To identify proteins that interact with the VDR we used a two-hybrid system which was based on the yeast GAL4 transactivator [Fields and Song, 1989]. Full length cDNA of the VDR fused to the GAL4 DNA binding domain in pGBT9 was used to screen a human skeletal muscle cDNA library in pGAD10 (GAL4 activation domain) in the presence of 10^{-6} M vitamin D. Approximately 30000 clones were screened, of which 30 clones showed an interaction with the VDR. The plasmids were rescued from yeast and retransformed in yeast strain SFY526 containing pGBT9-VDR. In this rescreen all interactions were confirmed. By Southern Blot analysis 18% of the recovered plasmids were shown to harbour either VDR or RXR cDNA inserts. One positive clone, pGAD-L7, containing an insert of 900 bp was proven to contain the entire open reading frame of protein L7 [Hemmerich et al., 1993; Seshadri et al., 1993]. The previously identified L7 protein was shown to associate in the cytoplasm with the 60S subunit of the ribosome and to interact specifically with mRNA [Lin et al., 1991; Hemmerich et al., 1997]. But until now it has no proven function. Histochemical approaches showed that L7 also was located in the nucleus [von Mikecz et al., 1994]. To further analyze interaction domains of L7, a *Pst*I/*Xho*I fragment was deleted from pGAD-L7. The resulting plasmid, pGAD-L7, contained the first 267 bp of L7 in frame with the GAL4 activation domain and therein the basic region leucine zipper domain (bZip) of L7 [Landschultz et al., 1988; Hemmerich et al., 1993]. Yeast cotransformants harbouring pGBT9-VDR and pGAD-L7 exhibited β-galactosidase activity indicating a VDR-bZIP interaction (Table I). To analyze interaction domains within the VDR, we cotransformed pGBT9-VDR-DBD or pGBT9-VDR-LBD together with pGAD-L7 or pGAD-L7 into yeast (Table I). Neither full length L7 nor the truncated L7 protein interacted with the DNA-binding domain or the ligand binding domain of the VDR indicating that the interaction face was not exclusively located in these VDR domains.

Expression of the L7 Protein in Yeast Interferes With VDR-RXR Heterodimerization

We have shown previously that VDR and RXR dimerized in the presence of vitamin D in the yeast two-hybrid system. Induction of β-galactosidase activity in the liquid assay was approximately three-fold in the presence of 10^{-6} M vitamin D [Berghöfer-Hochheimer et al., 1997]. We constructed the shuttle vector pTM175-L7, a constitutive expression system and cotransformed it with the VDR-RXR two-hybrid system into yeast cells to test the influence of L7 on heterodimerization. Constitutive expression of L7 strongly reduced β-galactosidase activity in the presence of vitamin D.

TABLE I. Colony Lift Filter Assay of SFY526 Co-Transformants, Demonstrating VDR-L7 Interaction*

	β-galactosidase activity	
SFY526 transformants	+ Vitamin D	− Vitamin D
pGBT9-VDR (GAL4-DBD-VDR)	−/+	−
pGBT9-VDR and pGAD10-L7 (GAL4-AD-L7)	+	−
pGBT9-VDR and pGAD10-L7bZip	+	−
pGBT9-VDR-DBD and pGAD10-L7	−	−
pGBT9-VDR-LBD and pGAD10-L7	−	−

*Strain SFY526 was transformed with the expression plasmids encoding Gal4-VDR and Gal4-L7 fused cDNAs. Co-transformants were incubated on solid media in the absence or in the presence of 10^{-6} M vitamin D. Activity of the β-galactosidase was detected by development of a blue colour of the transfered colonies in the filter assay, as described in Materials and Methods. The plasmid pGBT9-VDR-DBD contains amino acids 4-124 of the VDR fused to the Gal4-DNA binding domain. The plasmid pGBT9-VDR-LBD contains amino acids 125–423 of the VDR. −/+, blue colonies were detected after 16 h incubation with X-Gal on the filters. +, Co-transformants developed a blue colour after 4 h, indicating VDR-L7 protein interaction.

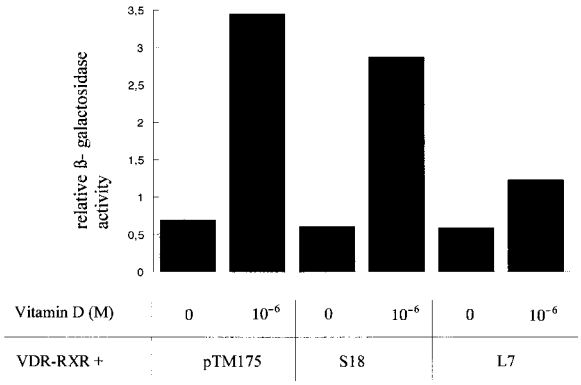
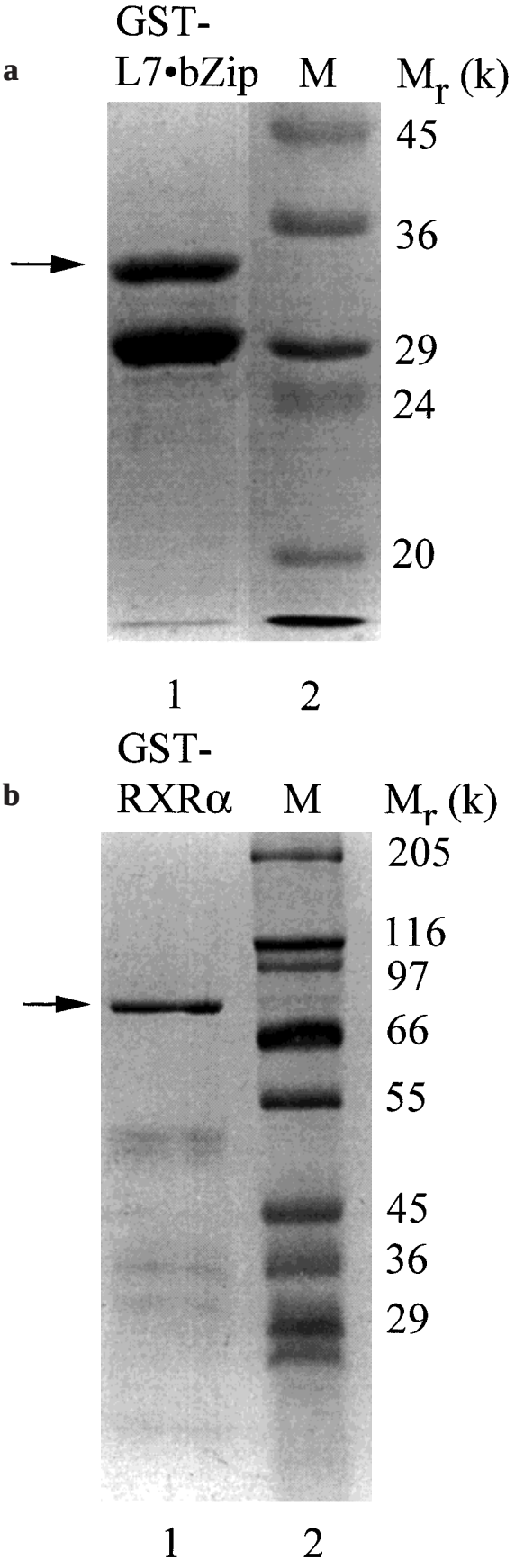


Fig. 1. Repression of β-galactosidase activity by the interaction of L7 with VDR-RXR heterodimers in the yeast two-hybrid assay. Co-transformants of strain SFY526, containing the *GAL1* promoter-*lacZ* transcription unit and the pGBT9-VDR and pGAD424-RXRβ expression plasmids, additionally were transformed with the expression plasmids pTM175 (empty vector), pTM175-S18, or pTM175-L7. The cells were subsequently grown in the absence or in the presence of 10^{-6} M vitamin D and afterwards lysed. The *lacZ* activity was quantified at $A_{415\ nm}$ in 96-well dishes and corrected for cell density at $A_{600\ nm}$ (Materials and Methods). The standard deviation was less than 5%.

Cotransformation of empty pTM175 did not influence VDR-RXR mediated transactivation of β-galactosidase activity (Fig. 1). These results indicated that L7 interfered with VDR-RXR heterodimerization, presumably by binding to the VDR either in the cytoplasm or in the nucleus. To exclude an unspecific negative effect on β-galactosidase activity by the overexpression of a heterologous protein we also co-transformed pTM175–S18. The human protein S18 exhibits structural similarities with the L7 protein. It contains a basic region leucine zipper domain and is about the size of the L7 protein [Chassin et al., 1993]. Constitutive expression of S18 had only a minor effect on transactivation by VDR-RXR heterodimers (Fig. 1). Thus, we excluded that repression of reporter gene activity by the L7 protein was caused by unspecific effects on cell viability such as protein overexpression and squelching.

VDR and the L7 Protein Interact In Vitro

The basic region leucine zipper domain of the L7 protein and full length human RXRα were expressed as GST-fusion proteins (Fig. 2). The purified fusion proteins were incubated with crude extracts from yeast transformants which contained the Yepubstu-VDR expression plasmid or with extracts from the cell line U937 either in the absence or in the presence of vitamin D. The mammalian cells were stimulated with vitamin D prior to protein preparation. VDR content in the extracts was confirmed by Western blot analysis (Fig. 3). The protein complexes were precipitated with glutathione sepharose and analyzed for VDR content by Western blot. Figure 3a shows that GST-RXR and GST-L7bZip (1.6-fold molar excess refering to RXR) efficiently precipitated VDR from the yeast extract, while a 1.7-fold molar excess of GST did not pull down the VDR from the crude extract. Precipitation of the VDR by RXRα was strongly enhanced in the presence of 10^{-6} M vitamin D. In contrast, the VDR-L7 interaction was not as strongly influenced by the ligand. Interestingly, L7bZip precipitated endogenous VDR from U937 cell extracts very efficiently in the presence of ligand in contrast to VDR selection in the absence of ligand (Fig. 3b). In summary, these data not only confirmed the VDR-L7 interaction found in yeast by an in vitro interaction. Moreover, they showed that the L7 protein selectively bound to the receptor even in the high background of other cellular components which were contained in the crude cell extracts.



L7 Represses Binding of VDR and RXR to a Vitamin D Response Element

The fusion proteins MBP-VDR (Fig. 4) and GST-RXR with apparent molecular masses of 85 kD and 75 kD were used in a gel shift assay to test binding of both receptors to a DR3-vitamin D response element (VDRE). In addition, His_6-L7 (26 kD) or His_6-bZipL7 (P. Hemmovich, unpublished results) were added to investigate their influence on DNA-binding of the receptors. Previous studies in yeast had shown, that transactivation from this response element was only induced in the presence of both receptors [Berghöfer-Hochheimer et al., 1997]. Addition of both receptors to the DNA resulted in a DNA-shift, which was enhanced by increasing the amount of MBP-VDR in the heterodimeric complex (Fig. 5a, lanes 3 and 4). In the presence of unlabeled competitor DNA the protein-DNA complex disappeared (Fig. 5a, lanes 6 and 7). MBP-VDR alone did not shift the VDRE, whereas GST-RXR alone resulted in weak DNA-binding (lane 2). On the other hand, the response element was not shifted by the RXR alone, from which the GST moiety had been removed by proteolytic cleavage (data not shown). The size of the protein-DNA complex indicated binding of GST-RXR homodimers to the DNA. Furthermore heterodimeric DNA-binding was not enhanced upon the addition of vitamin D (lane 5). Interestingly, heterodimeric complex formation with the VDRE was fully repressed in the presence of equimolar amounts of full length L7 or of the C-terminus, when the proteins were preincubated prior to the addition of DNA (Fig. 5b, lanes 2 and 6). Preincubation of the heterodimer and the L7 together with anti-L7 antibody (P. Hemmovich, unpublished results) restored heterodimer-DNA complex formation, presumably by sequestering the

Fig. 2. Expression of human L7 protein and human RXRα as glutathione (GST)-fusion proteins. *E. coli* strain BL21 was transformed with pGEX-5X-1::L7bZip or pGEX-5X-1::RXRα and proteins were purified from the transformants as described in Materials and Methods. **a**: SDS-polyacrylamide gelelectrophoresis (Coomassie-stained 10% gel) of purified GST-L7bZip. The arrow **(lane 1)** marks the product of the GST-L7bZip fusion construct. The smaller protein product represented a truncated fusion protein, as confirmed by Western blot analysis (not shown). **Lane 2:** Protein marker. **b**: Ten percent SDS-PAGE of purified GST-RXRα. The expected fusion protein is marked by an arrow **(lane 1). Lane 2**: Marker. The gel was stained with Coomassie blue.

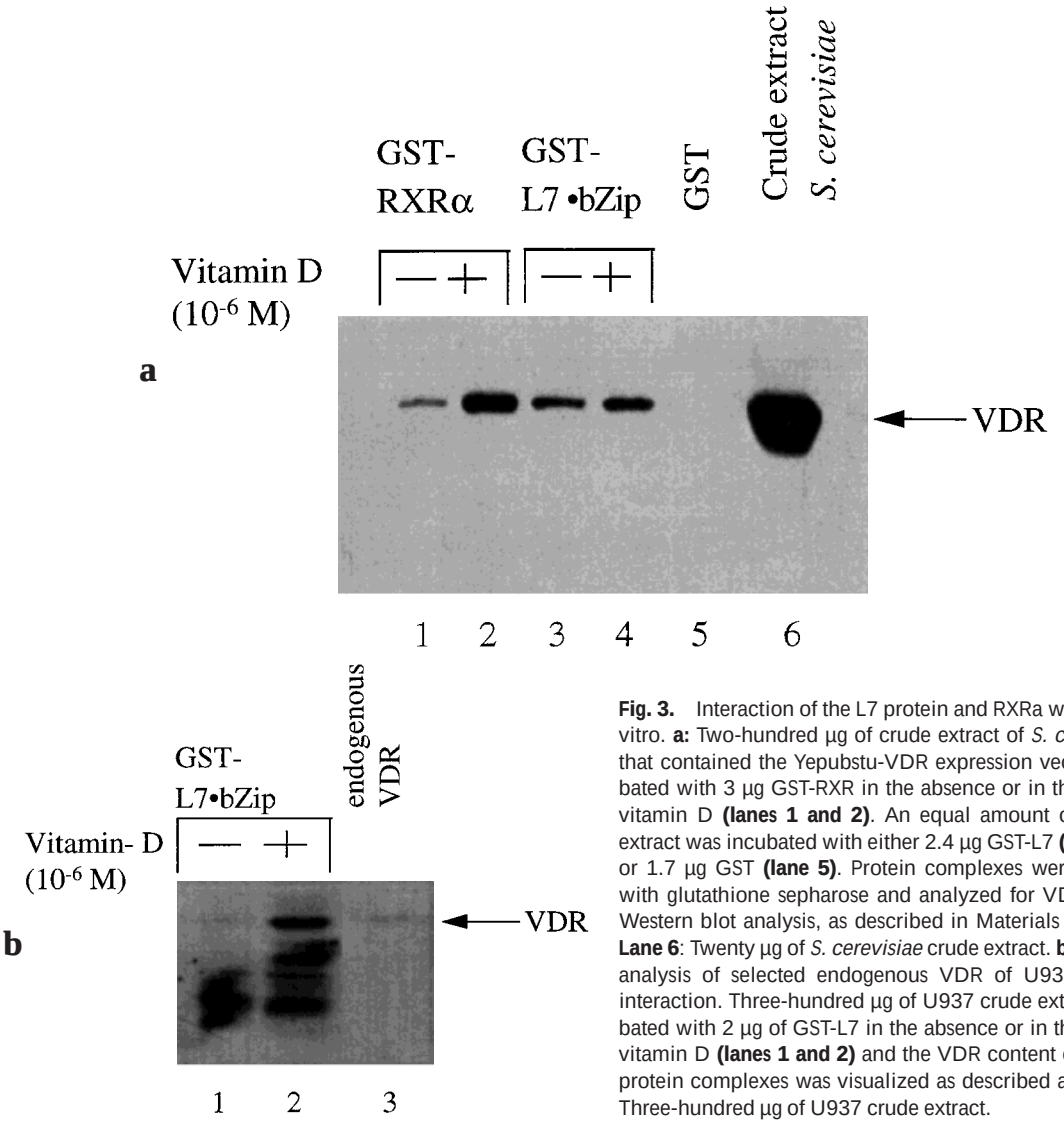


Fig. 3. Interaction of the L7 protein and RXRa with the VDR in vitro. **a:** Two-hundred µg of crude extract of *S. cerevisiae* cells that contained the Yepubstu-VDR expression vector was incubated with 3 µg GST-RXR in the absence or in the presence of vitamin D **(lanes 1 and 2)**. An equal amount of yeast crude extract was incubated with either 2.4 µg GST-L7 **(lanes 3 and 4)** or 1.7 µg GST **(lane 5)**. Protein complexes were precipitated with glutathione sepharose and analyzed for VDR content by Western blot analysis, as described in Materials and Methods. **Lane 6**: Twenty µg of *S. cerevisiae* crude extract. **b**: Western blot analysis of selected endogenous VDR of U937 by VDR-L7 interaction. Three-hundred µg of U937 crude extract was incubated with 2 µg of GST-L7 in the absence or in the presence of vitamin D **(lanes 1 and 2)** and the VDR content of the protein-protein complexes was visualized as described above. **Lane 3**: Three-hundred µg of U937 crude extract.

L7 protein (Fig. 5c, lane 6). If the antibody was added after incubation of DNA with the proteins, the signal strength of the retarded protein-DNA complex decreased (Fig. 5c, lane 7). In this experiment the interference of L7 with heterodimeric complex formation was partially compensated by sequestering L7 with antibodies. Together these findings again confirmed the repressive effect of L7 on DNA binding by VDR-RXR heterodimers. Repression probably was not due to DNA binding properties of the L7 itself, because L7 or its truncated product did not retard the VDRE (data not shown). Addition of anti-VDR antibody to the VDR and the RXR prior to incubation with DNA inhibited protein-DNA complex formation, confirming the specificity of the complex (data not shown). Surprisingly, incubation of full length L7 or of the truncated product with the RXR enhanced binding of the receptor to the VDRE in the presence of 9-cis retinoic acid (Fig. 5c, lanes 2 and 3). The position of the retarded complex in the gel, which was about the same size as the VDR-RXR-DNA complex, suggested binding of RXR-L7-dimer protein complexes to the VDRE. In contrast to this, the VDR did not bind to the VDRE in the presence of full length L7 (Fig. 5b, lanes 7 and 8). Different concentrations of KCl did not influence protein-DNA interaction in the presence of vitamin D. Also repression by L7 was not affected by these different experimental conditions.

DISCUSSION

Modulation of VDR activity and of many other nuclear receptors by the interaction with regu-

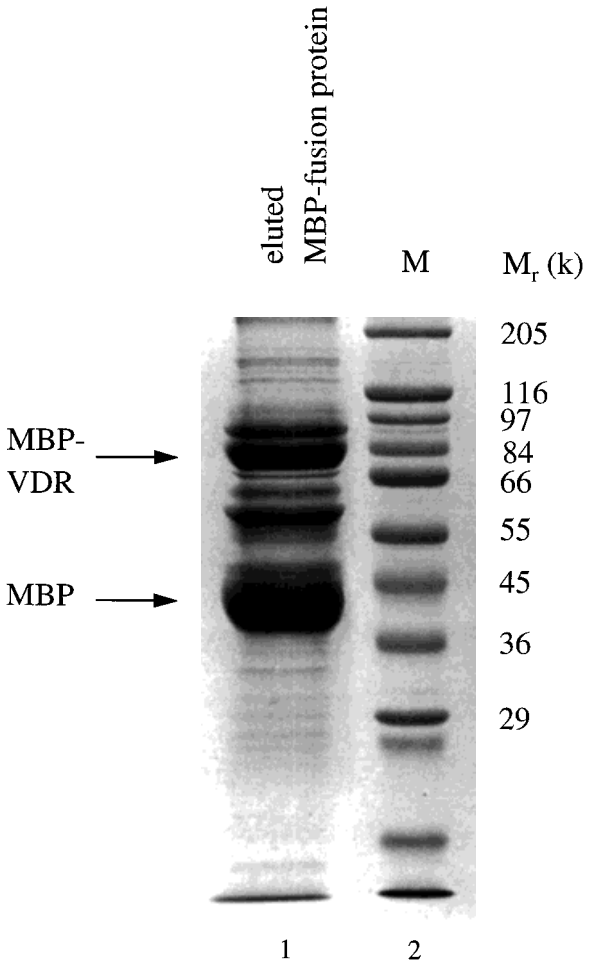


Fig. 4. Expression of the VDR as maltose binding protein (MBP)-fusion protein. *E. coli* strain JM109 was transformed with the expression vector pMAL-c2::VDR and fusion protein was purified from the cells by affinity chromatography as described in Materials and Methods. Arrows mark the expected MPB-VDR fusion and the endogenous MBP of *E. coli*, which also was enriched by binding to amylose **(lane 1)**. Western blot analysis with anti-VDR antibodies confirmed the identity of the fusion protein (not shown). **Lane 2:** Marker.

latory proteins has become a highly important aspect of gene regulation [Perlmann and Vennström, 1995; Torchia et al., 1997, and references therein]. Our results demonstrate the interaction between the VDR and the protein L7, which is an important autoantigen in different autoimmune diseases [Hemmerich et al., 1997, and references therein]. The L7 protein associates with the large subunit of ribosomes and in vitro and in vivo studies suggest that it is involved in translational regulation of distinct proteins due to its mRNA binding activities [Neumann et al., 1995]. The protein harbours an amino terminal basic region leucine zipper like domain, mediating RNA-binding, DNA-binding, and homodimerization [Hemmerich et al., 1993, 1997; Witte et al., 1996]. Furthermore, it has been demonstrated that L7 also was located in the nucleus [von Mikecz et al., 1994]. Thus, L7 protein is a prominent protein with potential separate activities in different cell compartments. Wiedmann et al. [1994] had reported on a similar example, the NAC-complex, which was shown to interact with nascent polypeptide chains at the ribosome and on the other hand to function as a transcription factor.

VDR-L7 interaction was ligand dependent in our yeast studies as well as in the in vitro interaction assays, using whole cell extracts. These results indicate a functional role of the L7 protein as a novel ligand dependent cofactor of the VDR. The yeast two-hybrid assay exhibited substantial reporter gene activity that was mediated by VDR-L7 interaction, only in the presence of vitamin D. Furthermore, we could show that vitamin D also was essential for the interaction of the VDR with the truncated form of the L7 protein, the L7bZip. Interaction studies with the truncated forms of the VDR revealed that amino acids relevant for L7 binding were neither restricted to the DNA-binding domain nor to the ligand binding domain of the receptor. Parts of the less conserved VDR-hinge region were contained in both VDR constructs [Baker et al., 1988]. Other coregulators have been shown to bind to the hinge-ligand binding domains of nuclear receptors [Danielian et al., 1992; Onate et al., 1995; Burris et al., 1995]. The in vitro interaction studies revealed that the selection of overexpressed VDR or endogenous VDR from U937 cells was susceptible to the influence of the hormone: Interaction of L7bZip with the mammalian receptor was strongly enhanced in the presence of vitamin D, but nevertheless also basically detected in the absence of hormone. In case of the VDR which was expressed in yeast, VDR-L7 interaction repeatedly showed only a minor influence of the ligand on the formation of the protein complex, which was in contrast to the hormone-stimulated VDR-RXR interaction. These differences in ligand dependency, which also opposed the ligand-dependent VDR-L7bZip interaction in viable yeast cells, presumably reflected altered conditions for protein-protein interaction, due to the features of crude extract preparation [Sone et al., 1990]. Nevertheless, another important aspect of VDR-L7 interaction was emphasized by the interaction studies: L7 binding to

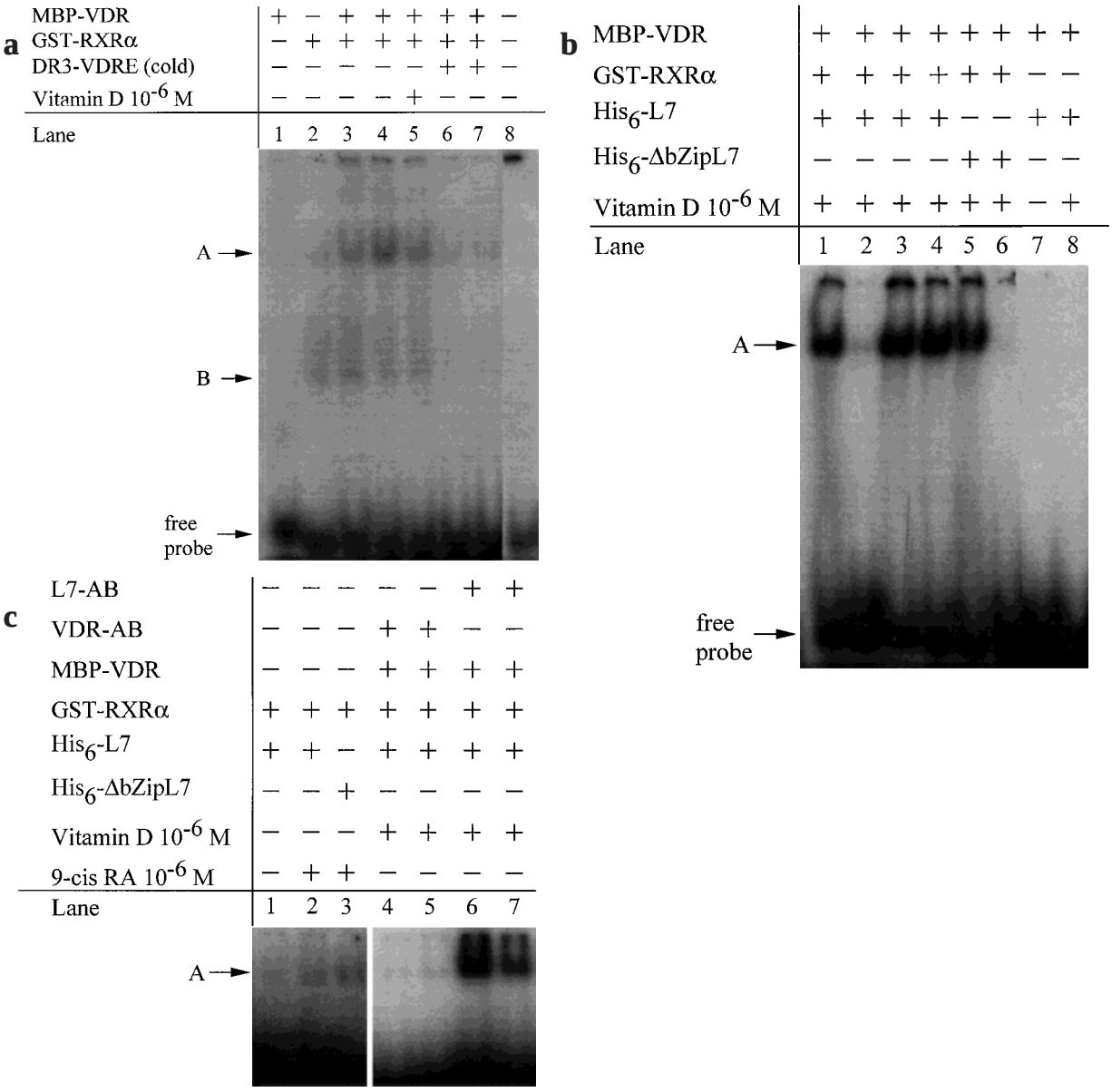


Fig. 5. **a**: Gel shift analysis of binding of MBP-VDR and of GST-RXRα to a vitamin D response element in the absence or in the presence of vitamin D. MBP-VDR and GST-RXR were preincubated 15 min at room temperature prior to the addition of radioactive labeled oligonucleotide, derived from the mouse SSP1 promoter (lanes 3–7). Binding of the proteins to the DNA was continued for 25 min at room temperature. The complexes were separated on a 5% polyacrylamide gel and the gels exposed to X-ray films (Materials and Methods). MPB-VDR or GST-RXRα alone were incubated with the response element **(lanes 1 and 2)**. Equal amounts of both fusion proteins were incubated with the DNA **(lane 3)**. In **lane 4**, a three-fold amount of the MPB-VDR was preincubated with GST-RXRα. **Lane 5:** Both fusion proteins were preincubated with 10^{-6} M vitamin D. The protein-DNA complexes were incubated with increasing amounts of unlabelled oligonucleotide **(lanes 6 and 7)**. **Lane 8:** Free oligonucleotide. Complex A represents RXR dimers or VDR-RXR dimers, bound to the VDRE. Complex B is unspecific. **b**: Gel shift analysis of binding of MBP-VDR and of GST-RXRα to a vitamin D response element in the presence of L7. Equimolar amounts of VDR and RXR were incubated with His_6-L7 or with His_6-bZipL7 in a ratio of 5:1 **(lanes 1, 3, and 5)** or in a ratio of 1:1 **(lanes 2, 4, and 6)**. The proteins were preincubated 15 min prior to the addition of DNA (lanes 1, 2, 5, and 6). Lanes 3 and 4: L7 was added 15 min after the addition of DNA to both receptors. **Lanes 7 and 8:** Equimolar amounts of VDR and L7 were preincubated 15 min prior to the addition of DNA in the absence or in the presence of vitamin D. The incubation of the proteins with DNA was performed in the presence of 50 mM KCl. **c**: Gel shift analysis of binding of MBP-VDR and GST-RXRα to a vitamin D response element in the presence of L7 and of anti-L7 antibody. Equimolar amounts of VDR, RXR, and L7 were preincubated 15 min prior to the addition of DNA in the presence of 50 ng/μl anti-L7 antibody (L7-AB, **lane 6). Lane 7:** Preincubated VDR, RXR, and L7 were added to the DNA and L7-AB was added 15 min after incubation of the proteins with DNA. Anti-VDR antibody (VDR-AB, 50 ng/μl) was preincubated with the proteins prior to the addition of DNA **(lane 4). Lane 5:** VDR-AB was added 15 min after incubation of the proteins with DNA. **Lanes 1–3:** Equimolar amounts of RXR and of L7 or bZipL7 were preincubated 15 min prior to the addition of DNA in the absence or in the presence of 9-cis retinoic acid. Gel shift analysis was performed with 50 mM KCl. Unbound oligonucleotides were covered by radioactive nucleotides, which had not been filled-in by Klenow fragment and therefore are not marked.

the receptor was neither impaired by the yeast cellular background nor by mammalian compounds, that were present in the crude extract of U937 cells. Repression of reporter gene activity by the interference of full length L7 with VDR-RXR heterodimerization in the yeast two-hybrid assay suggested that L7 functioned as a corepressor of transcription in vivo. The gel shift assays proved, that the L7 protein repressed binding of VDR-RXR heterodimers to DNA. Interestingly, L7 or its C-terminus obviously recruited RXR to the DNA in a ligand-dependent manner. This suggested, that repression could be enhanced by fixing RXR molecules to the VDRE, thereby shifting the equilibrium towards dissociation of VDR-RXR heterodimers. Ligand-dependent interaction of the RXR with L7 in the yeast two-hybrid system (data not shown) moreover contributed to the idea, that availability of the respective ligands at least controlled repression of VDR-RXR transactivation by L7. Transfection of full length L7 or L7bZip into the cell line MCF-7 revealed repression of vitamin D controlled reporter gene activity in the presence of ligand (not shown). Nevertheless, activity of a vitamin D independent reporter gene also decreased in the presence of L7, revealing an unspecific influence on transcription and/or on translation by L7 expression. These findings will be further investigated. In contrast to the recruitment of RXR to DNA in the presence of L7 and of 9-cis retinoic acid, DNA-binding of the VDR in the presence of vitamin D and of L7 was not detected. According to Kimmeljehan and coworkers [1997] we performed gel-shift experiments also in the presence of 150 mM KCl, which was proposed to reflect a physiological salt concentration. Binding of VDR-RXR-dimers to DNA in the presence of vitamin D was not enhanced. We therefore propose, that the VDR, which was expressed in *E. coli*, did not efficiently bind its natural ligand. Consequently, binding of VDR-L7 complexes to DNA in case of ligand-bound VDR cannot be excluded. Nevertheless, VDR-L7 interaction in the yeast assays and in the GST-pull down experiment was enhanced in the presence of vitamin D, indicating that inhibition of VDR mediated transcription was a ligand-dependent event. Burris and coworkers [1995] demonstrated the interaction of another ribosomal protein, L7a, with the thyroid receptor (TR) which resulted in silencing of TR controlled gene transcription. In contrast to our results, the interaction was shown to be independent of ligand. Recently, it has been demonstrated that corepressors which acted independently of ligand in case of retinoic acid/thyroid receptors, bound to the progesterone receptor (PR) and the estrogen receptor in the presence of particular antagonists [Jackson et al., 1997]. The same group reported, that the L7 protein was a co-activator for the PR and the glucocorticoid receptor in the presence of antagonists. Reporter gene activity controlled by cognate enhancers on the other hand was not enhanced in the presence of agonists. Consequently the absence or the presence of agonists or antagonists seemed to stabilize different conformations of nuclear receptors which are then prone to corepressor binding or not.

Ligand-dependent interaction of L7 with the receptors was reminiscent of co-activator-receptor complex formation. Recently it has been shown, that co-activators share an interaction motif of helical leucine-charged residue rich domains (LCDs) which are specific for receptor binding [Torchia et al., 1997; Heery et al., 1997]. We found a motif in L7-VLQLL-which was nearly identical to the consensus LXXLL. Mutation of the first conserved leucine to valine, as it has been found in L7, hardly affected the co-activator function of the RIP140 co-activator [Heery et al., 1997]. Although this motif was not comprised in our L7bZip constructs, we did not want to exclude a function of this putative binding site for the whole molecule. We propose, that the α-helicity of the L7bZip [Hemmerich et al., 1997] in addition to the conserved motif mediated binding of L7 to ligand-bound VDR, as it has been suggested for SRC1-estrogen receptor interaction [Danielian et al., 1992]. In contrast to the VDR, RXR did not interact with L7bZip in the yeast two-hybrid system (not shown). The gel shift assay demonstrated, that RXR-DNA interaction was modulated in the presence of the C-terminus of L7, containing the putative LCD. These data suggest, that L7 contains at least two distinct domains, which are responsible for differential binding to the receptors.

Our data suggest, that the L7 protein is a ligand-dependent coregulator of VDR-RXR-controlled genes. Thus, activity of the nuclear receptors in the cell is likely to be controlled at least by the availability of L7, which is determined by cell type specificity and by aging [Peacocke and Campisi, 1991; Seshadri et al.,

1993]. Taken together all current data, L7 binding activity not only is dedicated to an active VDR. Moreover, L7 turns out to be a general coregulator, which gears into different steroid hormone receptor controlled signal transduction pathways.

ACKNOWLEDGMENTS

We thank M.R. Haussler and T. Petri for the gift of human VDR cDNA. We also thank V. Güntzschel for providing us with the cell line U937. We thank C. Carlberg and B. Rudakoff for critical reading of the manuscript.

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