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Evolutionary origins of retinoid active short-chain dehydrogenases/reductases of SDR16C family

Olga V. Belyaeva^{a,*}, Chenbei Chang^b, Michael C Berlett^a, and Natalia Y. Kedishvili^a

Olga V. Belyaeva: obeliaeva@uab.edu

^aDepartment of Biochemistry and Molecular Genetics, University of Alabama – Birmingham, Birmingham, AL 35294, USA

^bDepartment of Cell, Developmental and Integrative Biology, University of Alabama – Birmingham, Birmingham, AL 35294, USA

Abstract

Vertebrate enzymes that belong to the 16C family of short-chain dehydrogenases/reductases (SDR16C) were shown to play an essential role in the control of retinoic acid (RA) levels during development. To trace the evolution of enzymatic function of SDR16C family, and to examine the origins of the pathway for RA biosynthesis from vitamin A, we identified putative SDR16C enzymes through the extensive search of available genome sequencing data in a subset of species representing major metazoan phyla. The phylogenetic analysis revealed that enzymes from protostome, non-chordate deuterostome and invertebrate chordate species are found in three clades of SDR16C family containing retinoid active enzymes, which are retinol dehydrogenase 10 (RDH10), retinol dehydrogenases E2 (RDHE2) and RDHE2-similar, and dehydrogenase reductase (SDR family) member 3 (DHRS3). For the initial functional analysis, we cloned RDH10- and RDHE2-related enzymes from the early developmental stages of a non-chordate deuterostome, green sea urchin *Lytechinus variegatus*, and an invertebrate chordate, sea squirt *Ciona intestinalis*. *In situ* hybridization revealed that these proteins are expressed in a pattern relevant to development, while assays performed on proteins expressed in mammalian cell culture showed that they possess retinol-oxidizing activity as their vertebrate homologs. The existence of invertebrate homologs of DHRS3 was inferred from the analysis of phylogeny and cofactor-binding residues characteristic of preference for NADP(H). The presence of invertebrate homologs in the DHRS3 group of SDR16C is interesting in light of the complex mutually activating interaction, which we have recently described for human RDH10 and DHRS3 enzymes. Further functional analysis of these homologs will establish whether this interaction evolved to control retinoid homeostasis only in vertebrates, or is also conserved in pre-vertebrates.

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*Corresponding author at: Department of Biochemistry and Molecular Genetics, School of Medicine, University of Alabama – Birmingham, 720 20th Street South, 466 Kaul Human Genetics Building, Birmingham, AL 35294, USA. Tel: +1 (205) 996 4024; fax: +1 (205) 975 2188.

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cbi.2014.10.026>.

Keywords

Short chain dehydrogenases/reductases; Evolution; Retinoic acid

1. Introduction

Mammalian genomes contain six genes encoding proteins that belong to the SDR16C family of short-chain dehydrogenases/reductases (SDR) (Fig. 1). While closely related 17 β -hydroxysteroid dehydrogenases HSD17B11 and HS17B13 (SDR16C2 and SDR16C3) are active toward steroids, retinol dehydrogenase 10 (RDH10, SDR16C4), epidermal retinol dehydrogenase 2 (RDHE2) and RDHE2-similar (SDR16C5 and SDR16C6) and dehydrogenase reductase (SDR family) member 3 (DHRS3, SDR16C1) are retinoid-active enzymes. Genes encoding RDHE2 and RDHE2-similar proteins represent a recent mammalian duplication event. Retinol dehydrogenases of SDR16C family are the only enzymes shown to control the rate-limiting step of all-*trans*-retinoic acid (RA) biosynthesis, namely oxidation of retinol to retinaldehyde, during vertebrate development [1–3]. Retinaldehyde reductase DHRS3 has recently been shown to counteract the action of RDH10 by reducing retinaldehyde back to retinol during embryogenesis [4,5]. Thus, SDR16C family contains enzymes essential for the control of RA levels during development, and the analysis of their evolution offers an opportunity to trace the origins of metabolic pathway of RA biosynthesis from retinol [6] (Fig. 2).

The function of RA as an important regulator of vertebrate development is well established [7]. However, recent studies in amphioxus and ascidian revealed that RA signaling also plays a role in the development of invertebrate chordates [8–14]. Endogenous retinoids, including RA and retinyl esters, were detected in protostome mollusks [15–17]. Extensive bioinformatics studies have identified elements of RA biosynthetic and signaling apparatus, including putative receptors, retinaldehyde dehydrogenases and enzymes of RA degradation outside the Chordate phylum, in non-chordate deuterostomes and protostomes [18–24]. These findings resulted in an intriguing hypothesis that the RA signaling originated early in the evolution of bilaterian animals. The much-needed experimental support for this hypothesis must include the evidence for both the functionality of RA receptors [17], and the existence of active metabolic pathway to synthesize their ligand, RA. A recent in-depth study addressed the evolution of substrate specificity in aldehyde dehydrogenases type 1, a group that contains retinaldehyde dehydrogenases catalyzing synthesis of RA from its immediate precursor, retinaldehyde [25]. However, the evolution of enzymes interconverting retinol and retinaldehyde is especially of interest, because the inclusion of the reversible rate-limiting step in the pathway offers a tighter control over the ligand levels (Fig. 2). Identification of proteins of SDR16C family as the enzymes essential for the control of RA levels during development provides a strong rationale for focusing on this group in search for the origins of RA signaling in embryogenesis, and in adult tissues. Studies of invertebrate homologs of vertebrate SDR16C proteins will shed light on whether these enzymes and RA signaling were co-opted in the developmental control before the emergence of the chordate body plan, and will contribute to understanding of how widespread RA signaling is in the animal kingdom.

2. Materials and methods

2.1. Identification of SDR16C enzymes and phylogenetic analysis

Homologs of mammalian SDR16C enzymes were identified in Ensembl and NCBI organism-specific genome databases, and NCBI non-redundant nucleotide database using *tblastn* BLAST algorithm with mouse protein sequences as queries. When the search retrieved predicted homolog derived from the automatic genome annotation that appeared to be incomplete, the EST and organism-specific whole genome shotgun (*wgs*) sequence databases were searched to recover the full-length sequences. For green sea urchin *Lytechinus variegatus*, BLAST search was performed on not annotated pre-publication release of genomic sequence available at <http://www.echinobase.org/Echinobase/Blasts>. Phylogenetic analyses were conducted in MEGA version 6 [26].

2.2. Cloning and constructs

RNA from ascidian and sea urchin embryos was isolated using Trizol and reverse-transcribed using Superscript III kit (Life Technologies). Coding sequences of predicted SDR16C enzymes were PCR-amplified using Pfx DNA polymerase (Life Technologies) and cloned into pCMV-Tag4a vector for expression of FLAG-tagged fusions in mammalian cells, and into pBluescript SK (–) vector for synthesis of sense- and antisense probes for *in situ* hybridization.

The construct encoding human RDHE2 (SDR16C5) protein with the C-terminal FLAG-tag was described previously [27]. The coding sequence of mouse SDR16C5 enzyme was PCR-amplified from mixed cDNA generated from mouse skin and liver RNA, and cloned into pCMV-Tag4a vector to generate the C-terminal FLAG-tagged fusion. Sequences encoding human and mouse FLAG-tag fusion proteins were re-amplified and transferred into pCS105 vector for expression in mammalian cells. Sequences of all PCR primers and cloning sites are provided in Supplemental Table 1.

2.3. Whole mount in situ hybridization

In situ hybridization was performed as described in [28] for ascidian, and in [29] for sea urchin embryos. Sense and antisense probes were synthesized with digoxigenin RNA labeling mix (Roche) and T3 or T7 RNA polymerase (Promega) using linearized constructs in pBluescript as templates. Probes were detected using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) and BM purple substrate (Roche).

2.4. Analysis of activity toward retinoids in cell culture

HEK293 cells were grown in 6-well gelatinized cell culture plates and transfected with 2 µg of the expression constructs per well using Lipofectamine 2000 (Life Technologies), according to the manufacturer's protocol. On the next day after transfections, cells were treated with 5 µM all-*trans*-retinol for 8 h, or 5 µM all-*trans*-retinaldehyde for 4 h. Cells were harvested, and retinoids were extracted, separated using normal-phase HPLC and quantified as described before [3,30]. The expression of recombinant proteins in cell homogenates was detected by Western blotting with anti-FLAG epitope antibody (M7425, Sigma Aldrich) at 1: 2500 dilution.

2.5. Invertebrate animals

Specimen of ascidian *Ciona intestinalis* were procured from the Ascidian Resource Center, University of California – Santa Cruz. Dissection to obtain gametes and *in vitro* cross-fertilization were performed as described in [31]. Embryos were cultivated in artificial sea water at ambient temperature, collected at different stages up to 48 h post-fertilization and either homogenized in Trizol reagent (Life Technologies) for RNA isolation, or fixed in 4% paraformaldehyde in 0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO₄ for *in situ* hybridization. After fixation, embryos were washed with phosphate-buffered saline, dehydrated by transferring into increasing concentrations of ethanol, and stored in 100% ethanol at –20 °C. Specimen of *L. variegatus* were provided by the Aquatic Animal Research Core within the Nutrition and Obesity Research Center at the University of Alabama – Birmingham. Sea urchin spawning and *in vitro* fertilization were performed as described in [32]; embryos were incubated in artificial sea water at ambient temperature, collected at different stages and processed as described above for ascidian embryos.

3. Results and discussion

3.1. Phylogenetic analysis

To trace the evolutionary origins of vertebrate SDR16C enzymes, we have identified their homologs in invertebrate genomes belonging to major Metazoan phyla. Relative phylogenetic positions of the covered phyla and species included in the analysis are shown in a simplified diagram in Fig. 3. Only the full-length sequences with over 30% protein identity to mammalian enzymes, a total of 94 proteins, were included in further analysis. Accession numbers and a full-length alignment of sequences used in this work are provided in Supplemental Fig. 1.

Phylogenetic analysis was conducted to determine the position of putative invertebrate homologs within SDR16C family. Two alternative methods, maximum likelihood and minimum evolution, were used to infer the phylogeny for 95 protein sequences with human SDR34C1 enzyme included as an outgroup. Phylogenetic tree constructed using minimum evolution method is shown in Fig. 4. To demonstrate in a compact dendrogram how widely SDR16C proteins occur in Metazoans, clades formed by sequences that belong to the same phylum were collapsed and are represented by triangles with width proportional to the number of individual sequences in the clade. The clades are labeled only with the name of the phylum that they represent, and a number of sequences in each clade. The complete tree with individual sequence labels is shown in Supplemental Fig. 2. Phylogenetic analysis placed non-chordate deuterostome and protostome sequences into clades containing each of the vertebrate SDR16C forms (RDHE2-, RDH10-, HSD17B11/HSD17B13, and DHRS3). This implies that the common ancestor for all bilaterians had already possessed all four major forms of SDR16C. While the bootstrap support [37] for the clades containing only vertebrate enzymes was strong (96–100% out of 1000 replicates), the bootstrap values for clustering of protostome enzymes with the particular clade containing vertebrate proteins were generally low, except for the DHRS3-containing clade (77%). Low bootstrap support does not allow for good resolution at the base of each clade, and for unambiguous assignment of some invertebrate proteins to either RDH10 or RDHE2 clade. The topology

of the obtained tree shows that several invertebrate sequences (from cnidarian, ecdyzoans, non-chordate deuterostomes and larvacean urochordate) occupy the basal position for the RDHE2 and RDH10 clades instead of clustering with any one of the two groups. This implies that these basal sequences might have evolved from a common ancestral form for RDHE2 and RDH10, before the duplication generating these two separate forms occurred. The phylogeny estimated using maximum likelihood method (not shown) resulted in a similar topology, supporting the presence of invertebrate homologs in each of the four major groups of SDR16C family.

3.2. Functional analysis of invertebrate homologs of RDH10 and RDHE2

To complement the phylogenetic analysis with functional studies, we cloned predicted homologs of vertebrate RDH10 and RDHE2 enzymes from the invertebrate chordate, ascidian *C. intestinalis*, and from a non-chordate deuterostome, green sea urchin *L. variegatus*. Transcripts encoding sequences of two predicted ascidian homologs, corresponding to cDNA clones AK115133 and AK114164, were amplified from mixed cDNA of early (blastula through neurula) and late (tailbud and tadpole) stages, suggesting the expression throughout embryonic development. These ascidian homologs match predicted *Ciona* proteins XP_2120364 and XP_002130984 automatically annotated as “RDH10 isoform” and “RDHE2-like” enzymes, therefore, here we designated them as *Ci* RDH10 and *Ci* RDHE2. The deduced coding sequences of three homologs from sea urchin were also amplified from both earlier (through gastrula) and later (pluteus larvae until 48 h post fertilization) stages. As these *L. variegatus* sequences had not been reported and characterized previously, we deposited them in the Genbank under the accession numbers KM358166, KM358167 and KM358168, and designated them as retinol dehydrogenase 10 isoforms A, B and C (RDH10-A, RDH10-B, RDH10-C). Fig. 5 shows a protein alignment of the newly cloned homologs with human RDH10 and RDHE2 sequences. All ascidian and sea urchin sequences contain the conserved glycine-rich motif GxxxGxG typical of SDR NAD(P) cofactor binding site, and the conserved substrate binding motif YxxxK.

Whole mount *in situ* hybridization revealed that ascidian and sea urchin RDH10 homologs were expressed in specific domains (Fig. 6), implying a possible role in tissue development. Antisense probes for all three of the sea urchin RDH10 and RDHE2 homologs detected similar expression pattern (shown for RDH10-B), suggesting some functional redundancy. Hybridization with sense probes was used as a control, and detected a much weaker background staining.

To determine whether invertebrate proteins have the same enzymatic activity as their vertebrate homologs, ascidian and sea urchin proteins were expressed in HEK293 cells as FLAG-tagged fusions, and their expression was verified by Western blotting with anti-FLAG epitope antibody. Their ability to metabolize retinoids was determined in the assays that we routinely use to characterize mammalian retinoid-active enzymes. After treatment with all-*trans*-retinol, cells transfected with the expression constructs produced significantly more retinaldehyde and RA than the cells transfected with an empty vector (Fig. 7). After treatment with all-*trans*-retinaldehyde, however, cells transfected with expression constructs did not show an increased conversion of retinaldehyde to retinol (data not shown). These

findings are similar to those previously reported for vertebrate RDH10, RDHE2 and RDHE2-similar enzymes [3,30]: in intact cells, these proteins function as retinol dehydrogenases, and contribute to the oxidation of retinol, but not to the reduction of retinaldehyde. Thus, the invertebrate proteins belonging to the RDH10-RDHE2 branch of SDR16C family appear to have evolved retinol dehydrogenase activity similar to vertebrate homologs before the emergence of chordate body plan.

These findings support the pre-chordate origins of a two-step pathway of RA biosynthesis, and have a broader implication in the emergence RA signaling early in bilaterian evolution. However, the presence of RDH10-RDHE2 homologs in non-bilaterian Cnidarians and Placozoans, which do not have RA receptors, suggests that the function of the ancestral metazoan form was unrelated to the RA signaling. Interestingly, genome of the larvacean urochordate *Oikopleura dioica* encodes at least six RDH10-RDHE2 homologs, which form a separate clade on the phylogenetic tree, suggesting several lineage-specific duplications (Fig. 4 and Supplemental Fig. 2), but this organism is thought to lack RA signaling due to the loss of RA receptors [38]. It is yet to be determined whether enzymes from the animals that lack RA receptors can oxidize retinol, but *in vivo* they are likely to be involved in pathways other than the synthesis of RA ligand for retinoid nuclear receptors.

3.3. Evolution of DHRS3 group

As established by phylogenetic analysis, DHRS3 (SDR16C1) branch of SDR16C family contains putative homologs from invertebrate chordates (amphioxus), non-chordate deuterostomes (hemichordate acorn worm) and from protostome annelid and mollusk (Figs. 4 and 8A). This suggests that the common ancestor of protostome and deuterostome animals (Urbilaterian) possessed a DHRS3-similar enzyme.

In light of our recently reported findings that human DHRS3 acts as a regulator of the retinol-oxidizing activity of RDH10 [5], it is interesting to establish when the enzymes of DHRS3 group have evolved their catalytic properties. Among the characterized vertebrate SDR16C enzymes, only DHRS3 protein prefers the phosphorylated NAD cofactor and functions as a reductase in intact cells. Although the functional analysis of predicted invertebrate DHRS3 homologs was beyond the scope of the current study, we compared their primary structures with proteins belonging to other clades of SDR16C family to evaluate their cofactor preferences. As shown in the partial alignment in Fig. 8B, enzymes from RDH10, RDHE2 and HSD17B11/13 groups all have an aspartate in position 37 of this alignment, while none of DHRS3-similar proteins, including predicted invertebrate homologs, has an aspartate at this position. Instead, putative DHRS3 enzymes have a glycine residue immediately followed by the positively charged amino acid, arginine (Fig. 8B). In NAD⁺-dependent enzymes, the acidic residue (aspartate) often occupies the position at the C-terminus of the second strand of Rossmann fold and is critical for the interaction with NAD⁺. In NADP⁺-preferring enzymes, the acidic residue is absent, and this position is followed by a basic amino acid [39,40]. Thus, although we did not assess their enzymatic properties experimentally, we can predict that the putative enzymes belonging to the DHRS3 clade of SDR16C are likely to prefer NADP⁺ as a cofactor, and function in the reductive direction *in vivo*, similar to vertebrate DHRS3 enzymes.

A remarkable property of human DHRS3 enzyme is that it requires the activation by RDH10 to display its full retinaldehyde reductive activity [5]. In turn, it modulates the retinol-oxidizing activity of RDH10. The interaction between these two enzymes forms a finely tuned mechanism for the control of RA levels, which likely involves a direct protein–protein binding. To establish whether this mechanism is unique for RDH10-DHRS3 pair of enzymes, or DHRS3 can be activated by related enzymes of RDHE2 group, and thus display a broader regulatory control over the oxidative SDR16C enzymes, we transfected HEK293 cells with human and mouse RDHE2 (SDR16C5) alone, or together with DHRS3 expressing construct, and treated the cells with all-*trans*-retinaldehyde. In analogous experiment described in Fig. 2B in Ref. [5], neither RDH10, nor DHRS3-transfected cells showed increased production of retinol, whereas in the cells expressing both RDH10 and DHRS3 proteins, DHRS3 became active leading to the increased conversion of retinaldehyde to retinol. However, as shown in Supplemental Fig. 3, coexpression of neither human, nor mouse RDHE2 enzyme with human DHRS3 leads to increased synthesis of retinol. Hence, it appears that the mutually activating interaction with DHRS3 is specific for RDH10 enzymes and either evolved in RDH10 clade of SDR16C family, or was lost in RDHE2 clade.

The intriguing question to be addressed in the future functional studies is whether the invertebrate forms of DHRS3 can reduce retinaldehyde, or they were recruited in retinoid metabolism later, in conjunction with the appearance of a sensitive mechanism to control the levels of RA during chordate, or, specifically, vertebrate development. Additional studies will also establish whether invertebrate DHRS3 enzymes require activation by RDH10 homolog, and whether the interaction between RDH10 and DHRS3 enzymes is a feature specific for vertebrates, or it is more widespread in the animal kingdom.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

SDR16C proteins encoded by human and mouse genomes. Mouse and human orthologs occupy syntenic areas in the genomes. In human genome, genes encoding RDHE2 and RDHE2-similar, are located on the same chromosome as RDH10, while in mouse genome, Rdhe2 and Rdhe2-similar are located on chromosome 4 together with DhRs3. This suggests that in the common ancestor of mouse and human lineage genes encoding RDH10, DHR3, RDHE2 and RDHE2-similar enzymes were located on the same chromosome. RDHE2 and RDHE2-similar represent a recent mammalian duplication, because amphibian genome contains only one gene at this position [3].

Fig. 2.

Diagram showing the main components of RA signaling. RA signaling consists of two modules: metabolic pathways leading to the biosynthesis of RA and RA-mediated regulation of transcription by nuclear receptors. RA serves as a ligand for RA receptors and peroxisome proliferator-activated receptors (PPAR), which function as heterodimers with retinoid X receptors (RXR). RA is synthesized from its immediate precursor, retinaldehyde (RAL) in an irreversible reaction catalyzed by aldehyde dehydrogenases type I (ALDH1A). Biosynthesis of RA from retinol (ROL), esterified form of which (RE) is the main vitamin A storage form invertebrates, includes a reversible step of retinol oxidation, where a balance between oxidative and reductive activities offers a tighter control over the RA levels. While several enzymes of SDR and alcohol dehydrogenase (ADH) superfamilies can catalyze the oxidation of retinol, and several SDRs and aldo-keto reductases (AKRs) can catalyze the reverse reaction, only enzymes of SDR16C family were shown to be essential for RA levels during normal vertebrate development [1–5].

Fig. 3.
A simplified diagram of phylogenetic relationship of Metazoan groups included in the analysis.

Fig. 4.

Evolutionary history of SDR16C family. Minimum Evolution method [33] was used. The optimal tree with the sum of branch length = 26.45033940 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method [34] and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [35] at a search level of 1. The Neighbor-joining algorithm [36] was used to generate the initial tree. All positions containing gaps were eliminated. There were a total of 238 positions in the final dataset. Ascidian (*Ci*) and sea urchin (*Lv*) homologs used for functional analysis in this study are shown next to the branches to which they belong.

Fig. 5.

Protein alignment of newly characterized enzymes. Prefixes *Ci* and *Lv* designate *C. intestinalis* and *L. variegatus* sequences. Amino acid positions that are identical in all sequences, are shown on the black background, similar in all sequences – on gray. BLOSUM62 protein weight matrix was used to determine amino acid similarity.

Fig. 6. Expression pattern of sea urchin *Rdh10-b* in pluteus larvae and ascidian *Rdh10* in early tadpole embryo. Whole mount *in situ* hybridization was performed with digoxigenin-labeled antisense RNA probes, and developed using alkaline-phosphatase conjugated anti-digoxigenin antibody and BM purple substrate. *In situ* hybridization with a sense probe is shown as a control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Conversion of all-*trans*-retinol by HEK293 cells expressing ascidian and sea urchin proteins. All-*trans*-retinaldehyde (RAL, panel A) and retinoic acid (RA, panel B) produced by cells transfected with empty vector, or constructs expressing sea urchin and ascidian proteins after treatment with 5 μ M all-*trans*-retinol. The observed differences between mock-transfected and invertebrate enzymes expressing cells were significant under two-tailed *t*-test with *p*-values < 0.05, with the exception of retinaldehyde produced by cells expressing ascidian proteins (*p* = 0.09 for *Ci* RDH10, and *p* = 0.06 for *Ci* RDHE2).

Fig. 8.

Analysis of phylogeny and cofactor preferences of DHRS3 homologs. (A) DHRS3 clade of the minimum evolution tree of SDR16C family. (B) Partial alignment of cofactor-binding domains of SDR16C. Asterisk marks the amino acid position occupied by aspartate in NAD⁺-preferring enzymes.