A conserved retinoid X receptor (RXR) from the mollusk Biomphalaria glabrata transactivates transcription in the presence of retinoids

D Bouton, H Escriva1, R L de Mendonça, C Glineur2, B Bertin, C Noël, M Robinson-Rechavi1, A de Groot3, J Cornette, V Laudet1 and R J Pierce

INSERM U 547, Institut Pasteur, 1 rue du Professeur Calmette, 59019-Lille, France
1CNRS UMR 49, École Normale Supérieure de Lyon, 46 allée d’Italie, 69364-Lyon, France
2INSERM U 545 Institut Pasteur, 1 rue du Professeur Calmette, 59019-Lille, France
3Laboratoire d’Écologie Microbienne de la Rhizosphère (LEMiR), Département d’Écophysiologie Végétale et de Microbiologie (DEVLM), CEA Cadarache, 13108 Saint Paul Lez Durance, France

Abstract

Retinoid X receptors (RXR) are members of the nuclear receptor superfamily of ligand-activated transcription factors that have been characterized in a wide variety of metazoan phyla. They act as heterodimer partners of other nuclear receptors, and in vertebrates also activate transcription as homodimers in the presence of a ligand, 9-cis retinoic acid. In order to test the hypothesis that retinoic acid signaling pathways involving RXRs are present in the Lophotrochozoa, we have sought to isolate conserved members of this family from the platyhelminth parasite Schistosoma mansoni and its intermediate host, the mollusk Biomphalaria glabrata. Here we report that an RXR ortholog from B. glabrata (BgRXR) is better conserved, compared with mouse RXRα, both in the DNA-binding domain (89% identity) and in the ligand-binding domain (LBD) (81% identity), than are arthropod homologs. In EMSA, BgRXR binds to the direct repeat response element DR1 as a homodimer or as a heterodimer with mammalian RARα, LXR, FXR or PPARα. When transfected alone into mammalian cell lines, BgRXR transactivated transcription of a reporter gene from the Apo-A1 promoter in the presence of 9-cis retinoic acid or DHA. Constructs with the Gal4 DNA binding domain fused to the hinge and LBDs of BgRXR were used to show that ligand-dependent activation of transcription by BgRXR required its intact AF-2 activation domain, and that the LBD can form homodimers. Finally, the binding of 9-cis retinoic acid preferentially protected the LBD of BgRXR from degradation by trypsin in a proteolysis protection assay. Our results show that BgRXR binds and is activated by retinoids and suggest that retinoid signaling pathways are conserved in the Lophotrochozoa. The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession no. AY048663.

Journal of Molecular Endocrinology (2005) 34, 567–582

Introduction

Retinoic acids (RA) play a key role in embryo patterning and organogenesis in vertebrates (reviewed in Ross et al. 2000), but there is growing evidence that their role in cellular signaling is ancient within the Metazoa. Retinoids and carotenoids were detected in sponges and 13-cis RA was found to regulate gene expression (Biesalski et al. 1992). In the freshwater sponge Ephydatia muelleri, retinoic acid initiates morphogenetic events (Imiecke et al. 1994) and the downregulation of a homeobox-containing gene (Nikko et al. 2001).

Moreover, in the marine sponge Suberites domuncula, incubation with all-trans RA leads to reversible tissue regression in intact individuals (Wiens et al. 2003). However, the only evidence for the influence of RA in protostomes is its active uptake by the parasitic nematode Brugia malayi and its influence on the development of the worm within its mammalian host (Wolff & Scott 1995). There are no reports of the eventual role of RA in lophotrochozoan invertebrates.

Retinoid signals are transduced in vertebrates by two families of nuclear receptor, the retinoic acid receptors RARα, β and γ, which preferentially bind all-trans RA,
and the retinoid X receptors RXR α, β and γ, which bind 9-cis RA. The latter play a central role in a variety of nuclear signaling pathways (Mangelsdorf & Evans 1995) and, in vertebrates, modulate the activities of other nuclear receptors (including RAR, the vitamin D receptor or the peroxisome proliferator-activated receptor (PPAR)) by forming heterodimers. These bind specifically to direct repeats (DR) of the consensus response element PuGGTCA spaced by 1–5 nucleotides (DR1 to DR5) (Mangelsdorf et al. 1995). The spacing determines the specificity of the binding of the various heterodimers.

Whereas the RAR family has been found only in vertebrates, arthropods possess homologs of RXR, including Drosophila ultraspiracle (USP), but none of these receptors has been conclusively shown to bind 9-cis RA, or indeed any ligand. However, an RXR-like nuclear receptor has been characterized in the marine sponge S. domuncula, and although its capacity to bind RA was not demonstrated, treatment of the sponge with all-trans RA strongly upregulated the expression of its mRNA (Wiens et al. 2003). An RXR in the jellyfish Tripedalia cystophora has a well-conserved ligand-binding domain (LBD) and has been shown to bind 9-cis RA (Kostrouch et al. 1998), but transactivation of transcription by T. cystophora RXR in the presence of the ligand was not demonstrated.

Among the non-moulting triploblastic invertebrates, the Lophotrochozoa (Aguinaldo et al. 1997), RXR family members have been characterized only in the platyhelminth parasite of man, Schistosoma mansoni. However, both receptors described (Freebern et al. 1999, De Mendonça et al. 2000b) have poorly conserved LBDs and are unlikely to be able to bind 9-cis RA. Moreover, they also show atypical DNA-binding properties and in their native forms do not bind to conventional DR elements. Nevertheless, RA signaling remains a possible element in the molecular dialog that exists between the parasite and its definitive and intermediate hosts (reviewed in De Mendonça et al. 2000a), and in order to test this hypothesis we have sought to characterize novel RXRs, both from the parasite and from its intermediate host, the freshwater snail Biomphalaria glabrata.

Here we describe a structurally and functionally conserved RXR from B. glabrata (BgRXR) that both binds to conventional DR elements as a homodimer or as a heterodimer partner with mammalian nuclear receptors, and transactivates transcription from these response elements in the presence of 9-cis RA when transfected alone into mammalian cell lines. We present evidence showing that the transcriptional activity of BgRXR observed is due to the direct binding of retinoids by its LBD. Our results argue both for the conservation of nuclear receptor–cofactor interactions in the mollusk, but also for the importance of RA signaling and its conservation in the Lophotrochozoa.

Materials and methods

Biologic material

B. glabrata snails were maintained in a mixture (1:1, v/v) of deionized water and mineral water at 24 °C and fed with fish pet food maintained solid with alginate, under an approximately natural diurnal light cycle. Snails were starved for 6 h before use. The B. glabrata embryonic cell line (Bge) (Hansen 1976) was maintained in Schneider Drosophila medium (Invitrogen), 22% (v/v); lactalbumin hydrolysate (Invitrogen), 0.45% (w/v); D+ galactose (Sigma), 0.13% (w/v); phenol red (Sigma) 0.01% (w/v); fetal bovine serum (Invitrogen), 10% (v/v); and double-distilled water 68% (v/v). S. mansoni cercariae were released from infected snails and harvested on ice. Balb/c mice were obtained from D. Dombrowicz (INSERM U 547, Institut Pasteur de Lille, France) and killed by cervical dislocation before removal of livers. Total RNA was obtained by the guanidine thiocyanate/cesium chloride method, as described by Chirgwin et al. (1979), from mollusk hepatopancreas, Bge cells, mouse liver or S. mansoni cercariae. Poly A+ RNA was purified with the Oligotex mRNA kit (Qiagen). Genomic DNA was extracted by standard techniques (Sambrook & Russell 2001) from Bge cells, whole mollusk bodies, S. mansoni cercariae and mouse liver.

Touchdown RT-PCR

Touchdown RT-PCR was performed by the method of Don et al. (1991). Briefly, reverse transcription of 5 μg total RNA was carried out with 110 pmol random primers (Promega) and the Superscript Kit (Invitrogen). cDNA (2 μl) was then amplified with 40 pmol forward (5′-ggI TgY AAR ggI TTY TTY AA-3′) and reverse (5′-ARC TCI gWI ARI ACI CKR TCR AA-3′) degenerate primers in a total volume of 50 μl with 2.5 U of TagGold DNA polymerase (Applied Biosystems, Courtaboeuf, France), the supplied buffer and 2 mM MgCl₂. After 10 min of denaturation at 95 °C, four sets of five cycles of 95 °C for 15 s, at annealing temperatures of respectively 55 °C, 50 °C, 45 °C and 40 °C for 30 s and 72 °C for 90 s were performed, followed by 25 cycles of 95 °C for 15 s, 37 °C for 30 s and 72 °C for 90 s with an Applied Biosystems 9700 Thermocycler. Nested PCR was performed with forward (5′TgY gAR ggI TgY AAR ggI TTY TTY AA-3′) and reverse (5′-TKS IKI CgI SWR TRC TCY TC-3′) primers, 2 μl of the previous reaction and the same amplification program. Analysis of the product was carried out on 1% agarose gels in TAE buffer stained with ethidium bromide. Fragments of interest were excised from the gel, purified on silica beads (GeneClean kit, BIO 101, QBiogene, Irvine, CA, USA) and cloned into pCR 2.1-TOPO (Invitrogen). Top 10F′ electrocompotent cells were transformed with an Equibio (Boughton, Kent,
UK) electroporator under 2500 V and 25 µF in a 2-mm-wide cuvette. Plasmids from positive clones were prepared by alkaline lysis (Birnboim & Doly 1979). Sequencing was performed on an ABI 377 automated sequencer (Applied Biosystems), by the methods and reagents of the supplier. Full-length cDNA was obtained by performing 5’ and 3’ RACE with cDNA prepared from Bge cells with the SMART RACE kit (Clontech) as a template (a gift from Colette Dissous, INSERM U 547, Institut Pasteur de Lille, France), and primers derived from the sequence of the initial fragment as follows: 5’-gAA AgA gAA Agg gggTgA ggg gAg AgC-3’ and 5’-gATCCA gTg ACC AAT TgC CAg gCA gCT g-3’ for 3’-RACE and 5’-ATg gAg Cgg AgT gCA ggT AAT CgA AgA Agg Ag-3’ and 5’-Tgg CCA CCA gCT CAg TCA ggA CAC ggT-3’ for 5’-RACE.

**Sequence analysis and phylogenetic tree**

The protein sequence of BgRXR was manually aligned with homologs with Seaview (Galtier et al. 1996). All other sequences than BgRXR were taken from the non-redundant database of nuclear receptors NUREBASE (Duarte et al. 2002), families NRB010202 and NRB010201. These data can be accessed at www.ens-lyon.fr/LBMC/laudet/nuibase/nuibase.html. The tree was rooted with *T. cystophora* RXR as an outgroup. Only complete sites (no gap, no X) were used for phylogenetic analysis. Trees were constructed by Maximum Likelihood, as implemented in PhyML (Guindon & Gascuel 2003), with the JTT substitution model; rate heterogeneity between sites was corrected by a gamma law with eight parameters (alpha estimated by PhyML). The reliability of branches was evaluated by bootstrap with 1000 replicates (Felsenstein 1985), and by likelihood tests as implemented in Tree-Puzzle (Strimmer & Von Hessler 1996).

**Northern and Southern blotting**

Electrophoresis of poly A+ RNA from the mollusk was carried out alongside RNA size markers from New England Biolabs (Beverley, MA, USA) in a 1% agarose/3% formaldehyde gel in MOPS buffer (Lehrach et al. 1977). The gel was then blotted onto a Hybond N+ membrane (Amersham). The full-length cDNA probe was α32P-labeled by the Random Primers DNA Labelling System (Invitrogen). A probe corresponding to the complete coding sequence of *B. glabrata* actin (Lardans et al. 1997) was labeled in the same way and used as a control. Hybridization was carried out as described elsewhere (Sambrook & Russell 2001), and blots were exposed overnight to X-Omat AR film (Kodak). Genomic DNA (25 µg) was digested with EcoRV and BamHI and separated on a 1:2% agarose gel in TAE buffer. After transfer to a Hybond N+ membrane, hybridization was carried out by a standard method (Sambrook & Russell 2001) with a 474 bp probe (nucleotides 589–1062 of the BgRXR cDNA) radiolabeled as above. After stringent washes, the blot was exposed overnight to X-Omat AR film.

**Antibodies**

Two ovalbumin-coupled peptides (supplied by Syntem, Nimes, France) covering the residues 177–191 and 371–386 of BgRXR were used to immunize two New Zealand rabbits (IFFA-Credo, Larbresle, France) (Vaitukaitis et al. 1971). Sera were collected 2 months after the first injection and tested for the presence of specific antibodies by ELISA against uncoupled peptides adsorbed onto Maxisorp immunoplates (Nunc, Roskilde, Denmark). The purified immunoglobulin (Ig) G fraction of these sera, as well as commercial antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), directed against the LBD of human RXRα (residues 198–468), was also tested by Western blotting.

**Western blotting and immunohistochemistry**

Mollusks were frozen in liquid nitrogen and homogenized in sperms buffer containing NH4Cl 150 mM, EDTA 100 mM, NaHCO3 100 mM and PMSF 1 mM with an Ultraturax (IKA, Labortechnik, Staufen, Germany). Bge cells were resuspended in the same buffer, and both were sonicated three times for 10 s (maximum power; Mircroson XL, Misonix, Farmingdale, NY, USA). The homogenates were centrifuged 20 800 g for 30 min, and the protein content of the supernatant was measured with the BCA assay kit (Pierce, Rockford, IL, USA). Each extract (1·4 mg) was separated on a 10% SDS/polyacrylamide gel and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Bge cells were resuspended in the same buffer, and both were sonicated three times for 10 s (maximum power; Mircroson XL, Misonix, Farmingdale, NY, USA). The homogenates were centrifuged 20 800 g for 30 min, and the protein content of the supernatant was measured with the BCA assay kit (Pierce, Rockford, IL, USA). Each extract (1·4 mg) was separated on a 10% SDS/polyacrylamide gel and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) (Towbin et al. 1979). Blots were developed with either the purified IgG fraction of anti-peptide antisera or commercial antibodies at a dilution of 1/4000 in PBS/skim milk 1% (w/v) and peroxidase-coupled antirabbit IgG antiserum (Sigma) at a 1/20 000 dilution in PBS/skim milk 1% (w/v) and peroxidase-coupled antirabbit IgG antiserum (Sigma) at a 1/20 000 dilution in PBS/skim milk 1% (w/v). Detection was carried out by chemiluminescence with the West-Pico detection kit (Pierce). Immunofluorescence detection of recombinant BgRXR expressed in transfected cells was performed as described elsewhere (De Mendonça et al. 2000b).

**Electrophoretic mobility shift assay (EMSA)**

BgRXR cDNA was cloned into XhoI/KpnI digested pTL1 (a modified version of pSG5; Stratagene, La Jolla, CA, USA), for transient transfection assays and in vitro translation. Vectors expressing potential heterodimer partners were as follows: human vitamin D receptor and human RARα, cloned in pSG5, were gifts from...
Transactivation assays

Transactivation experiments with intact BgRXR or murine RXRα cloned into pT1L1 were done with the Apo-AI-tk-luc reporter gene (de Urquiza et al. 2000) (a gift from T Perlmann, Ludwig Institute, Stockholm, Sweden). In order to test the activity of the LBD independently of the DNA-binding properties of the receptors, constructs allowing fusion of the LBD to the Gal4 protein were used. An EcoRI fragment encoding amino acids 1–143 of the Saccharomyces cerevisiae Gal4 protein was cloned into pT1L1 (pT1L1-Gal4). The oligonucleotide primers 5′—ggA AgC TTA AgC gAg AAg CTg TCC Agg Agg AA-3′ and 5′—AAg Cgg CCg Cgg ATT ggT TgC TgAg CTg CAT CCT-3′ or 5′—AAg Cgg CCg CCT AAA ATg TgT gTC AAT ggg CTg gTC A-3′ were used respectively to amplify the regions encoding BgRXR hinge and ligand domains (amino acids 183–436) and the hinge and ligand domain truncated at the C-terminal end (amino acids 183–424; ΔAF2). Resulting PCR products were cloned into pCR 2·1-TOPO and sequenced. Inserts containing no errors were excised with HindIII and NotI and cloned into the corresponding sites in pT1L1-Gal4 in frame with the Gal4 DNA-binding domain (DBD) and LBD of murine RXRα. 

Limited proteolytic digestion

For limited proteolytic digestions (Vivat et al. 1997), in vitro synthesized 35S-labeled BgRXR (TNT kit, Promega) was used. Briefly, after incubating on ice for 30 min with ligands, receptor proteins were digested at 25 °C for 10 min with 50 mg/ml trypsin. The proteolytic fragments were separated on a 15% SDS-PAGE polyacrylamide gel and visualized by autoradiography.

Results

Isolation of BgRXR from the mollusk B. glabrata

Touchdown PCR followed by nested touchdown PCR on cDNA from Bge cells with degenerate primers flanking the DNA-binding domain (DBD) and LBD of RXR allowed us to obtain a single band of 833 bp, which contained an open reading frame (ORF), including the nuclear receptor signature DNA and LBDs, sharing a high level of peptide sequence identity (89% and 81% respectively) with those of mouse RXRα and β. Bovine aortic endothelial cells (BAE cells) were obtained by scraping the aorta excised from a freshly slaughtered cow. The different clones of cells were cultured in DMEM containing fibroblast growth factor (1 ng/ml) with 10% newborn calf serum. The cultures used in the present study had undergone six passages. Cells were transfected with 1–1·2 µg total DNA per assay and polyethyleneimine (4 µl Ex Gen 500, Euromedex, Mundolsheim, France) under the conditions recommended by the supplier. The pT1L1 plasmid was used as a carrier when necessary. Ligands (ethanol solution of 9-cis retinoic acid 10 mM; ethanol solution of cis-4,7,10,13,16,19-docosahexanoic acid (DHA) (de Urquiza et al. 2000) (Sigma) (100 mM); a DMSO solution of the RXR specific agonist LG268 (10 mM; a gift from B. Staels) or a 10 mM solution of WY-14643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid) (Alexis Biochemicals, Lausen, Switzerland) in DMSO were incubated with the cells overnight. Cells were lysed in the commercial reporter lysis buffer (Promega) and assayed for luciferase (assay kit, Promega) in a Wallac Victor 1420 multilabel counter (Perkin-Elmer, Wellesley, MA, USA).
then designed, flanking this ORF, and RT-PCR was carried out to obtain a unique clone encompassing the full-length coding sequence of BgRXR. Although this clone carried the complete ORF, it did not represent the entire mRNA sequence, since Northern blotting of snail poly A+ RNA revealed a unique band of 5 kb (Fig. 1A, lane 1), while a blot probed with the B. glabrata actin cDNA gave a band at the expected size of about 3 kbp (lane 2). This indicates that about 3280 bases of UTR are missing from the BgRXR cDNA. Although the resulting sequence terminated with a poly A tail, the absence of a consensus polyadenylation signal (AAG AAA instead of AATAAA) suggested that the 3’UTR, as well as the 5’UTR, was incomplete. No variant transcripts were identified during RACE-PCR experiments, suggesting that the BgRXR gene does not produce alternatively spliced or truncated forms of the mRNA encoding protein isoforms, although this phenomenon has been described for RXRs in other organisms (Guo et al. 1998, Kostrouch et al. 1998).

The snail origin of the sequence was further confirmed by Southern hybridization using EcoRV and BamHI-digested genomic DNA from Bge cells and, as controls, DNA from S. mansoni cercariae and from mice. No signal was obtained with either mouse or schistosome DNA, whereas a single band of about 4·5 kbp was obtained with Bge cell DNA (not shown). Neither restriction enzyme used cuts within the BgRXR cDNA sequence.

The BgRXR peptide sequence is highly conserved compared with mammalian RXRs

The sequence we obtained encodes a protein of 436 amino acids with an estimated size of 48 kDa. This protein shares the modular structure characteristic of the nuclear receptor superfamily with a divergent N terminal A/B domain, a highly conserved DBD and a less well conserved LBD. As usual in other RXRs, there is no F domain following the LBD (Chung et al. 1998).

The DBD of BgRXR is extremely well conserved (Fig. 2A) compared with Drosophila USP and vertebrate RXR DBDs, whereas the LBD shows greater identity to vertebrate RXRs than to Drosophila USP (Fig. 2B). Within the BgRXR DBD, the P-box, involved in determining DNA binding specificity, is 100% identical with those of other RXRs and USPs, while the D-box, which contributes to the homodimerization interface, contains an amino acid substitution compared with vertebrate homologs (D156 substituted for N in mouse RXRα) (Fig. 2A). Furthermore, in another helix known to participate in this interface (Lee et al. 1993) (amino acids 170–180), Q175 and A179 are replaced by M and S respectively (Fig. 2A). These differences could explain specific dimerization properties of BgRXR as described below.

The LBD displays a higher level of identity to vertebrate RXRs than to arthropod USPs (75-81·5% and 43·8–68·7% respectively (Fig. 2B), except for the locust Locusta migratoria USP: 80·1% (Hayward et al. 1999). The overall sequence conservation of the BgRXR LBD is consistent with the maintenance of the canonical nuclear receptor fold (Wurtz et al. 1996) with 11 helices (H1, H3–H12) and two short strands (s1 and s2). Several subdomains, such as the helix-turn zipper in helix H4 and the regulatory zipper (helices H7–H8), are well conserved. The identity or I-box, which is involved in determining the specificity of heterodimerization, (Zhang et al. 1994, Perlmann et al. 1996) and other residues involved in interactions with heterodimer partners (Bourget et al. 2000) are perfectly conserved (Fig. 2B). The turn, a subdomain involved in binding pocket formation, mainly in association with helices H3, H5, H7 and H11 (Egea et al. 2000), is completely conserved, as are the amino acids involved in the ligand entering and anchoring in the ligand-binding pocket (Fig. 2B). The helix H11 is also 100% identical to those of vertebrate RXRs. The consensus signature of the LBD ((F,W,Y)A(S,L))[(K,R,E,G)XXX(F,L)XX(I,V,L)]
XXX(D,S)(Q,K)(X,L,V)(L,I,F)) (Brzozowski et al. 1997, Xu et al. 1998, Kraichely et al. 1999), extending from the C terminal of H3 to the middle of H5, is completely conserved in BgRXR. All these elements suggest that, unexpectedly, BgRXR may have a similar ligand-binding pocket to that of mouse RXR/afii9825. This is also indicated by the perfect conservation of residues that have been shown to interact with 9-cis RA in mouse RXR/afii9825 (Egea et al. 2000) (Fig. 2B). Moreover, apart from one substitution, helix H12, and within it the activating domain AF2–AD, are completely conserved compared with vertebrate RXRs. The consensus core motif of the activating domain, designated ϕϕXEϕϕ where ϕ represents a hydrophobic amino acid, is present in the mollusk receptor. The AF2–AD is known to interact in a ligand- or phosphorylation-dependent manner with the transcriptional machinery through transactivation intermediary factors (TIF) (Durand et al. 1994, Voegel et al. 1996, vom Baur et al. 1996, Hammer et al. 1999), and its conservation could indicate that BgRXR may interact with TIFs that are closely related to those identified in vertebrates. The only major difference in the LBD structure of BgRXR compared with mouse RXR concerns the loop connecting helix H1 to helix H3, which is eight residues shorter in the former (Fig. 2B). However, this region is often found to be extremely...
variable in length and in sequence, consistent with its nature as a flexible and loosely structured region (Billas et al. 2001).

**Phylogenetic analysis**

Because of the similarity between RXR LBDs, a simple distance phylogenetic reconstruction, with rate heterogeneity between sites not taken into account in this reconstruction, groups BgRXR with vertebrate RXRs (data not shown). With a more realistic model under maximum likelihood, BgRXR is placed in a manner consistent with known relationships between metazoans (Fig. 3). In addition to the bootstrap test, likelihood phylogenetic tests significantly support the position of BgRXR as being neither an arthropod nor a vertebrate RXR (Expected likelihood weight test (Strimmer & Rambaut 2002) \( P < 0.011 \)). The relationships we recover are consistent with previous analyses (Laudet et al. 1992, Gronemeyer & Laudet 1995, Laudet 1997), notably including rapid evolution of the USPs. This fast evolution is again consistent with the higher sequence and functional resemblance between vertebrate RXRs and BgRXR, rather than between BgRXR and USPs.

**The protein BgRXR**

We raised antibodies against synthetic peptides derived from the sequence of BgRXR in order to detect the expression of the BgRXR protein by Western blotting.
In addition, in view of the marked sequence identity of the LBD with mouse RXRα, we also tested whether commercial antibodies specific for the C terminal portion of this NR could recognize BgRXR. After in vitro transcription and translation, the 58 kDa protein produced was recognized by both commercial and BgRXR-specific antibodies (not shown) on a Western blot (Fig. 1B). This size does not correspond to the theoretical molecular mass of the unmodified BgRXR protein (48 kDa) and may be due to incorrect folding of the molecule synthesized in vitro. Western blotting (Fig. 1B) using either our antibodies (not shown) or commercial antibodies with the soluble protein fraction of mollusk homogenate as antigen led to the detection of a single band of 52 kDa, closer to the theoretical molecular mass. Moreover, we always obtained a unique band indicating that the presence of several Kozak consensus sequences (Kozak 1991) in the ORF do not interfere with the translation mechanism.

**BgRXR binds to direct repeat elements as a homo- or heterodimer**

In order to determine whether BgRXR could bind to consensus RXR response elements, electrophoretic mobility shift assays (EMSA) were carried out with labeled probes containing the DR response element incubated with recombinant protein transcribed and translated in vitro. A weak band was obtained with BgRXR alone on the DR1 response element (Fig. 4), whereas mouse RXRα used as a control bound strongly to DR1 as expected (Mader et al. 1993). Various mammalian nuclear receptors were tested as heterodimer partners since, so far, no other *B. glabrata* nuclear
receptors have been characterized. BgRXR heterodimerizes weakly with human RARα and LXR and more strongly with human FXR and PPARα (Fig. 4). Both direct binding and competition experiments showed that binding was strongest with both RARα and PPARα as partners on the DR1 element, was weaker on DR2 and DR5, and was much weaker on DR3, DR4 and a palindromic response element (data not shown). Unexpectedly, BgRXR was unable to form heterodimers on the DR1 element with one other common partner of vertebrate RRRs, VDR (Fig. 4), in contrast to Drosophila USP, which does form heterodimers with this receptor.

**BgRXR transactivates reporter gene transcription in the presence of 9-cis retinoic acid**

In order to determine whether BgRXR was capable of transactivating a reporter gene in mammalian cell lines, we constructed a pTL-1-based plasmid expressing BgRXR under the control of the SV40 promoter. Several different cell lines were used in this study in order to eliminate line-specific effects in these heterologous expression systems. Immunolocalization showed that the BgRXR protein was expressed in the nucleus of transfected CV-1 cells (not shown). Surprisingly, preliminary experiments showed that BgRXR was unable to promote transactivation of a reporter gene as a heterodimer partner of PPARα, either with the ligand of the latter, WY-14643, or with the RXR ligand 9-cis RA, despite the strong binding of PPARα to BgRXR shown in EMSA. In contrast, BgRXR on its own consistently transactivated transcription in the presence of 9-cis RA or LG268, an RXR-specific agonist, despite the fact that it failed to form homodimers on DR elements in EMSA. We therefore concentrated our efforts on determining whether this transactivation was specific and could be unambiguously attributed to functional interaction between the vertebrate RXR ligands and the BgRXR LBD.

We therefore sought to confirm the transactivation observed with BgRXR alone in the presence of RXR ligands by determining the dose-dependency of transactivation with the Apo-A1-tk-luc reporter gene (de Urquiza et al. 2000) in the 293T and Cos-1 cell lines. Figure 5A shows the results obtained in the 293T cell line (Cos-1 cells gave identical results) and the dose-dependent activation of transcription by 9-cis RA obtained, whether with mouse RXRα or BgRXR. A clearly dose-dependent activation was obtained with the latter, although it was significantly lower than that observed with mouse RXRα. Activation above the background level was significant at a concentration of 10⁻⁸ M 9-cis RA. We next used the specific RXR ligand DHA (de Urquiza et al. 2000) at a concentration of 10⁻⁴ M in the same cell lines (Fig. 5B). Activation by mouse RXRα was much greater, but again a significant activation of transcription in the presence of BgRXR was noted.

Despite the absence of ligand-dependent transactivation by control cells transfected with the reporter plasmid alone, the observed transactivation by BgRXR could still have been due to the formation of a permissive heterodimer with an endogenous RXR, the expression of which had been induced by that of BgRXR. In order to test our hypothesis that the ligand domain of BgRXR binds 9-cis RA and that it is this binding that allows transactivation of transcription, two chimeric proteins were expressed in mammalian cells. The first comprises the hinge and LBD of BgRXR (amino acids 183–436) fused to the Gal4 DBD (Gal4–BgRXR) and the second comprises the hinge domain and a C-terminally truncated LBD of BgRXR (amino acids 183–424) that is deleted for the AF2-AD, again fused to the Gal4 DBD (Gal4–BgRXR ΔAF2). The plasmids encoding these proteins were cotransfected into Cos-1 or BAE cells together with a reporter plasmid containing the Gal4 recognition sequence upstream of the luciferase gene. Figure 6 shows results obtained for a representative experiment using BAE cells. Results obtained with Cos-1 cells were very similar. Transactivation of transcription above the background level obtained with the vector expressing the Gal4 DBD alone occurred only in the presence of 9-cis RA and with the intact BgRXR LBD. The ΔAF2 construct failed to transactivate transcription in the presence of the ligand. Indeed, the levels of luciferase expression obtained were consistently below those produced by the Gal4 DBD alone. This indicates that the ΔAF2 construct squelches the background transcription level due to the Gal4 DBD. More importantly, the results demonstrate that transactivation obtained in the presence of intact BgRXR is due to the ligand-dependent interaction between the AF2–AD and the cellular transcriptional machinery. Moreover, although, again, we cannot rule out heterodimer formation, the activation we observed is clearly not due to heterodimerization with an endogenous retinoid receptor in the mammalian cells that itself binds the ligand and drives transcription. A construct expressing a Gal4 DBD fusion protein with the mouse RXRα hinge and LBD (Gal4-mRXRα) also transactivated transcription exclusively in a ligand-dependent manner in BAE cells (Fig. 6) and Cos-1 cells. The levels of activation above the background were superior (about threefold) to those obtained with BgRXR (about twofold), but this is consistent with the results obtained with the complete receptors on their response element (Fig. 5) and may indicate that 9-cis RA is not an optimal ligand for BgRXR.

Since BgRXR bound to DR1 elements only weakly as a homodimer, we sought to demonstrate homodimer formation after transfection into mammalian cells. Using the mammalian two-hybrid system, with the BgRXR hinge and LBD fused to the Gal4 DBD, on the one hand,
and to the VP16 activation domain, on the other hand, we were able to show that homodimerization of the LBD did indeed occur (Fig. 7). Increasing amounts of the BgRXR-VP16 constructs increased the transactivation of the luciferase reporter gene in a dose-dependent manner up to threefold over the background obtained with the Gal4-BgRXR construct alone and in the absence of ligand. In contrast, the heterodimerization between the BgRXR-VP16 construct and a fusion between Gal4 DBD and the human RARα LBD produced a stronger signal up to sixfold background. The stronger interaction with a heterodimer partner in part supports the EMSA results and agrees with the generally observed stronger interaction of mammalian RXR and heterodimer partners, compared with homodimerization of these receptors (Vivat-Hannah et al. 2003).

**BgRXR binds 9-cis RA in vitro**

To demonstrate that BgRXR physically binds 9-cis RA, we carried out a proteolysis protection assay. BgRXR produced in vitr0 in rabbit reticulocyte lysate was preincubated with 9-cis RA, DHA or other fatty acids at concentrations from $10^{-4}$ to $10^{-6}$ M and then incubated with trypsin. Only the addition of 9-cis RA stabilized the protein (LBD) against proteolysis at all concentrations (Fig. 8). The failure of DHA to do so, despite the fact that this ligand did have a moderate effect on transactivation of transcription by BgRXR may be linked to the high concentrations necessary for the latter, and the low affinity of BgRXR for DHA implied by this. Other fatty acids (but not juvenile hormone III) had a stabilizing effect only at the highest concentration, perhaps indicating that they can enter the ligand-binding pocket, but are not bound with a high affinity.

**Discussion**

The RXR family nuclear receptor from the freshwater snail *B. glabrata* that we describe is highly conserved, both in structure and function, particularly with respect
to vertebrate RXRs, and is the first RXR from an invertebrate that has been shown to interact functionally with vertebrate RXR ligands. This suggests that retinoid signaling, which evolved early in the Metazoa but has not been demonstrated in the ecdysozoan moulting invertebrates, is shared between the lophotrochozoan branch of the protostomes (Aguinaldo et al. 1997) and the deuterostomes.

The observed sequence conservation which extends over both the DBD and the LBD is particularly high.

Figure 6 Activation of transcription in BAE cells transfected with Gal4 constructs. Plasmids expressing the hinge and ligand-binding domains of BgRXR (Gal4–BgRXR), or mouse RXRa (Gal4–mRXRa), or the hinge and ligand domains of BgRXR deleted for the AF2-AD (Gal4–BgRXR ΔAF2) as fusion proteins with Gal4 DNA binding domain, or the Gal4 DBD alone were cotransfected with the pGal5-TK-pGL3 reporter plasmid into BAE cells incubated in the presence or absence of 10⁻⁶ M 9-cis RA. Results are presented as luciferase units and are representative of two separate triplicate assays.
On the phylogenetic tree obtained, it is notable that BgRXR has evolved much more slowly than either USPs or the RXRs from the lophotrochozoan parasite S. mansoni, SmRXR1 and SmRXR2 (De Mendonça et al. 2000b), and is thus much less divergent from vertebrate RXRs (Fig. 3). Both vertebrate and Biomphalaria RXRs appear to evolve slowly, and seem to represent the most conserved form of RXR. Given the structural conservation of the DBD of BgRXR, it is surprising that it fails to bind more strongly to the DR response elements as a homodimer in EMSA and differs in this respect from vertebrate RXRs. This might be due to the residue substitution described above (Fig. 2A) in part of the DBD known to be important for homodimer formation. However, it is well known that some other members of the RXR subfamily, vertebrate or arthropod, are dependent on a heterodimer partner to bind to their response element. For example, Drosophila

Figure 7 BgRXR LBD forms homodimers in mammalian cells. (A) Dose–response of interaction between the Gal4–BgRXR and BgRXR–VP16 constructs, expressing the BgRXR LBD fused to the Gal4 DBD or the VP16 activation domain respectively, in the mammalian two-hybrid assay (CV-1 cells). Amounts of 200, 500 and 800 ng BgRXR–VP16 construct were transfected in the presence of 200 ng Gal4 BgRXR and 200 ng reporter plasmid. Amounts of DNA were adjusted to 1-2 µg with the pVP16 plasmid. Results are presented as fold activation compared with the pVP16 plasmid cotransfected with Gal4–BgRXR and are representative of two separate triplicate assays. (B) The BgRXR LBD heterodimerizes with the human RARα LBD in the mammalian two-hybrid assay. A construct expressing the human RARα LBD fused to the Gal4 DBD (Gal4-hRARα, 200 ng) was cotransfected with the BgRXR-VP16 construct (800 ng) and reporter plasmid (200 ng) in CV-1 cells. Results are presented as fold activation compared with the pVP16 plasmid cotransfected with Gal4–hRARα.

Journal of Molecular Endocrinology (2005) 34, 567–582.
USP can only bind as a heterodimer with a variety of partners (Yao et al. 1992). Similarly, Danio rerio RXRβ can bind to DR1 as a heterodimer with D. rerio RARγ and produce a shifted band in EMSA but is unable to do so alone (Jones et al. 1995). It is also noteworthy that BgRXR is not able to bind as a heterodimer with one of the vertebrate nuclear receptors, VDR, that interacts with Drosophila USP (Yao et al. 1992). The very strong interaction noted with PPARα probably does not indicate the presence of a PPAR in B. glabrata, however, as this subfamily (as is the case for RAR) has been detected only in vertebrates (Laudet 1997), even if the presence of subfamily I nuclear receptors in lophotrochozoans cannot be excluded (Bertrand et al. 2004). The heterodimeric complexes containing BgRXR were able to bind to either the DR1 or the DR5 response elements. Vertebrate RXR–RAR heterodimers bind to each element with an opposite polarity and this in turn determines different responses to ligands and coactivators. This property is associated with a switch in activity from repression to activation of retinoic responsive genes (Kurokawa et al. 1994, 1995). The BgRXR–PPARα heterodimer should bind only to the DR1 element (Kliewer et al. 1992) and it is possible that the amino-acid substitution in the D box of BgRXR may induce a change in binding specificity due to a modification of the dimerization interface.

In mammalian RXR the major dimerization interface is present on the LBD and implicates residues from helices H7 to H10 and loops L8–9 and L9–10 (Bourguet et al. 2000). Among these, the I-box in H10 is critical for the determination of identity in heterodimeric interactions (Perlmann et al. 1996), whereas the mutation of a single Tyr residue in H9 to Ala favors the formation of homo- over heterodimers (Vivat-Hannah et al. 2003). Both the I-box and the H9 Tyr residue are perfectly conserved in BgRXR, suggesting that, like its mammalian counterparts, the mollusk receptor functions primarily as a heterodimer partner. However, while the results obtained by the mammalian two-hybrid assay confirm that the heterodimeric interactions of the BgRXR LBD with partners are more stable, possibly explaining why they are stronger in EMSA, they also support the assertion that BgRXR can function as a homodimer.

The transactivation of transcription observed in three different cell lines by BgRXR on its own in the presence of 9-cis RA or of specific RXR agonists is the first such demonstration for an invertebrate RXR. However, an alternative explanation for the observed transactivation with BgRXR alone is the presence of an endogenous nuclear receptor in the mammalian cells that formed a heterodimer with BgRXR, resulting in a 9-cis RA activated complex. This explanation applies, provided that these heterodimers are permissive; that is, that they could be activated by ligands of both receptors regardless of the order of binding (Kliewer et al. 1992, Willy et al. 1995, Willy & Mangelsdorf 1997). However, this hypothetic partner is not an RXR or an RAR since no transactivation was detected in cells transfected with the reporter plasmid alone in the presence of ligand. Moreover, the fact that DHA activates BgRXR is of particular interest since it has been proposed as a natural ligand of mammalian RXR (de Urquiza et al. 2000). Moreover, it is specific for RXR and will not activate RAR, unlike 9-cis RA, which does have some effect on the latter and this indicates that the observed transactivation is indeed due to the activation of BgRXR. This conclusion is further supported by the results obtained with chimeric Gal4-BgRXR constructs transfected into mammalian cells. In this system, transactivation of a reporter gene was dependent on the presence of the ligand and the AF2-AD of the receptor. This shows that transactivation occurred via the ligand-dependent interaction of the BgRXR AF2 domain with the host cell transcriptional machinery and was not due to the formation of an active heterodimer with an endogenous receptor. In the latter case transactivation should have occurred with the ΔAF2 construct, whereas in fact, in this situation, the reporter gene expression was greatly reduced compared with the background level obtained with the Gal4 DBD alone. Moreover, we have shown that the BgRXR LBD can form homodimers in transfected mammalian cells with
the two-hybrid assay, supporting the view that the transactivation of transcription in cells transfected with the full-length BgRXR is due to this receptor alone. Finally, the observation that 9-cis RA stabilizes BgRXR toward trypsin proteolysis further supports the hypothesis that it binds this ligand.

The transactivation of transcription by BgRXR in the presence of both 9-cis RA and DHA was clearly significant, but much less marked than that obtained with mouse RXRa. This could be due in part to the use of heterologous mammalian cell lines and the consequent suboptimal interaction between the mollusk receptor and the endogenous transcriptional coactivators. However, it may also suggest that neither of the compounds tested represents the natural BgRXR ligand. In mammals, the status of 9-cis RA as a natural RXR ligand is controversial since it is undetectable in normal tissues in vivo, even using highly sensitive techniques (Ulven et al. 2001). DHA has since been suggested as a natural ligand in mammals (de Urquiza et al. 2000) although it is active at much higher concentrations than 9-cis RA. Its presence or absence in B. glabrata, as is the case for other ‘rexinoids’, is at present unknown and requires urgent study.

The only other invertebrate RXR that has been shown to interact with 9-cis RA is the T. cystophora receptor, which was shown to bind the ligand, but for which no ligand-dependent transactivation of transcription was demonstrated (Kostrouch et al. 1998). The fact that BgRXR was able to transactivate transcription in a ligand-dependent manner may be due to the high degree of conservation, not only of the LBD itself, including residues known to interact with the ligand in mouse RXRa (Egea et al. 2000), but also of the interfaces responsible for cofactor binding, allowing its activity in a heterologous cell system. Our results favor the conservation of RA signaling in triploblastic invertebrates, but this may have been lost in the Ecdysozoa. In the arthropods the LBD sequences can be divided into two groups (Hayward et al. 1999), with RXR/USP family members from crabs, ticks and hemimetabolous insects showing far greater similarity to vertebrate RXR LBDs than do those from Diploptera and Lepidoptera (Fig. 3). However, the RXR/USPs from the former group do not activate transcription in the presence of either 9-cis RA or a synthetic retinoid (Guo et al. 1998). It is clear that vertebrate-like RXRs are absent from both Drosophila (Adams et al. 2000) and the nematode Caenorhabditis elegans (Sluder et al. 1999), and the latter even lacks a USP homolog, although one is present in the parasitic nematode Dirofilaria immitis (Sluder & Maina 2001, Shea et al. 2004).

The presence of a highly conserved, functional, vertebrate-type RXR in a mollusk poses the problem of the evolution of ligand binding in the RXR family, and in the nuclear receptor superfamily in general. Recently, an estrogen receptor (ER) α-related receptor was isolated from another mollusk, Aplysia californica (Thornton et al. 2003), but it failed to bind estradiol and was constitutively active in transactivation assays. However, the structure of a hypothetic ancestral ER was predicted and the corresponding molecule was synthesized. This did bind estradiol, albeit feebly, suggesting that the bona fide ancestral ER may have bound a steroid ligand, and that this capacity was lost in Aplysia ER. There is no need to reconstruct an ancestral RXR since BgRXR is both highly conserved and transactivates transcription under the control of ligand. Taken together with the diploblast T. cystophora RXR which binds 9-cis RA, and the RXR family receptor in the sponge S. domuncula, the expression of which is upregulated on treatment with RA, our results support the view that ligand binding and its function in the control of transcription was acquired during the evolution of the nuclear receptor superfamily (Escriva et al. 1997).

Acknowledgements

This work was supported by the Institut National de la Santé et de la Recherche Médicale (U 547), the Centre National de la Recherche Scientifique (UMR 49), the Institut Pasteur de Lille, the École Normale Supérieure de Lyon and the microbiology program of the Ministère de l’Education Nationale de la Recherche et de la Technologie. D B benefited from the Fondation des Treilles and by the Institut Pasteur de Lille, the Région Nord-Pas de Calais and the Fondation pour la Recherche Médicale. R M was supported by the Fondation des Treilles and by the Institut Pasteur de Lille. H E was supported by the European Molecular Biology Organization. B B was supported by a grant from the Ministère de l’Education Nationale de la Recherche et de la Technologie.

References


Billas IM, Moulinier L, Rochel N & Moras D 2001 Crystal structure of the ligand-binding domain of the ultraspiracle protein.


Mader S, Chen Y, Chen Z, White J, Chambon P & Gronemyer H 1993 The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding...
specificities of the DNA binding domains. EMBO Journal 12 5029–5041.


Sluder AE, Mathews SW, Hough D, Yin VP & Maina CV 1999 The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. Genome Research 9 105–120.


Towbin H, Stachelin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. PNAS 76 4350–4354.


Willy PJ & Mangelsdorf DJ 1997 Unique requirements for retinoid-dependent transcriptional activation by the orphan receptor LXR. Genes and Development 11 289–298.


Accepted 5 January 2005

Received 15 December 2004

Made available online as an Accepted Preprint 12 January 2005