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Retinoid X receptor and retinoic acid response in the marine sponge *Suberites domuncula*

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Summary

To date no nuclear receptors have been identified or cloned from the phylogenetically oldest metazoan phylum, the Porifera (sponges). We show that retinoic acid causes tissue regression in intact individuals of the demosponge *Suberites domuncula* and in primmorphs, special three-dimensional cell aggregates. Primmorphs were cultivated on a galectin/poly-L-lysine matrix in order to induce canal formation. In the presence of 1 or 50 μmol l^{-1} retinoic acid these canals undergo regression, a process that is reversible. We also cloned the cDNA from *S. domuncula* encoding the retinoid X receptor (RXR), which displays the two motifs of nuclear hormone receptors, the ligand-binding and the DNA-binding domains, and performed phylogenetic analyses of this receptor. RXR expression undergoes strong upregulation in response to treatment with retinoic acid, whereas the expression of the sponge caspase is not increased. The gene encoding the LIM homeodomain protein was found to be strongly upregulated in response to retinoic acid treatment. These data indicate that the RXR and its ligand retinoic acid play a role in the control of morphogenetic events in sponges.

Key words: sponge, Porifera, *Suberites domuncula*, retinoid X receptor, retinoic acid, canal formation, morphogenesis, primmorphs, functional molecular evolution.

Introduction

Sponges (phylum Porifera) represent the oldest, still extant taxon of the common ancestor of all metazoans, the Urmetazoa (Müller, 2001). The sponge body is highly organized, particularly the aquiferous system composed of inhalant and exhalant canals, passing the choanocyte chambers (Müller, 1982; Simpson, 1984). This complex body plan in Demospongiae and Hexactinellida is organized around a siliceous and in Calcarea around a calcareous skeleton. Such a structural organization only became possible because the animals developed a sequential differentiation pattern, starting from the toti/omni-potent sponge archaeocytes (see Koziol et al., 1998). Archaeocytes have an (almost) unlimited proliferation potency (Koziol et al., 1998; Müller, 2002), which results in the formation of differentiated, functionally determined cells, e.g. collencytes, which synthesize collagen, or sclerocytes, which form the spicules (reviewed in Simpson, 1984; Cha et al., 1999; Krasko et al., 2000; Uriz et al., 2000). We have previously described sponge molecules, cell-surface bound receptors and extracellular matrix molecules, which are required for the tuned interaction of cells. In sponges the establishment of a bodyplan is organized through these proteins (reviewed in Müller, 1997; Müller et al., 2001; Wimmer et al., 1999a,b).

Sponges require morphogenetic factors acting extracellularly, e.g. myotrophin (Schröder et al., 2000), and nuclear receptors, acting intracellularly, e.g. allograft inflammatory factor in conjunction with the Tcf-like transcription factor, for their differentiation (Müller et al., 2002). Sponges even possess specific homeodomain proteins, e.g. a LIM/homeobox protein (Wiens et al., in press). The introduction of primmorphs, special three-dimensional aggregates composed of proliferating cells that have retained their differentiation potency and thus represent an *in vitro* cell culture system (Müller et al., 1999), was another very important step towards the elucidation of potential morphogenetic factors/processes. This system provided proof that homologous cell substrate molecules, e.g. galectin, cause signaling events in non-structured primmorphs that ultimately result in the formation of canals, a process very likely correlated with the expression of LIM/homeobox transcription factors (Wiens et al., in press). Canal formation not only occurs in primmorphs but is also one of the major morphogenetic processes observed during hatching of sponges from gemmules, the asexual reproduction bodies (Imsiecke et al., 1994), and during embryogenesis (see Simpson, 1984; Weissenfels, 1989).

Gemmules are formed by many sponges in response to adverse physiological or ecological factors (see Simpson, 1984). For example, gemmulation occurs in response to an increased bacterial load (Rasmont, 1963) or change in light

intensity (Rasmont, 1970); in addition to exogenous factors, endogenous factors are also known to initiate gemmule formation (Simpson and Gilbert, 1973). Focusing on *S. domuncula*, under experimental conditions (Böhm et al., 2001) and in the field, the formation of gemmules is very frequently seen after infection of the specimens with bacteria.

Retinoids and carotenoids have been detected in sponges (Biesalski et al., 1992). In *Geodia cydonium* retinoic acid reduces cell metabolism (Biesalski et al., 1992) but does not induce gemmule formation. In the freshwater sponge *Ephydatia muelleri*, however, retinoic acid initiates morphogenetic events leading to formation of buds/gemmules (Imsiecke et al., 1994). At micromolar concentrations, retinoic acid causes a downregulation in the expression of the *EmH-3* homeobox-containing gene in *E. muelleri* (Nikko et al., 2001), which led the authors to conclude that retinoic acid and the responsive *EmH-3* gene are involved in differentiation and re-differentiation of archaeocytes to choanocytes, and hence in formation of a functional aquiferous system.

In the coral *Acropora millepora*, receptors were detected that share some similarity to retinoid X receptors (RXRs) (Grasso et al., 2001), but to date molecular data on the presence of nuclear hormone receptors in the Porifera, the phylogenetically oldest metazoan phylum, are missing. Several RXRs are activated by known regulatory ligands; receptors for whom the ligands have not yet been identified were termed 'orphan receptors' (Aranda and Pascual, 2001). Experimental identification of nuclear receptors in sponges would reinforce the theory of monophyletic evolution of animal genomic regulatory systems, as with e.g. cell surface receptors and the receptor tyrosine kinases (Schäcke et al., 1994), both establishing the basis for common cell–cell/cell–matrix regulatory networks present in all Metazoa (Müller et al., 1994).

For an initial search of a nuclear hormone receptor in the phylogenetically oldest animals we used the primmorph system of *S. domuncula* and concentrated on the retinoid X receptor (RXR). The decision to use RXR was based on the facts that (i) in sponges, retinoic acid causes morphogenetic responses, (ii) related receptors had already been isolated from the coral *Acropora millepora* (Grasso et al., 2001) and the Cnidarian *Tripedalia cystophora* (Kostrouch et al., 1998) and (iii) RXR heterodimerizes with other nuclear receptors. RXRs form heterodimers with orphan receptors involved in lipid metabolism; these receptors bind their ligands with lower affinity than the endocrine steroid receptors and function as lipid sensors/regulators (Chawla et al., 2001). It is well established that in several biological systems retinoic acid is involved in the induction of apoptosis (see Sato et al., 1999).

In the present work we have studied the morphogenetic effect of retinoic acid *in vivo* and *in vitro*, particularly in the primmorph system of the sponge *S. domuncula*. The amino acid sequence of RXR from *S. domuncula* is described and we demonstrate its modulated expression in response to retinoic acid. We investigated whether the morphogenetic effect of retinoic acid in the primmorph system occurs *via* caspase-mediated apoptosis or *via* a controlled expression of homeotic genes, by studying expression of the *S. domuncula* caspase gene (*SDCASPR;* accession number AJ426651*;* Wiens et al., 2003a) and the LIM/homeobox protein (*SDLIM4*; AJ493059) in parallel. The *S. domuncula* caspase is also involved in apoptosis (Wiens et al., 2003).

Finally, the potential effect of retinoic acid on the expression of *SDCYP4* (accession number Y17616; Müller et al., 1999), a gene belonging to the cytochrome P450 superfamily (Goksøyr and Förlin, 1992), was investigated.

Materials and methods

Chemicals and enzymes

The sources of chemicals and enzymes used have been given previously (Kruse et al., 1997; Wimmer et al., 1999a; Krasko et al., 2000). The 'Cell Death Detection ELISA plus' kit and the PCR-DIG-Probe were from Roche (Mannheim, Germany), and all-*trans* retinoic acid and poly-L-lysine (M_r 30 000–70 000) from Sigma (Deisenhofen, Germany).

Sponges

Live specimens of *Suberites domuncula* Olivi (Porifera, Demospongiae, Hadromerida) were collected near Rovinj (Croatia). The sponges (20–40 specimens) were kept in 200 litre large aquaria in Mainz (Le Pennec et al., 2003). *S. domuncula* lives in symbiosis with a hermit crab *Pagurites oculatus* Herbst (Decapoda: Paguridea), which resides in the snail shell *Trunculariopsis trunculus* L. (Gastropoda: Muricidae; Herland-Meewis, 1948) on which the sponge settles.

In the incubation experiments 3–5 specimens were kept under conditions of optimal aeration in 20 litre aquaria containing 1–50 μmol l^{-1} of retinoic acid for up to 10 days.

Dissociation of cells and formation of primmorphs

Primmorphs from dissociated cells were obtained as described (Müller et al., 1999). After dissociation and washing, the dissociated cells at a density of 3×10^6 cells ml^{-1} were added to each well of 12-well tissue culture test plates (2 ml per well) in natural seawater (Sigma), supplemented with 0.1% of RPMI1640-medium, silicate to the optimal concentration of 60 μmol l^{-1} (Krasko et al., 2000) and 30 μmol l^{-1} Fe^{3+} (added as ferric citrate) (Krasko et al., 2002).

To stimulate primmorph formation the cells were cultivated either on non-coated dishes, or on tissue culture test plates coated with poly-L-lysine (M_r 30 000–70 000) or galectin (Wiens et al., in press). Homologous galectin (rGALEC1_SUBDO) was obtained by recombinant techniques from a complete cDNA, termed *SDGALEC1* (EMBL/GenBank accession number AJ493055; Wiens et al., in press). To obtain coated plates, 500 μl of poly-L-lysine solution (10 μg ml^{-1}) or a recombinant galectin solution (10 μg ml^{-1}) were added per well. After 3 h at room temperature the wells were washed and the dissociated cells added.

Primmorphs formed after 5–8 days; those that developed on

non-coated plates had diameters of 3–6 mm as observed by inspection with an Olympus OSP-MBI binocular light microscope. When cultivation was continued on non-coated dishes the size and the shape of the primmorphs remained unchanged during a prolonged period of more than 3 weeks. If however, primmorphs were transferred to galectin- or poly-L-lysine-coated tissue culture test plates after an initial period of 5 days, and cultured for up to 10 days, they developed canal-like structures; these canal-containing primmorphs were approximately 10 mm in diameter.

Treatment of primmorphs with retinoic acid

The potential effect of retinoic acid on canal-formation in primmorphs was studied by first cultivating primmorphs for 5 days on non-coated plates, followed by transfer to either galectin- or poly-L-lysine-coated tissue culture test plates; 2-3 primmorphs were retained per well. At day 5 after transfer, retinoic acid was added at concentrations of 1–50 μmol l^{-1}. At the end of the incubation the three-dimensional-cell aggregates were collected for further analysis.

Determination of apoptosis

The amount of apoptotic cells was assessed using the photometric immunoassay 'Cell Death Detection ELISA plus' kit. Primmorphs were treated with Ca^{2+}- and Mg^{2+}-free artificial seawater containing EDTA (Rottmann et al., 1987) to facilitate dissociation into single cells. Five parallel determinations from two primmorphs were performed for each experiment. As described by the manufacturer (Roche), the cells were centrifuged (500 ***g***; 5 min) and the pellets treated with lysis buffer (30 min; room temperature). After collecting the nuclei by centrifugation (2000 ***g***; 5 min), the released nucleosomes were assayed using the one-step sandwich immunoassay. The nucleosomes in the lysate were immobilized on streptavidin-coated wells and the amount of nucleosomes determined using the peroxidase substrate 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]. The absorbance of the colored product, i.e. immobilized nucleosomes, at 405 nm was determined and corrected for the background absorbance at 480 nm. The values obtained were correlated with the amount of DNA present in the samples before lysis. The mean values and standard deviations were analyzed by paired Student's *t*-test (Sachs, 1984). DNA concentration was determined by standard assay (Kissane and Robins, 1958).

Isolation of the S. domuncula *RXR-related cDNA clone*

One complete cDNA, encoding the putative retinoid X receptor, was isolated from a cDNA library obtained from *S. domuncula* (Kruse et al., 1997) by polymerase chain reaction (PCR). A forward primer was designed against the conserved amino acids (aa) within the first zinc finger module, P-box, of RXR and RXR-related receptors. The degenerate primer is located with the sponge receptor between aa 74–80 and reads: YKRAARAAIVHIGYRCAISC-3′ (Y=C,T; K=G,T; R=A,G; V=A,G,C; H=A,C,T; I=inosine). It was used in conjunction with a vector-specific reverse primer. PCR was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer, Boston, MA, USA), with an initial denaturation at 95°C for 3 min, then 35 amplification cycles each at 95°C for 30 s, 51°C for 45 s, 74°C for 1.5 min, and a final extension step at 74°C for 10 min as described (Ausubel et al., 1995; Pancer et al., 1997). The amplified product (approx. 0.8 kb) was purified and used for screening of the library (Ausubel et al., 1995). The plasmid DNAs were sequenced using an automatic DNA sequenator (Li-Cor 4200; Lincoln, NE, USA). The sequence obtained was termed *SDRXR* and was 1949 nt long, excluding the poly(A) tail.

Sequence analysis

The sequences were analyzed using computer programs BLAST (1997; ncbi.nlm.nih.gov: 80/cgi-bin/BLAST/nph-blast) and FASTA (1997; http://www.ebi.ac.uk/fasta33/). Multiple alignments were performed with CLUSTAL W Version 1.6 (Thompson et al., 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbour-joining, as implemented in the 'Neighbor' program from the PHYLIP package (Felsenstein, 1993). Distance matrices were calculated using the Dayhoff PAM matrix model as described (Dayhoff et al., 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). Graphic presentations were prepared using GeneDoc (Nicholas and Nicholas, 2001).

Northern blotting

Primmorphs were grown first in the non-attached state (for 5 days) and then for 15 days on a galectin matrix with or without retinoic acid (1–50 μmol l^{-1}). RNA was then extracted and subjected to northern blot analysis to determine the steady-state level of expression of the following sponge genes: *SDRXR*, *SDCASPR*, *SDLIM4* or the sponge *SDCYP4* gene (see below). The level of expression of the genes in canal-forming primmorphs from *S. domuncula* was determined semiquantitatively by northern blotting.

RNA was extracted from primmorphs pulverized in liquid nitrogen with TRIzol Reagent (GibcoBRL, Grand Island, NY, USA). 5 μg of total RNA was then electrophoresed through a 1% formaldehyde/agarose gel and blotted onto Hybond-N^+ nylon membrane following the manufacturer's instructions (Amersham, Little Chalfont, Bucks, UK) (Wiens et al., 1998). Hybridization was performed with 400–600 bp probes, derived from the following *S. domuncula* cDNAs: *SDRXR*, encoding the putative sponge retinoid X receptor; *SDCASPR*, encoding the caspase CASPR_SD (accession number AJ426651); *SDLIM4*, encoding the LIM/homeobox protein (accession number AJ493059) or *SDCYP4* (Y17616), encoding the putative CYP4_SD protein, a polypeptide belonging to the CYP4A (clofibrate) subfamily cytochrome *P*-450 proteins. These probes were labeled with the 'PCR-DIG-Probe-Synthesis Kit' according to the manufacturer's instructions. After washing, digoxygenin (DIG)-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase; diluted 1:10,000) and

visualized by a chemiluminescence technique using CDP-Star: disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.-1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate, the chemiluminescence substrate alkaline phosphatase, according to the manufacturer's instructions (Roche).

Northern blot signals were quantitated by a chemiluminescence procedure (Stanley and Kricka, 1990). The screen was scanned with the GS-525 Molecular Imager (Bio-Rad, München, Germany). The relative values for expression of the four genes selected were correlated with the intensities of the bands measured in controls (primmorphs not treated with retinoic acid).

Results

Effect of retinoic acid on intact S. domuncula *animals*

The specimens of *S. domuncula* were kept in aquaria (see Materials and methods) (Fig. 1A). On treatment with 50 μmol l^{-1} of retinoic acid for 10 days the tissue clearly regressed (Fig. 1B) and eventually gemmules were formed (Fig. 1C). In this series of experiments all nine treated specimens exhibited tissue regression. The gemmules were embedded in the grooves of the snail shell (Fig. 1C). There were no obvious morphological differences between gemmules developed after treatment of the specimens with bacteria (Böhm et al., 2001) and those formed in response to retinoic acid.

From these observations we conclude firstly that retinoic acid caused an involution *in vivo* and secondly, in consequence, that the respective receptor, the RXR, must be present. To verify the effect seen *in vivo* under controlled cell culture conditions, the potential morphogenetic effect of retinoic acid was studied in the *in vitro* primmorph system.

Induction of canals in primmorphs through attachment to the substratum

Primmorphs are characterized by a compact smooth and 'waxy' surface (Fig. 1D). Recently, we succeeded in inducing canal formation in primmorphs following adhesion to substrata. We first used homologous recombinant galectin as the matrix (Fig. 1E). Under these conditions the primmorphs attached to the matrix and formed canal-like structures, which could also be visualized in cross sections of the primmorphs. Poly-L-lysine was also a suitable substrate for canal formation in primmorphs (Fig. 1F). Fig. 1G shows the canal system in the primmorphs at higher magnification. As outlined in Materials and methods, canal formation generally starts approximately 10 days after transfer of round-shaped, non-attached primmorphs (incubated for 5 days) to galectin/poly-L-lysine-coated plates.

Effect of retinoic acid on canal formation in primmorphs

For these studies we used primmorphs that had already formed canals after incubation on non-coated plates followed by 10 days of incubation on galectin-coated plates. Retinoic acid was applied at 1 μmol l^{-1} (Fig. 1H,I) or 50 μmol l^{-1} (Fig. 1J–L) to determine its effect on the integrity of canals.

The canals disappeared after 5 days of incubation with 1 μmol l^{-1} retinoic acid in every one of the ten primmorphs studied (Fig. 1H). Additionally round shaped bodies of a size (<1 mm) smaller than the gemmules (diameter 1–3 mm) were released (Fig. 1I). After incubation with 50 μmol l^{-1} retinoic acid the canals in the primmorphs disintegrated (Fig. 1J) and most of the primmorph tissue involuted (Fig. 1K,L). To determine whether the change in primmorph morphology caused by retinoic acid was reversible, the compound was washed out after a total incubation period of 20 days. Approximately 5 days later all the primmorphs studied started to form canals again (N=6; data not shown).

Effect of retinoic acid on cell viability

We determined whether the sponge primmorphs, cultivated on a galectin-coated matrix for up to 15 days (after the initial incubation period of 5 days in the non-attached state), and in the presence of retinoic acid, undergo apoptotic cell death. We used a photometric immunoassay to quantitate the amount of nucleosomes as outlined in Materials and methods. Untreated primmorphs showed low apoptotic degradation of DNA, with absorbance values (correlated to 50 ng of DNA) between 0.7 (day 3 after transfer to the galectin matrix) and 1.3 (day 15; Fig. 2). Similar values were found for primmorphs that had been transferred after 5 days to a galectin-coated matrix and were further cultivated for 10 or 15 days, with retinoic acid (1 μmol l^{-1} or 50 μmol l^{-1}) during the last 2 days of incubation. In these primmorphs the amount of released nucleosomes (measured as absorbance at 405 nm) varied non-significantly (P>0.1) around values of 0.8–1.2, irrespective of the retinoic acid concentration.

These data suggest that retinoic acid does not cause apoptosis, which features fragmentation of chromatin to nucleosomes.

Reversibility of the retinoic acid effect

The reversibility of the effects caused by retinoic acid on primmorphs was tested by washout experiments. After the total incubation of 15 days the primmorphs were transferred into the normal culture medium (seawater/RPMI1640/silicate/Fe^{3+}). Primmorphs restored their original morphology regardless of whether they had been treated at low (1 μmol l^{-1}) or higher (50 μmol l^{-1}) retinoic acid concentrations. In more than 80% of the 3D-cell aggregates tested (N=12), the canal organization reappeared after 5 days (not shown). We therefore conclude that the retinoic acid-mediated effect on canal formation is reversible.

Cloning of the S. domuncula *retinoid X receptor*

The complete clone for the sponge RXR cDNA, termed *SDRXR*, was isolated using PCR and degenerate primers. The 1949 nt cDNA has one open reading frame (ORF) with the start-methionine at nt 180–182 and the stop codon at nt 1824–1826. The 548-aa deduced protein has a calculated size of 61346 (PC/GENE 1995; Data Banks CD-ROM; Release 14.0. Mountain View, CA, USA: IntelliGenetics, Inc.). The

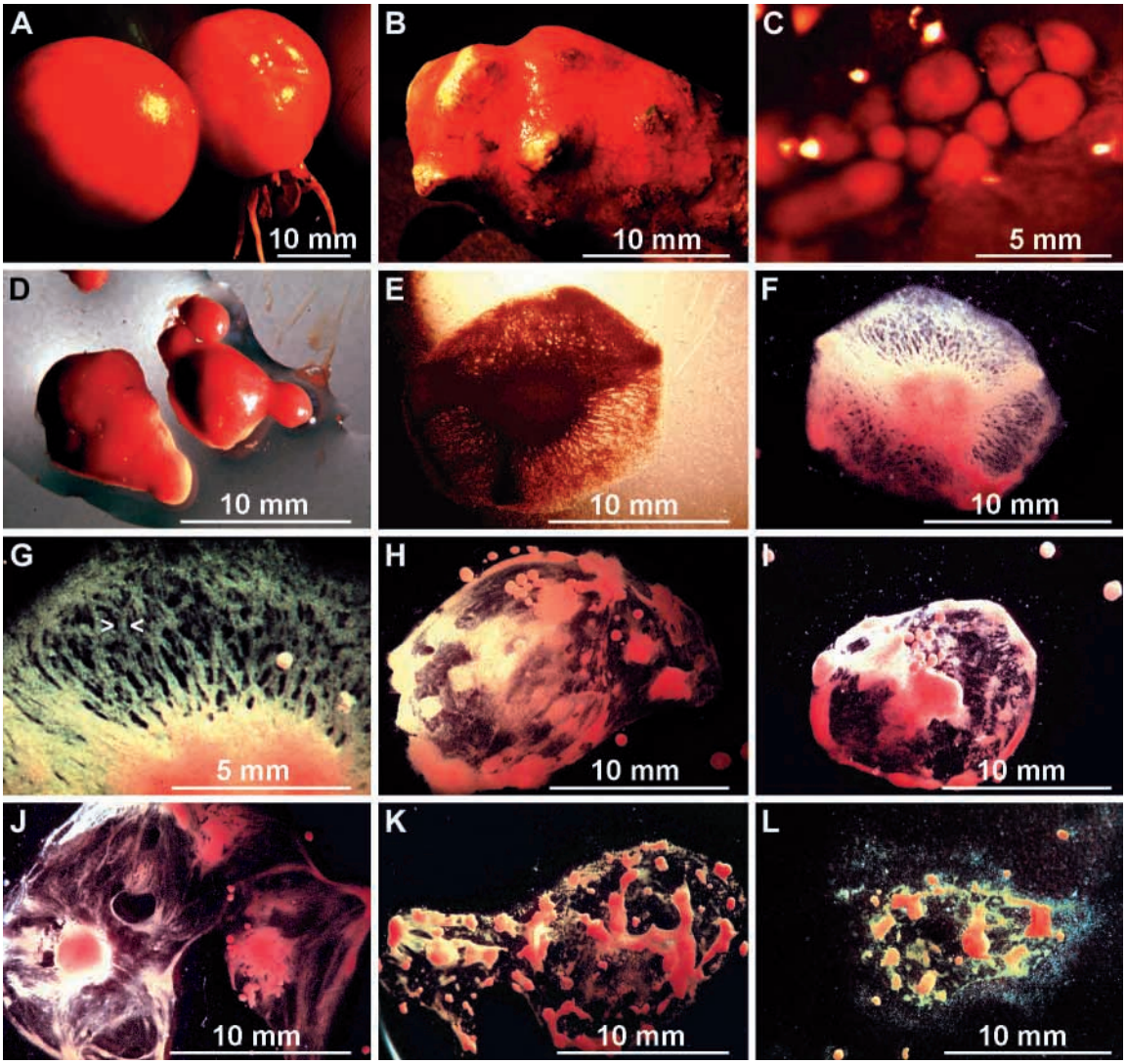

Fig. 1. Effect of retinoic acid on whole sponges and primmorphs. (A) Untreated specimens of *S. domuncula*. (B,C) Induction of gemmules in animals by retinoic acid (50 μmol l^{-1}). (B) Gemmules formed on a shell of the snail *Trunculariopsis trunculus* (incubation: 10 days) after retinoic acid treatment. (C) Group of gemmules arranged on the grooves of a shell. (D–G) Induction of canals in primmorphs. (D) Primmorphs formed in the absence of any organic matrix in the culture dish. The 3D-aggregates remained round and without canals. (E) Formation of canals by incubation of cells for 5 days on non-coated plates followed by 10 days on plates coated with galectin matrix. (F) Primmorphs with canals formed after for 5 days on non-coated plates followed by 10 days on poly-L-lysine plates. (G) Higher magnification of the canal system (> <) in the primmorphs developed for 20 days on the galectin matrix following 5 days incubation on non-coated plates. (H–L) Effect of retinoic acid on the canal system in primmorphs. (H,I) Incubation of canal-containing primmorphs (formed after a total incubation period of 15 days), cultured on the galectin matrix for 10 days, + 1 μmol l^{-1} of retinoic acid for an additional 5 days. (J–L) Incubation of the canal-containing primmorphs at the higher concentration of retinoic acid (50 μmol l^{-1}) for the same period of time. For further details, see Materials and methods. Scale bars: 10 mm (except for C and G, 5 mm).

instability index was computed as 65.16, indicating an unstable protein. The putative protein, the *S. domuncula* retinoid X receptor RXR_SUBDO, comprises two motifs with a high significance score, the DNA-binding (hormone-receptor) domain including the zinc finger (C4 type; two domains) modules (PFAM00105) between aa 153 and 228, and the ligand-binding domain of nuclear hormone receptors [PFAM00104 (www.ncbi.nlm.nig.gov)] spanning the protein sequence between aa 361 and 545 (Fig. 3). The nuclear receptor signature, W-a-b[basic residue]-x-h[hydrophobic]-P-x-F-x-x-L-x-x-x-D-Q-x-x-L-L (Wurtz et al., 1996), is completely present in the sponge sequence between helices 3 and 4, aa 370–389 (Fig. 3). Northern blot studies revealed that the *S. domuncula SDRXR* transcript is 2.0 kb in size (see below), indicating that the complete cDNA was isolated. Furthermore, genomic DNA prepared from *S. domuncula* was used successfully to perform Southern blots with *SDRXR* (not shown).

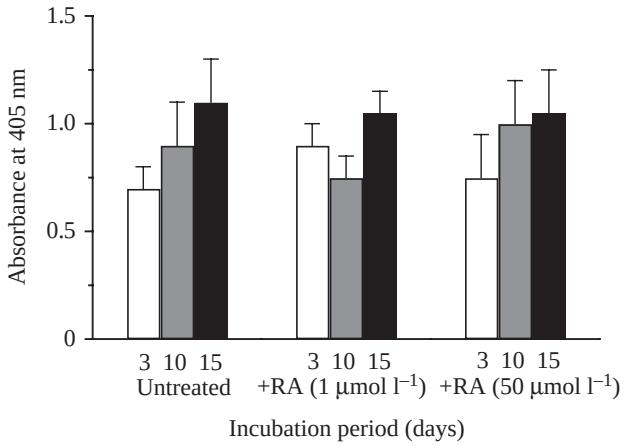

Fig. 2. Extent of nucleosome formation in primmorphs, determined by photometric immunoassay. Primmorphs were grown for the initial period of 5 days in the non-attached manner, followed by an additional 3 days (total 8 days), 10 days (total 15 days) or 15 days (total 20 days) on galectin-coated culture dishes. The cultures remained either non-treated or were treated with 1 μmol l^{-1} or 50 μmol l^{-1} of retinoic acid (RA) for the last 2 days of incubation. Then the cells (100–200 mg protein) were lysed and the released nucleosomes were quantitated immunochemically as described in Materials and methods. The amount of colored product was measured spectrophotometrically. The absorbance values were correlated to 50 ng of DNA (present in the samples before lysis). Values are means ± S.D., from five parallel determinations for each sample, and show the amount of nucleosomes released in primmorphs grown on the galectin matrix for 3 days (white bars); 10 days (gray bars) and 15 days (black bars).

Sequence analysis of the sponge RXR

It is important to note that the two domains, the ligand-binding domain of nuclear hormone receptors and the DNA-binding (zinc finger) domain, exist exclusively in proteins from metazoans. For comparative analysis we list the highest similarity score values 'Expect value' (*E*; Blast-NCBI; Coligan et al., 2000) of the *S. domuncula* domains to sequences from Protostomia, Deuterostomia, fungi and plants.

The ligand-binding domain for the nuclear hormone receptor: from two protostomians, the hormone receptor/zinc finger protein of *Caenorhabditis elegans* (NM_058702; $E=1\times10^{-16}$) and the *Drosophila melanogaster* hepatocyte nuclear factor 4 (S36218; $E=2\times10^{-24}$), the deuterostomian (human) retinoid X receptor, gamma (BC012063; $E=9\times10^{-35}$) and, in comparison, the fungus glutathione reductase from *Saccharomyces cerevisiae* (D37871; $E=3.6$) as well as from the plant *Arabidopsis thaliana* an unknown protein (AY086748; $E=5.0$).

A likewise exclusively high relationship of the *S. domuncula* zinc finger (C4 type) domain is found to that in metazoan proteins; the scores are: for the *C. elegans* nuclear hormone receptor (NM_077038; $E=8\times10^{-15}$), the *D. melanogaster* steroid receptor protein svp 2 (NM_079601; $E=5\times10^{-27}$), the human retinoic acid receptor RXR, alpha (X52773; $E=9\times10^{-27}$), as well as for the conserved hypothetical protein of the fungus *N. crassa* (AL390189; $E=5.4$) and the putative cellulose synthase catalytic subunit from *A. thaliana* (NM_128111; $E=1.9$).

Based on these comparisons it can be concluded that the sponge nuclear hormone receptor must be grouped with the other metazoan receptors of this class and that it shares highest similarity to the corresponding human sequences.

A more detailed analysis was elaborated for the ligand-binding domain of nuclear hormone receptors (reviewed in Giguère, 1999). This part of the sequence is involved in ligand-binding, dimerization, interaction with heat shock proteins, nuclear localization and transactivation. Based on X-ray crystallographic experiments, the ligand-binding domain can be subdivided into up to 13 helices that bury the ligand-binding site; these helices H1–H5 and H7–H10 are shown in Fig. 3. At the carboxy-terminal end of the ligand-binding domain the sponge RXR also shows the activator function-2 stretch, which is involved in the recruitment of coactivators (Onate et al., 1998).

The zinc finger (C4 type) domain represents the DNA-binding domain (see Glass, 1994). It comprises two zinc finger modules, which are encoded in the sponge RXR by 76 aa (or 92 aa, according to Giguère, 1999) (Fig. 3). The four determinant cysteine residues exist at the same positions/distances as those found in other nuclear hormone receptor proteins (Giguère, 1999) (Fig. 3). The zinc finger modules are further subdivided into subdomains that are involved in recognition of the core half-site sequences (P-box; Umesono and Evans, 1989) and the dimerization determinants (D-box; Zechel et al., 1994); also their conserved residues are present in the sponge protein (Fig. 3). The zinc finger modules are followed by the carboxy-terminal extension stretch, which in RXR_SUBDO comprises the consensus length. Between the DNA-binding (zinc finger) domain and the ligand-binding domain is the hinge region which, in comparison to other receptors of this group, is very variable. The function of this region is to support dimerization (Glass, 1994). At the amino-terminal end of the sponge RXR is the modulator domain, containing highly variable amino acid residues (Giguère, 1999). Here the transcriptional activation function (AF-1) resides.

Phylogenetic analysis of sponge RXR: total sequence

The sequence alignment of the sponge RXR, RXR_SUBDO, with the related metazoan sequences in Fig. 3 also shows the nuclear receptor AmNR8 (from the coral *Acropora millepora*). Based on the Expect value (*E*), sequences were selected with the highest similarity between *S. domuncula* RXR and the corresponding sequences from human, *D. melanogaster* and *C. elegans* as well as from *A. millepora*. The overall protein sequence similarity/identity of the sponge RXR to these metazoan sequences is around 20–35%. No nuclear receptor/nuclear hormone receptor molecule other than in Metazoans has been found in, for example, plants or yeast (see Grasso et al., 2001), indicating that the sponge RXR is phylogenetically the oldest one known.

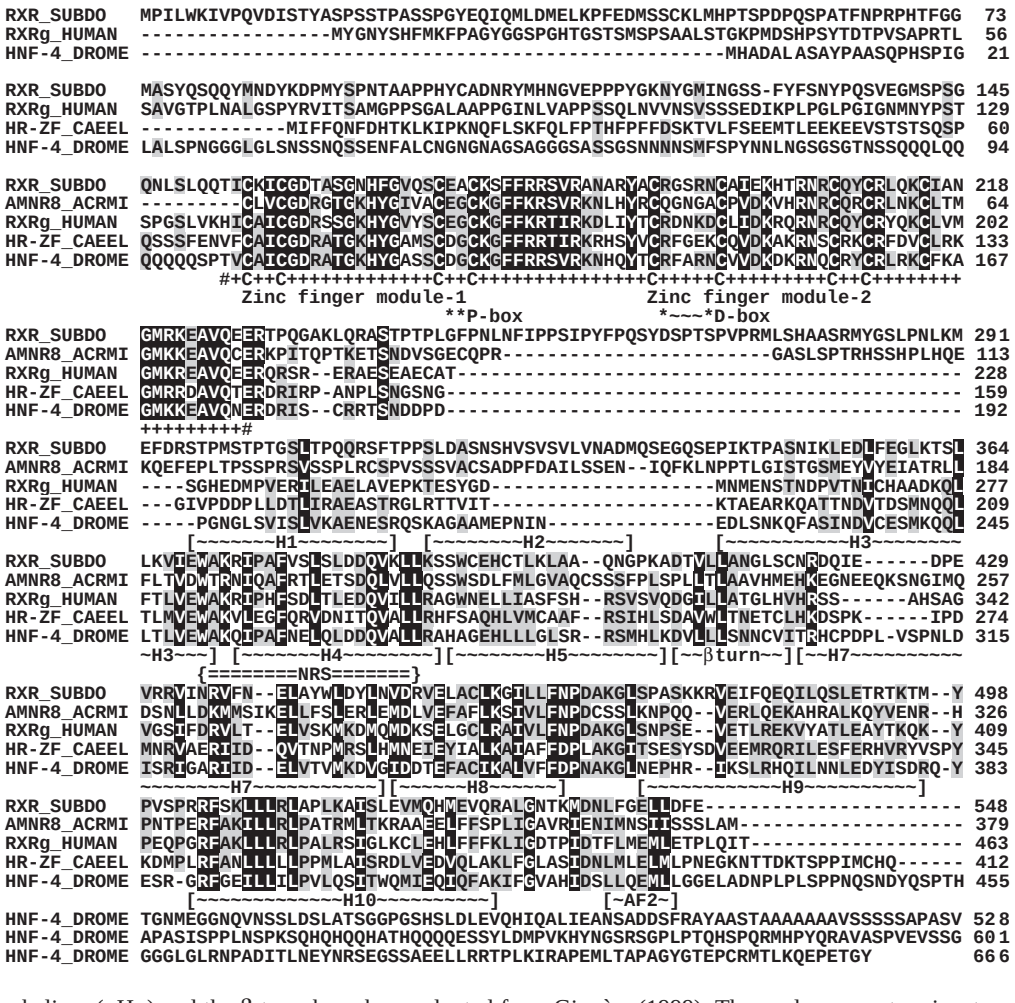

Fig. 3. The *S. domuncula* retinoid X receptor (RXR). The deduced RXR from *S. domuncula*, RXR_SUBDO, was aligned with other most similar sequences from human, retinoid X receptor gamma (RXRg_HUMAN; accession number NP_008848), *C. elegans*, hormone receptor/zinc finger protein (HR-ZF_CAEEL; NP_491103) and *D. melanogaster*, hepatocyte nuclear factor 4 (HNF-4_DROME; S36218), as well as the partial sequence from a coral (*Acropora millepora*), the nuclear receptor AmNR8 (AMNR8_ACRMI; AAL29201). Residues conserved (similar or related with respect to their similar physico-chemical properties) in all sequences are shown in white on black and those occurring in at least three sequences in black on gray. The characteristic DNA-binding domain, the two zinc finger modules (#++#) with the conserved cysteine residues (C), are marked. The DNA-binding domain is provided with the conserved residues of the P-box as well as of the D-box (marked). In addition, the borders of the putative secondary structures within the ligand-binding site, the ten α-helices (~H~) and the β-turn, have been adopted from Giguère (1999). The nuclear receptor signature (NRS) and the activator function-2 (AF2) are indicated.

In 1999 a unified nomenclature system for the nuclear receptor superfamily (Nuclear Receptors Nomenclature Committee; NRNC, 1999) was proposed. In accordance with this subfamily grouping, representative members from the different groups were selected from the databases and analyzed. After alignment an obvious classification of the *S. domuncula* protein became possible based on an unrooted tree (Fig. 4). The sponge polypeptide does not belong to the nuclear receptors of the subfamilies 1, 5 or 6 but to subfamily 2 (retinoic acid receptors; NRNC, 1999; Escriva et al., 2000; Chawla et al., 2001). This subfamily is subdivided again into six fractions: group 2A (e.g. human hepatocyte nuclear factor 4), group 2B (e.g. human retinoic acid receptor-like protein), group 2C (e.g. the human steroid receptor TR2-11), group 2D (e.g. the insect THR6 nuclear receptor), group 2E (e.g. the photoreceptor-specific nuclear receptor PNR) and group 2F (e.g. the human v-erbA related ear-2 receptor); Fig. 4. Other receptors identified apart from those of Protostomia or Deuterostomia are from the coral *A. millepora* (Phylum Cnidaria; Anthozoa), from which a series of nuclear receptors has been cloned (Grasso et al., 2001); among those the NR7 receptor falls into group 2F, the TLL receptor to group 2E, while NR8 does not significantly belong to a known group. A further diploblast receptor identified is the retinoic acid X receptor from *Tripedalia cystophora* (Cnidaria; Cubozoa; Kostrouch et al., 1998); this protein belongs to group 2B. The new, herein described sponge polypeptide, which represents the phylogenetically oldest nuclear hormone receptor known to date, falls in the branch of group 2A (with the human hepatocyte nuclear factor) / group 2B (including the retinoid X receptors) with a high significance (>98%). In general, the receptors of subfamily 2, comprising the mentioned groups 2A–2F, are adopted orphan receptors that bind with low-affinity dietary lipids (Chawla et al., 2001).

Expression of selected genes in response to retinoic acid incubation

Differential expression of the genes, in response to retinoic acid was seen (Fig. 5). Expression of the putative sponge retinoid X receptor at low retinoic acid concentration (1 μmol l^{-1}; Fig. 5, lane b) was 1.5-fold higher than that of the controls (primmorphs cultivated in solution; Fig. 5, lane a); the

size of the transcript was 2 kb. At higher retinoic acid concentrations, of 3 μmol l^{-1} (Fig. 5, lane c) and 50 μmol l^{-1} (Fig. 5, lane d), expression levels increased even to 5- and 22-fold, respectively.

A different expression pattern was seen for the caspase gene *SDCASPR*, where the steady-state expression remained almost unchanged even after the different retinoic acid treatments; the transcript size was 1.6 kb. In contrast, the expression of *SDLIM4* (transcript size: 1.7 kb) the sponge LIM/homeobox protein, increased at 1 μmol l^{-1} retinoic acid; higher concentrations of retinoic acid caused a further upregulation of the expression of this gene. A similar pattern as for the caspase gene was seen for the gene encoding the CYP4A related cytochrome *P*-450 protein, *SDCYP4* (transcript size: 1.8 kb); no significant change in the expression pattern was seen in the primmorphs after treatment with different concentrations of retinoic acid (Fig. 5).

From these data we conclude that retinoic acid (at

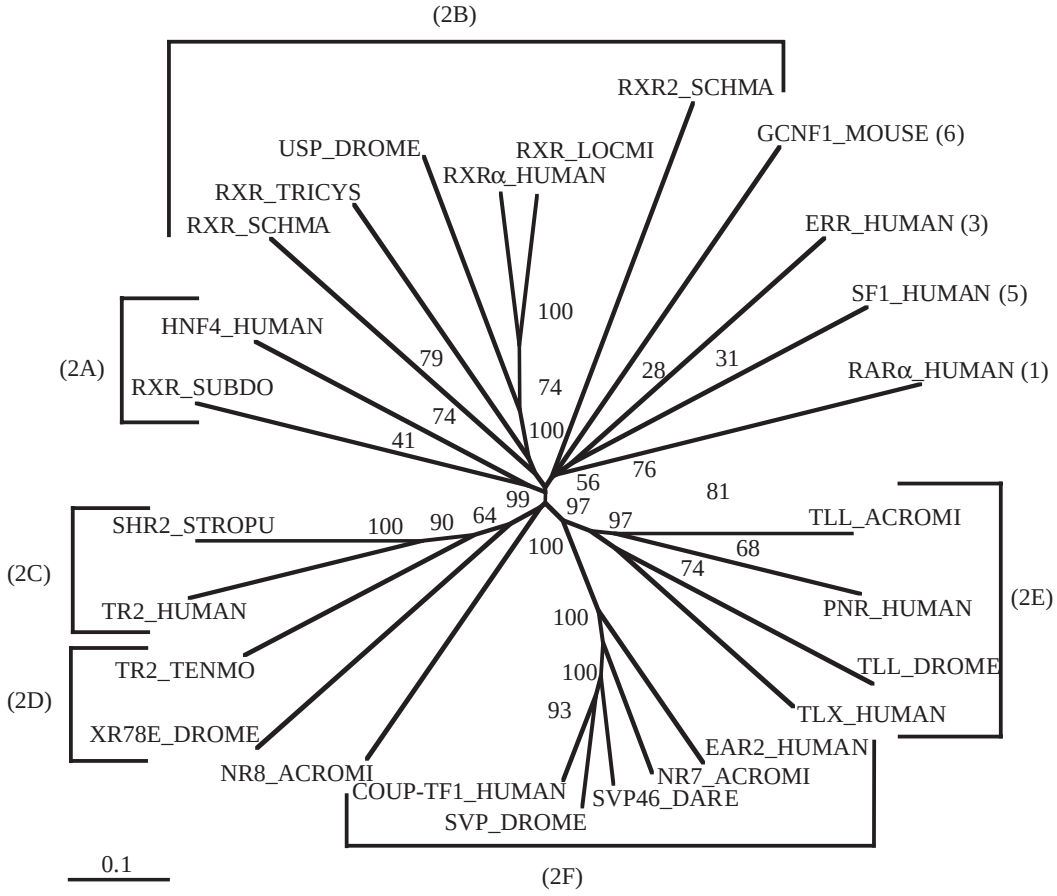

Fig. 4. Phylogenetic relationships of the *S. domuncula* RXR, RXR_SUBDO, with the related sequences from the coral *Acropora millepora* nuclear receptors TLL (TLL_ACROMI; AF323680), NR7 (NR7_ACROMI; AF323687) and NR8 (NR8_ACROMI; AF323688), Cnidaria *Tripedalia cystophora* retinoic acid X receptor (RXR_TRICYS; AF091121), Platyhelminthes *Schistosoma mansoni* retinoic acid receptor RXR (RXR_SCHMA; AF094759) and *S. mansoni* retinoid X receptor RXR-2 mRNA (RXR2_SCHMA; AF129816). In addition, relationships are shown with sequences from the Protostomia: *D. melanogaster*, seven-up protein (svp) type 1 (SVP_DROME; M28863), tailless (TLL_DROME; M34639), mRNA for XR2C ultraspiracle gene (USP_DROME; X53417), *D. melanogaster* nuclear receptor XR78E/F (XR78E_DROME; U31517); insects *Locusta migratoria* RXR mRNA (RXR_LOCMI; AF136372) and *Tenebrio molitor* THR6 (nuclear receptor) gene (TR2_TENMO; AJ005765); and from Deuterostomia: *Danio rerio* svp 46 mRNA for steroid receptor homologue (SVP46_DARE; X70300); human upstream promoter transcription factor (COUP-TF1_HUMAN; X58241), v-erbA related ear-2 gene (EAR2_HUMAN; X12794), the steroid hormone receptor hERR2 (ERR_HUMAN; X51417), hepatocyte nuclear factor 4 (HNF4_HUMAN; X76930), photoreceptor-specific nuclear receptor (PNR) (PNR_HUMAN; AF121129), receptor of retinoic acid-alpha (RARα_HUMAN; X06614), retinoic acid receptor-like protein (RXRα_HUMAN; X52773), steroidogenic factor 1 mRNA (SF1_HUMAN; U76388), tailless gene homologue (TLX_HUMAN; Y13276) and the steroid receptor (TR2-11) (TR2_HUMAN; M29960); from *Mus musculus* germ cell nuclear factor (GCNF1_MOUSE; U14666) as well as the orphan steroid hormone receptor from *Strongylocentrotus purpuratus* (SHR2_STROPU; U38281). The different sequences were classified according to the unified proposition (NRNC, 1999). The tree was constructed as described in Materials and methods and remained unrooted. The numbers at the nodes indicate the level of confidence (%) for the branches as determined by bootstrap analysis (100 bootstrap replicates). The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence. Numbers in parentheses refer to the respective subfamilies/groups.

1–50 μmol l^{-1}) causes a dose-dependent increase in the expression of the sponge retinoid X receptor gene and *SDLIM4*, the LIM/homeobox protein. The expression levels of the *P*-450 protein gene and the caspase gene remained unaffected.

Discussion

Retinoic acid is a major biologically active derivative of retinol (vitamin A) and plays a key role during embryonal development and the balance of the normal cellular function (Morris-Kay, 1997; Redfern, 1997). At the protein level, retinoic acid (9-*cis*-retinoic acid) binds to the retinoid X receptor. 9-*cis*-retinoic acid is isomerized from all-*trans* retinoic acid and hence, likewise, a natural derivative of vitamin A (see Heery et al., 1993; Egea et al., 2000). 9-*cis*-retinoic acid binds selectively to the RXRs, while all-*trans* retinoic acid binds primarily to RARs (see Egea et al., 2000).

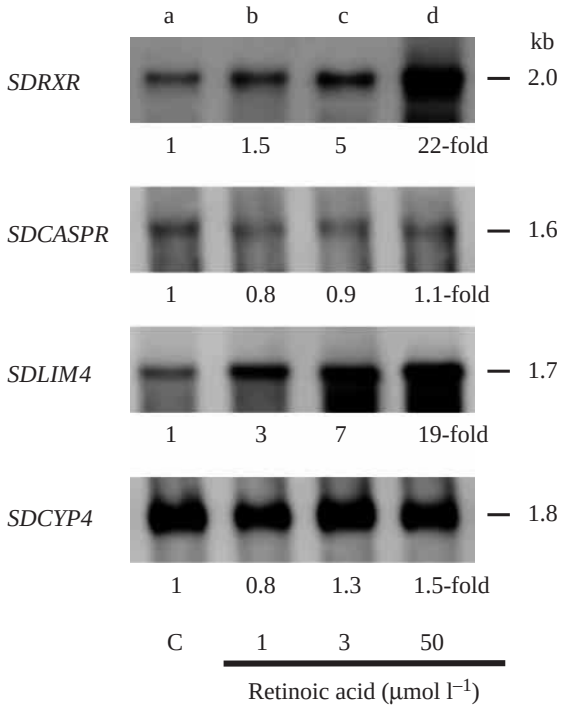

Fig. 5. Expression of selected genes in canal-forming primmorphs in response to retinoic acid. Primmorphs were first incubated for 5 days on non-coated dishes and then, in order to induce canals, on galectin-coated dishes for 10 days. Subsequently, these primmorphs remained either untreated for 5 days (C; lane a) or were treated with 1 μmol l^{-1} (lane b), 3 μmol l^{-1} (lane c), or 50 μmol l^{-1} of retinoic acid (lane d) as indicated. RNA was then extracted and 5 μg of total RNA per lane were size-separated. After blot transfer, hybridization was performed using the following probes: *SDRXR* (the putative sponge retinoid X receptor), *SDCASPR* (*S. domuncula* caspase), *SDLIM4* (sponge LIM/homeodomain protein) or the sponge *SDCYP4* (CYP4A related cytochrome *P*-450 protein). The levels of expression of the respective genes were estimated by northern blot and the intensities of the different transcript bands were determined relative to the intensity of the controls (cultivated for a total of 15 days in solution).

Both RXRs and RARs can, however, not only homodimerize but also heterodimerize with each other and also with other related receptors (Mangelsdorf et al., 1995; Osburn et al., 2001).

Retinoic acid was previously found to trigger sponges to undergo involution (gemmule or bud formation; see Simpson 1984). We show using the primmorph system that retinoic acid causes both an involution of the intact *S. domuncula* organisms under formation of the asexual reproduction bodies, the gemmules, and a similar regression *in vitro*. Using retinoic acid concentrations that cause similar effects in other sponge systems, especially in freshwater sponges (Imsiecke et al., 1994; Nikko et al., 2001). Sponges, individuals and primmorphs, reacted to concentrations of 1–50 μmol l^{-1} retinoic acid by tissue regression.

In order to substantiate the observed effect of retinoic acid, a cDNA library from the sponge *S. domuncula* was screened for the presence of RARs and RXRs. So far we have been unable to detect an RAR in the library, but the search for the RXR was successful. The full-length clone of a RXR was identified, the deduced protein comprising the characteristic features of other metazoan RXRs, e.g. the zinc finger (C4 type) domain and the activator function-2 stretch, a segment that is important for the recruitment of coactivators (see Results). The *S. domuncula* RXR showed high similarity to the subfamily 2 of the nuclear receptor superfamily and, more specificly, to group 2A/2B of these receptors. Hence this *S. domuncula* RXR is phylogenetically the oldest nuclear hormone receptor identified to date. The RXR from the Cnidarian jellyfish *Tripedalia cystophora* (Kostrouch et al., 1998), which until now has been the phylogenetically oldest one identified, must be evolutionarily younger. With these data in hand it is now possible to answer the question of the origin of the nuclear hormone receptors and their possible existence in sponges (Mendoza et al., 1999). Our data demonstrate that with the evolutionary transition to the Metazoa and with the Porifera as the oldest metazoan phylum, the nuclear hormone receptors had already appeared. This finding is amazing since sponges do not contain a circulation vessel system that would allow an efficient transport of hormones, e.g. steroids, thyroid hormones or ecdysones.

The data, however, clearly demonstrate the existence of the RXR gene in *S. domuncula*. In addition, the data show that retinoic acid exerts a morphogenetic effect on this sponge both *in vivo* and *in vitro*. In order to investigate the most likely mode of action of retinoic acid/RXR at the subcellular level, gene expression studies were performed using the northern blot technique. It was found that retinoic acid causes a strong upregulation of the expression of the *RXR* gene, a finding that is in accordance with the related receptors present in triploblasts; in these animals a tissue- and stage-dependent expression of RXRs is known (see Rana et al., 2002). Since this effect is observed in *S. domuncula* with all-*trans* retinoic acid we assume that the morphogen is isomerized in the animal to 9-*cis*-retinoic acid, the characteristic ligand for RXRs.

Further genes were analyzed in parallel with the expression

studies of RXR in the *in vitro* system of *S. domuncula*. A caspase gene was included, since retinoic acid is known to cause apoptosis in some systems, e.g. leukemia cells (Nervi et al., 1998). In a recent study it was established that in the sponge system caspases are also involved in the induction of apoptosis (Wiens et al., 2003). Surprisingly it was found that in the presence of retinoic acid the expression level of the caspase gene does not alter, and this finding was also supported by a series of experiments which showed that there is no parallel increase in the formation of free nucleosomes during the retinoic acid-mediated regression of tissue.

Another gene involved in detoxification of drugs (Nebert et al., 1991; Chawla et al., 2001), the sponge *CYP4_SD* gene, was selected together with *SDLIM4* (encoding LIM/homeobox protein), which mediates differentiation events. Using the northern blot approach, it could be demonstrated that the steady-state expression of the caspase gene remained unchanged, irrespective of the retinoic acid concentration used. However, a strong increase in expression of the *SDLIM4* gene was observed. In triploblasts, both in Protostomians and in Deuterostomians, the Lim-class homeodomain proteins are involved in organogenesis (see Bach, 2000). The Lim-class HD proteins still await identification in Cnidaria, but the finding presented in this report is in agreement with our recent observation that the LIM/homeobox protein gene is upregulated after induction of canal-like structures in primmorphs (Wiens et al., in press) and emphasises that the LIM/homeobox protein, like the Paired-class homeobox genes, is involved in the control of the patterning (Galliot and Miller 2000). Likewise, elucidation of the role of the HOX-like molecules (e.g. Degnan et al., 1995) in sponges will provide further clues on the regulation of the bodyplan formation in these animals.

The results presented in this paper suggest that RXR(s), retinoic acid, and its derivatives may play important roles during morphogenesis in sponges. Identifying the proteins/receptors which ‘transduce/facilitate’ the retinoic acid signal to the transcription factor(s), and in turn are involved in the cytological/morphological changes, will be the next major steps forward in the understanding of the molecular biology of retinoid responses in the earliest metazoan phylum, which branched off from the common animal ancestor, the Urmetazoa.

The sequence from *Suberites domuncula* reported here is deposited in the EMBL/GenBank database; retinoid X receptor *SDRXR* accession number AJ493057. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung (project: Center of Excellence *BIOTEC*marin) and the European Commission (project: SPONGES).

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