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Identification and Characterization of Retinoid-Active Short-Chain Dehydrogenases/Reductases in *Drosophila melanogaster*

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Abstract

Background—In chordates, retinoid metabolism is an important target of short-chain dehydrogenases/reductases (SDRs). It is not known whether SDRs play a role in retinoid metabolism of protostomes, such as *Drosophila melanogaster*.

Methods—*Drosophila* genome was searched for genes encoding proteins with ~50% identity to human retinol dehydrogenase 12 (RDH12). The corresponding proteins were expressed in Sf9 cells and biochemically characterized. Their phylogenetic relationships were analyzed using PHYLIP software.

Results—A total of six *Drosophila* SDR genes were identified. Five of these genes are clustered on chromosome 2 and one is located on chromosome X. The deduced proteins are 300 to 406 amino acids long and are associated with microsomal membranes. They recognize all-*trans*-retinaldehyde and all-*trans*-3-hydroxyretinaldehyde as substrates and prefer NADPH as a cofactor. Phylogenetically, *Drosophila* SDRs belong to the same branch of the SDR superfamily as human RDH12, indicating a common ancestry early in bilaterian evolution, before protostome-deuterostome split.

Conclusions—Similarities in the substrate and cofactor specificities of *Drosophila* versus human SDRs suggest conservation of their function in retinoid metabolism throughout protostome and deuterostome phyla.

General Significance—The discovery of *Drosophila* retinaldehyde reductases sheds new light on the conversion of β-carotene and zeaxantine to visual pigment and provides a better understanding of the evolutionary roots of retinoid-active SDRs.

Keywords

retinaldehyde; retinol; dehydrogenase; reductase; *Drosophila*; visual pigment

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INTRODUCTION

Short-chain dehydrogenases/reductases (SDR) represent one of the largest known families of proteins [reviewed in refs. 1 and 2]. To date, well over 3000 primary structures from various species have been annotated in sequence databases as members of the SDR superfamily based on SDR signature features such as the TGX3GXG motif of the nucleotide binding region and the catalytically active tetrad N-S-Y-K, which constitutes the active site [1]. At least 70 SDR genes have been identified in the human genome database [3]; and about two-thirds of the human SDRs have been characterized to some extent with respect to their expression, substrate and cofactor specificities. A large number of these enzymes were found to exhibit catalytic activities toward retinoid substrates [reviewed in ref. 4], suggesting that retinoid metabolism is an important target of SDR enzymes.

Retinoid metabolism takes place in many types of cells and tissues, resulting in the production of visual pigment 11-*cis*-retinaldehyde [5] or bioactive retinoic acid, which serves as an activating ligand for nuclear transcription factors, retinoic acid receptors [6]. Two types of retinoid-active SDRs have been identified: the NAD^+- and $NADP^+$-preferring. The NAD^+-preferring SDRs, such as RDH5 and RDH10, participate primarily in the oxidative processes [4]. RDH5 catalyzes the oxidation of 11-*cis*-retinol to 11-*cis*-retinaldehyde in the visual cycle [7], whereas RDH10 oxidizes all-*trans*-retinol to all-*trans*-retinaldehyde and contributes to retinoic acid biosynthesis [8]. The importance of RDH5 and RDH10 as retinol dehydrogenases is supported by *in vivo* observations: mutations in human *RDH5* gene cause a delay in the regeneration of cone and rod photopigments [9], whereas mutation in RDH10 results in embryonic lethality, consistent with its role in retinoic acid biosynthesis [10]. On the other hand, the $NADP^+$-preferring RDH12 appears to function in the reductive direction, catalyzing the conversion of all-*trans*-retinaldehyde to all-*trans*-retinol in the eye [11,12], and protecting photoreceptors from excessive all-*trans*-retinaldehyde that forms from 11-*cis*-retinaldehyde upon eye illumination [5]. Mutations in human *RDH12* gene lead to severe early-onset autosomal recessive retinal dystrophy [13] and autosomal dominant retinitis pigmentosa [14], indicating that the reductive activity of RDH12 is essential for vision.

SDRs are found in all forms of life, but it is not yet clear when they acquired the ability to recognize retinoids as substrates. Recent studies have identified genes encoding key components of retinoic acid signaling pathway in non-chordate deuterostomes, such as hemichordates and echinoderms [15-17], and even in protostomes, such as mollusks and annelids [18]. Whether these non-chordate animals produce and utilize retinoic acid remains to be shown. However, the use of retinaldehydes for vision in protostomes is well established [19]. *Drosophila*, a well-characterized model organism that belongs to ecdyzoan lineage of protostomes, is well known to utilize retinoid chromophore for generation of the visual pigment. In *Drosophila*, opsin is bound to the hydroxylated retinoid, 11-*cis*-3-hydroxy-retinal [reviewed in ref. 20]. 11-*cis*-3-hydroxyretinal can be derived from β-carotene or zeaxanthin [21,22], which represents the hydroxylated form of β-carotene at positions 3 and 3' of the β-ionone ring. It is not yet known whether SDRs have a role in retinoid metabolism in flies. To address this question and to better understand the evolutionary origins of retinoid-active SDRs, we examined whether proteins related to mammalian RDH12 exist in *Drosophila*, and then characterized the properties of identified proteins to determine whether their functions are conserved throughout the protostome and deuterostome phyla.

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl-α-D-glucoside, all-*trans*-retinaldehyde and all-*trans*-retinol were from Sigma-Aldrich (St. Louis, MO), all-*trans*-3-hydroxyretinal – from Toronto Research Chemicals

(Toronto, Canada). All-*trans*-3-hydroxyretinol was synthesized from all-*trans*-3-hydroxyretinal by reduction with potassium borohydride [8].

Drosophila SDR clones and expression constructs

Fruit fly homologs of mammalian SDRs were identified through the BLAST search of *Drosophila* sequences using human RDH12 protein sequence as a query. Berkeley Drosophila Genome Project EST clones encoding identified RDH12 homologs were obtained from Research Genetics (Huntsville, AL): clone SD23903 for homolog CG2064, clone RH23455 for homolog CG2065, clone LP06328 for homolog CG2070, clone GH10714 for homolog CG3842, and clone AT09608 for homolog CG30491. Constructs for expression of *Drosophila* SDRs in Sf9 cells were made as follows. The cDNA sequences were PCR-amplified using primers with restriction sites (underlined) matching those in baculovirus transfer vector pVL1393 (BD Biosciences Pharmingen, San Jose, CA): 5′-tattctagatgtgcattttcatcgattgct-3′ and 5′-cgtagatcttagttattagcttccagcttatc-3′ for CG2064, 5′-ttgtctagaaaatacatgcagggcggtcag-3′ and 5′-aaactgcaggttgcttaatcaactttggttga-3′ for CG2065, 5′-gtagaattcggagcggaaagatgagtg-3′ and 5′-atactgcagctcatatattaattcccgtccac-3′ for CG2070, 5′-atcgaattcatcgcttagccagctatgtcg-3′ and 5′-accagatctacgaacgattgaccacgacc-3′ for CG3842, and 5′-tttgaattcctaaaatgtcactatttgcgt-3′ and 5′-ctgctgcaggaaacagctatgaccatgtg-3′ for CG30491. The PCR products were cleaved with restriction endonucleases, purified, and cloned into the corresponding sites of pVL1393 vector (BD Biosciences Pharmingen) as follows: CG2064 into *Xba*I – *Bgl*II, CG2065 into *Xba*I – *Pst*I, CG3842 into *Eco*RI – *Bgl*II, and both CG2070 and CG30491 into *Eco*RI – *Pst*I restriction sites. The resulting constructs were sequence-verified and expressed in Sf9 insect cells using Baculovirus expression system as described for RDH12 [23].

Identification of non-Drosophila RDH12 homologs and phylogenetic analysis

Strongylocentrotus purpuratus, Ciona intestinalis and Caenorhabditis elegans genomic sequence databases available at NCBI website were searched using *tblastn* algorithm with human RDH12 and RDH14 protein sequences as queries. If only partial sequences were retrieved, the NCBI EST database was also screened for overlapping clones in order to recover full-length coding sequences. Accession numbers for all sequences used in this work are provided in Supplemental Table 1. If the predicted full-length homolog is derived from several sequences, all accession numbers are provided.

Protein sequences were aligned using ClustalW [24]; a maximum likelihood phylogenetic tree with Jones, Taylor, and Thornton model of amino acid substitution was obtained using PHYLIP package, version 3.68 [25,26]. TREEVIEW 1.6.6 [27] was used for graphic representation of the tree.

RT-PCR

Total RNA was isolated from heads or bodies of wild-type fruit flies (*Canton-S* strain) using RNeasy kit (Qiagen, Valencia, CA, USA) and reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI, USA). Transcripts were detected by RT-PCR with the following primers: 5′-tgtgtccagtctggcacatgc-3′ and 5′-aaggcaccagaagccacagg-3′ for CG2064, 5′-gtttccagcctggtgcacac-3′ and 5′-ctttccagtcttgtcgtcctg-3′ for CG2065, 5′-tgtgaagcttgttttgctcccg-3′and 5′-cccacggattacctgttctgc-3′ for CG2070, 5′-gattgttctgggcatattgctc-3′ and 5′-aagagatgtgccgccgaactg-3′ for CG3842, 5′-tgcgtttctcaagagccgcac-3′ and 5′-aggccttgccttcatcgtagg-3′ for CG30491, and 5′-gtttacatggcatgccgcaac-3′ and 5′-cctggctatctccgtgtctg-3′ for CG30495.

HPLC analysis of enzymatic activity

Enzymatic activity of Sf9 microsomes expressing *Drosophila* proteins was assayed with BSA-solubilized retinaldehyde substrates as described previously [28], except the reactions were incubated at ambient temperature. Reactions with all-*trans*-retinaldehyde were stopped by the addition of equal volume of cold methanol and extracted twice with double volume of hexane. For extraction of 3-hydroxyretinoids, the procedure described in reference [29] was modified as follows: the reactions were stopped by the addition of 0.7 volumes of methanol, and then extracted with a triple volume of dichlorometane : hexane (1 : 2). This step was repeated twice. The extraction efficiency of 3-hydroxyretinoids from aqueous reaction buffer was determined by performing triplicate extractions of all-*trans*-3-hydroxyretinaldehyde and all-*trans*-3-hydroxyretinol solubilized using equimolar BSA [28], followed by quantification of extracted 3-hydroxyretinoids using normal phase high performance liquid chromatography (HPLC). The difference in extraction efficiency between replicates was less than 5%; and the average recovery of all-*trans*-3-hydroxyretinaldehyde was 1.42-fold greater than that of retinol. This difference was taken into account whenever the enzymatic conversion was calculated. The extraction efficiencies for all-*trans*-retinol and all-*trans*-retinaldehyde were similar [23].

HPLC was performed using Waters Alliance Separation Module with Spherisorb S3W column (4.6 × 100 mm) and 2996 Photodiode Array Detector (Waters Corp., Milford, MA, USA). Mobile phase for the analysis of non-hydroxylated retinoids was hexane : ethyl acetate at 90 : 10, for 3-hydroxyretinoids – hexane : ethyl acetate : ethanol at 80 : 19 : 1. Peaks were identified by comparison to retention times of retinoid standards and evaluation of wavelength maxima. Retinoids were quantified by comparing their peak areas to a calibration curve constructed from peak areas of a series of standards.

α-Glucosidase activity assay

Colorimetric assay of α-Glucosidase activity was performed as described in [30]. One hundred micrograms of protein from different subcellular fractions were resuspended in PBS containing 1% Triton X-100, 0.1 mM glutathione, and 2.5 mM *p*-nitrophenyl-α-D-glucoside. Reaction mixtures (100-μl) were incubated at 37 °C for 5 hours and stopped by the addition of 0.7 ml of 0.2M Na_2CO_3. *p*-Nitrophenyl-α-D-glucoside cleavage was measured based on absorbance at 410 nm.

Determination of kinetic constants

The saturation curves for enzyme kinetics were performed under conditions when enzymatic activity was linearly dependent on the protein concentration and the incubation time. The apparent K_m values for the reduction of all-*trans*-retinaldehyde and 3-hydroxy-all-*trans*-retinaldehyde were determined at 1 mM NADPH and six concentrations of substrate (0.125−6 μM for all-*trans*-retinaldehyde, and 0.25−12 μM for all-*trans*-3-hydroxyretinaldehyde). With all-*trans*-retinaldehyde as substrate, the concentration of microsomal protein in activity assays was 0.5 μg/ml for CG2070, CG3842, CG30491, and 5−10 μg/ml for CG2064 and CG2065. With all-*trans*-3-hydroxyretinaldehyde, the microsomal protein concentration was 0.5−2.5 μg/ml for CG2070, CG3842, CG30491, and 5−10 μg/ml for CG2064 and CG2065.

Densitometric analysis of the protein bands in Coomassie-stained SDS-PAGE was done using UN-SCAN-IT software (Silk Scientific Corporation, Orem, UT).

RESULTS

Identification of RDH12–like *Drosophila* SDRs

To identify *Drosophila* homologs of RDH12, we carried out BLAST search of NCBI *Dr. melanogaster* genomic database using RDH12 protein sequence as a query. Surprisingly, this

search returned six putative proteins with 47−53% of pairwise identity to human RDH12 (Fig. 1) and 50−65% identity among themselves. The amino acid sequences of all deduced proteins contained motifs characteristic of the short-chain dehydrogenases/reductases (SDRs) [1,2] – the cofactor binding motif TGX_3GXG and the active site consensus sequence YX_3K (Fig. 1, inverted color). CG3842 was the longest among this group of SDRs with 406 amino acids. CG2065 polypeptide was the shortest with only 300 amino acids. The lengths of CG2064, CG2070, CG30491 and CG30495 polypeptides were between 325 and 331 amino acids (Fig. 1). Five of the *Drosophila* SDRs appeared to contain at least one N-terminally located transmembrane segment, as indicated by algorithms for prediction of transmembrane helices [31]. The existence of multiple homologs of RDH12 in *Drosophila* suggested potential redundancy in their functions.

Structural Organization of *Drosophila* SDR Genes

To better understand the relationships among the six RDH12-homologous genes, we compared their genomic structures. The genes encoding CG2064, CG2065, CG2070, CG30491 and CG30495 were located on chromosome 2, forming a tight cluster at the cytogenetic region 43E7−43E9. Alignment of predicted mRNAs with genomic sequences showed that CG2070 and CG30495 have similar exon-intron organization with four exons interrupted by three introns (Fig. 2). Exon 3 in CG2070 and CG30495 (417 bp) appeared to have split into two separate exons (157 bp and 260 bp) in three other genes CG2064, CG2065, and CG30491. In addition, the segment corresponding to exon 2 (264 bp) underwent another split (166 bp and 98 bp) in CG30491, adding the sixth exon to the gene structure. Interestingly, the ATG codon in the first exon of CG2065 gene and corresponding EST transcripts was followed by a stop codon. Thus, the open reading frame of CG2065 gene started at the ATG codon in the second exon, resulting in a shorter polypeptide chain (Fig. 2).

The remaining RDH12 homolog, CG3842, was located on X chromosome at 5F2 and consisted of non-coding exons 1 and 2 and a single coding exon 3, which suggests that this gene was derived from one of the homologs in chromosome 2 cluster via retroposition mechanism. This gene encoded a protein extended on both the N-terminus and C-terminus compared to other homologs (Figs. 1 and 2). Two transcripts for CG3842 were found in EST database, suggesting that CG3842 was alternatively spliced. The first transcript included exons 1 and 3, while the second transcript was comprised of exons 2 and 3. The open reading frame in both transcripts was identical.

To confirm that these SDR genes were expressed in fruit flies, we carried out RT-PCR analysis of total RNA isolated from abdomen, head and thorax of young adult and old flies, early larvae, third instar larvae and pupa using intron-spanning primers. This analysis detected transcripts of all six SDR genes in most of the samples (data not shown), suggesting an overlapping and broad expression pattern.

Expression of *Drosophila* SDRs in Sf9 Cells

To characterize the properties of *Drosophila* SDRs, the coding regions of CG2064, CG2065, CG2070, CG3842, and CG30491 were cloned into the Baculovirus vector for expression in insect cells. CG30495 was excluded because of uncertainty in its genomic sequence at the time when this study was initiated.

Initial assays were designed to determine whether the *Drosophila* SDRs possessed a retinaldehyde reductase activity, as predicted by their similarity to RDH12. These assays were carried out by incubating Sf9 cells infected with recombinant viruses in the presence of 5 μM all-*trans*-retinaldehyde and 1 mM NADPH. Interestingly, all cells infected with SDR expression constructs converted retinaldehyde to retinol at a greater rate than the cells infected

with the wild-type virus. Cells expressing CG2070 and CG3842 proteins exhibited the highest activity, exceeding the retinaldehyde reductase activity of control cells by 36-fold. The activity of these cells was closely followed by CG30491-producing cells, while CG2065 and CG2064 cells were significantly less active, exceeding the background by 8- and 3-fold, respectively.

CG2070 cells, which exhibited the greatest retinaldehyde reductase activity, were also tested for the oxidative activity toward all-*trans*-retinol with NAD^+ or $NADP^+$ as cofactors. These cells oxidized all-*trans*-retinol to all-*trans*-retinaldehyde in the presence of both cofactors, but the reaction rate was at least 17-fold greater in the presence of 1 mM $NADP^+$ than 1 mM NAD^+. Similarly, in the reductive direction, the reaction rate was greater with 1 mM NADPH than with 1 mM NADH. These assays demonstrated that, like RDH12, CG2070 was a bidirectional enzyme that catalyzed the oxidoreductive interconversions of retinoids and preferred $NADP^+$ and NADPH as cofactors.

It has been shown that several mammalian RDH12-related enzymes localize in endoplasmic reticulum, while RDH13, unlike other homologs, resides in mitochondria [23,32-34]. To determine the subcellular localization of *Drosophila* SDRs, Sf9 cells expressing CG2070 and CG3842 proteins were fractionated into nuclei, mitochondria, microsomes and cytosol by differential centrifugation as described previously [35]. The greatest retinaldehyde reductase activity for both CG2070 and CG3842-expressing cells was detected in 105,000 × *g* pellet (Table 1), suggesting that, like RDH12, *Drosophila* SDRs were associated with the microsomal membranes. To obtain further confirmation, the subcellular fractions were analyzed for α-glucosidase activity, which serves as a marker of endoplasmic reticulum membranes [30]. The highest specific activity toward *p*-nitrophenyl-α-D-glucoside was found in 105,000 × *g* pellet, confirming that this fraction is enriched in microsomal membranes and contains *Drosophila* SDRs.

Microsomal fractions from cells expressing fruit fly homologs were analyzed by SDS-PAGE followed by Coomassie R-250 staining. Each of the recombinant *Drosophila* proteins was clearly visible in the gel and appeared as a single protein band that was absent in the microsomes obtained from cells infected with the wild-type virus (Fig. 3). In general, electrophoretic mobilities of recombinant CG2064 (36.6 kDa), CG2065 (33.4 kDa), CG2070 (36.3 kDa), CG3842 (44.9 kDa) and CG30491 (37.1 kDa) correlated well with their predicted molecular masses. The amount of protein in the microsomal fraction was the highest for CG2070, with four other proteins present at lower and approximately equal levels.

This initial analysis demonstrated that all *Drosophila* homologs of RDH12 were catalytically active and exhibited both the subcellular localization and enzymatic activity similar to those of human RDH12.

Kinetic Analysis of *Drosophila* SDRs

Further characterization of *Drosophila* SDR proteins was carried out using the microsomal fractions of Sf9 cells as described previously for other microsomal SDRs [28,32]. Kinetic analysis showed that four of the homologs exhibited the apparent K_m values for all-*trans*-retinaldehyde in the submicromolar range, with only CG2064 having a higher K_m value of 4.2 μM (Table 2). CG2070, CG3842 and CG30491 microsomes exhibited greater reaction rates than CG2064 and CG2065 microsomes, but this could be due in part to the higher levels of the corresponding recombinant proteins in the microsomal fractions, especially in the case of CG2070 (Fig. 3). To account for the differences in expression levels of recombinant proteins, the activity of microsomal fractions was normalized per amount of each of the *Drosophila* proteins estimated using densitometric analysis of the scanned gel image. This resulted in more similar specific activities toward all-*trans*-retinaldehyde, ranging from 1 to 6.2 relative units (Table 2).

In addition to all-*trans*-retinaldehyde, we tested the activities of *Drosophila* SDRs toward all-*trans*-3-hydroxyretinaldehyde, since this hydroxylated form of retinaldehyde could be potentially produced by the cleavage of zeaxanthin, a major source of retinoids in *Drosophila* [21,22]. Interestingly, all five *Drosophila* SDRs recognized all-*trans*-3-hydroxyretinaldehyde as a substrate (Table 2). CG2070 was especially active, exhibiting the highest rate of 81 $nmol \cdot min^{-1} \cdot mg^{-1}$ at 8 μM all-*trans*-3-hydroxyretinaldehyde. The K_m value of this enzyme could not be determined because of the apparent substrate inhibition at higher concentrations, but the apparent K_m values of four other SDRs for the reduction of all-*trans*-3-hydroxyretinaldehyde were significantly higher than those for all-*trans*-retinaldehyde (Table 2). This resulted in somewhat lower catalytic efficiencies of *Drosophila* SDRs toward 3-hydroxyretinaldehyde (Table 2). Normalization of the rates per expression levels of different proteins reduced the range of activities somewhat, but still revealed great differences between CG30491 and CG3842 on one side and CG2064 and CG2065 on the other in their ability to metabolize 3-hydroxyretinaldehyde.

Thus, kinetic analysis demonstrated that the affinity of *Drosophila* SDRs for all-*trans*-retinaldehyde was very high and similar to that of RDH12. Furthermore, *Drosophila* SDRs were able to recognize another physiologically relevant form of retinaldehyde in *Drosophila*, the all-*trans*-3-hydroxyretinaldehyde.

Phylogenetic Relationships between *Drosophila* SDRs and RDH12

Similarities in the subcellular localization as well as the substrate and cofactor specificities of fruit fly SDRs and human RDH12 suggested common ancestry and, therefore, an ancient origin of these enzymes. While human RDH12 and other mammalian RDH12-related retinaldehyde reductases have been a focus of numerous studies [12-14,32,36-38], no analysis has been performed to trace the origins of this gene family. To address this question, we expanded our search of RDH12 homologs to non-vertebrate species, for which whole genome sequencing data became available. This search identified five predicted genes encoding proteins with over 40% identity to RDH12 in the genome of non-vertebrate chordate, ascidian *Ciona intestinalis*. Nine such homologs were identified in non-chordate deuterostome sea urchin *Strongylocentrotus purpuratus* (accession numbers in Supplemental Table 1), suggesting that this divergent family is not limited to chordates. To identify potential homologs in protostomes other than fruit fly, we searched the *C. elegans* genome using RDH12 and CG2070 proteins as queries. Interestingly, both searches returned the same two sequences encoding uncharacterized *C. elegans* SDRs dhs-22 and dhs-24 (NP_506570 and NP_507860). The deduced amino acid sequence of the two *C. elegans* proteins appeared to be equidistantly related to human and fruit fly homologs, with Dhs-22 sharing 37−40% identity with human RDH11−14 and 36−43% identity with *Drosophila* SDRs, and dhs-24 being somewhat less similar with 33−36% and 34−37% identity, respectively.

To determine the relative position of RDH12 homologous proteins in the SDR superfamily, we examined the phylogenetic relationships between mammalian RDH12-related proteins and their newly identified homologs from other species as well as their relationships with other SDRs. Mammalian SDR proteins included in this analysis were selected based on their known activities toward retinoids and/or steroids, since some retinoid-active SDRs exhibit dual retinoid/steroid substrate specificity [39]. The tree constructed using maximum likelihood algorithm revealed that fruit fly homologs clustered specifically with RDH12 and related mammalian RDH11 [35], RDH13 [33] and RDH14 [34], and not with other SDRs that exhibit NADPH-dependent retinaldehyde reductase activity, such as photoreceptor RDH (RDH8) or retina SDR1 (retSDR1) [40,41]. However, fruit fly paralogs formed a separate clade within this cluster (Fig. 4), indicating that their divergence occurred within the insect lineage. Ascidian and sea urchin SDR proteins identified through the search using RDH12 sequence were found

to belong to the same cluster as RDH11−14 and *Drosophila* retinaldehyde reductases, but they did not associate with RDH12 clade in the cluster (Fig. 4). Rather, some of these SDRs appeared to be more related to RDH13 and RDH14 proteins. For example, sea urchin Sp XP_798545 associated with RDH13 clade, whereas Sp XP_786908 associated with RDH14 clade (Fig. 4). Furthermore, among ascidian predicted homologs, five that had over 40% sequence identity to RDH12 formed a separate clade indicating a recent divergence (Fig. 4), but two others that were less similar to RDH12 (XP_002127240 and XP_002129539) and were not included in the final tree, were found within the RDH13 and RDH14-containing clades. This analysis suggested that RDH13 and RDH14 might have appeared earlier in evolution than RDH12. Finally, the phylogenetic placement of *C. elegans* predicted SDR proteins dhs-22 and dhs-24 in the same cluster as human RDH11−14 and their *Drosophila* homologs (Fig. 4) indicated that human RDH11−14, their fruit fly homologs, and nematode dhs-22 and dhs-24 all share a common ancestry early in bilaterian evolution, before protostome-deuterostome split.

DISCUSSION

This manuscript reports the first identification and characterization of retinoid-active SDRs in *Drosophila melanogaster*. The five proteins characterized in this study were identified based on their sequence similarity to human RDH12. As shown here, these fruit fly proteins also share remarkable similarities with human RDH12 in terms of their biochemical properties. First of all, fruit fly homologs are active toward all-*trans*-retinaldehyde. With the exception of CG2064, all other fruit fly SDRs exhibit the apparent K_m values for retinaldehyde between 0.3 and 0.7 μM. For comparison, the K_m value of RDH12 for retinaldehyde is 0.5 μM [22].

Secondly, both RDH12 and *Drosophila* CG2070 strongly prefer $NADP^+$ and NADPH as cofactors. The cofactor specificity of SDRs is believed to be determined by the presence of aspartate residue in the βαβ motif at the beginning of the Rossmann fold [42,43]. RDH12 and CG2070 lack this aspartate residue, instead, these two proteins as well as the rest of *Drosophila* SDRs have Cys in this position (Cys-70 in RDH12) (Fig. 1, vertical arrowhead), suggesting that all *Drosophila* RDH12 homologs prefer $NADP^+$ as a cofactor.

Finally, fruit fly SDRs are localized in the microsomal fraction similarly to RDH12 [22]. This is consistent with the prediction of a transmembrane segment in the N-terminus of five of the *Drosophila* homologs (Fig. 1). Surprisingly, CG2065, the only fruit fly homolog that lacks this N-terminal segment, is also associated with the microsomal membranes, suggesting additional means for membrane attachment of this protein.

The existence of multiple fruit fly SDRs with similar activities and overlapping expression patterns emphasizes the importance of their function. In *Drosophila*, retinoids are required for the biosynthesis of the visual pigments. Dietary β-carotene is the major substrate for production of retinoids, which are subsequently converted into the chromophore 11-*cis*-3-hydroxyretinaldehyde [20]. Many components of the pathway for the conversion of β-carotene to 11-*cis*-3-hydroxyretinaldehyde are still unknown, but the currently available data suggest that the pathway begins with the uptake of dietary carotenoids in the midgut mediated by the scavenger receptor NINAD. β-Carotene is then delivered to neurons and glia through circulation and taken up into these cells via the SANTA MARIA scavenger receptor. Here, β-carotene is cleaved to all-*trans*-retinaldehyde by the NINAB (β, β′-carotene-15,15′-monoxygenase), which functions in the same neurons and glia as SANTA MARIA [20]. Thus, the production of retinaldehyde by the NinaB monoxygenase occurs outside the retina [44]. It is not known whether retinaldehyde itself is transported to the retina or is converted to retinol before transport, but several observations argue in favor of retinaldehyde conversion to retinol. First of all, *Drosophila* has a retinoid binding protein, PINTA, which is capable of binding both retinol and retinaldehyde, but exhibits a stronger binding affinity for the retinol [45].

Secondly, the hydroxylated form of retinol (3-hydroxyretinol) accumulates in *Drosophila* mutants lacking NinaG, a glucose-methanol-choline oxidoreductase, which is proposed to act in the conversion of (3R)-hydroxyretinol to the 3S enantiomer [46,47]. The accumulation of 3-hydroxyretinol attests to the existence of the retinaldehyde reductase activity in fruit flies. Finally, because retinaldehyde is a highly reactive molecule, retinol appears to be better suited for transportation and is, in fact, the major transport form of retinoids in vertebrates [48].

A number of studies suggest that, in flies, dietary β-carotene is hydroxylated to yield zeaxanthin (3,3′-dihydroxy β,β-carotene) [21,22]. Therefore, zeaxanthin may be a major source of retinoids in these species. It remains to be shown whether NinaB monooxygenase in *Drosophila*, besides cleaving β-carotene [49], also cleaves zeaxanthin, but a recent study by Oberhauser *et al*. [50] indicates that the moth NinaB is capable of catalyzing the conversion of zeaxanthin into all-*trans* and 11-*cis*-3-hydroxy-retinaldehydes. If the same reaction occurs in *Drosophila*, these aldehydes may serve as substrates for the conversion to retinols by retinaldehyde reductases for transportation to retina.

The results of our study reveal that *Drosophila* possesses at least five SDR oxidoreductases with activities toward both all-*trans*-retinaldehyde and all-*trans*-3-hydroxyretinaldehyde. Therefore, these enzymes are well suited for catalyzing the conversion of retinaldehydes derived from either β-carotene or zeaxanthin. In addition, RDH12-like *Drosophila* homologs may have a role in the conversion of retinols back to retinaldehydes in retinal pigment epithelial cells for the final biosynthesis of 11-*cis*-3-hydroxyretinaldehyde, because they can catalyze reversible reactions, depending on the availability of substrates and redox potential of specific cell types. In support of this notion, gene knockout studies implicated RDH11, the $NADP^+$-dependent enzyme with close similarity to RDH12, in the oxidation of 11-*cis*-retinol to 11-*cis*-retinaldehyde in mouse retinal pigment epithelium [36].

Phylogenetic analysis shows that RDH12 related proteins have ancient origins. Identification of ecdyzoan SDRs homologous to human RDH12 suggests that the ancestral form for RDH12-related enzymes existed at least in Urbilaterian, a predecessor to all bilaterian animals, before the split of protostome and deuterostome lineages occurred ~580 millions years ago. Further divergence of this family to predecessors of mammalian RDH13 and RDH14 predated the emergence of chordates, because the genome of echinoderm sea urchin already contains genes closely related to these human proteins. Multiple intra-lineage or intra-species duplications both in protostomes (fruit fly) and deuterostomes (sea urchin and ascidia) contributed to additional diversification of this group of SDRs. It is unclear when the clade containing mammalian RDH11 and RDH12 emerged in evolution. Neither of the invertebrate SDRs identified in this study belong to RDH12 clade. On the other hand, we found multiple zebrafish SDRs that associate with RDH12/RDH11 clade (data not shown). Thus, based on the available data, this clade appears to be vertebrate-specific. It is not clear whether it emerged as a vertebrate innovation, in association, perhaps, with the appearance of a vertebrate visual cycle, or it emerged earlier but was subsequently lost in echinoderms and urochordates.

The retinoid activities of fruit fly SDRs have physiological meaning because these activities can contribute to the visual pigment biosynthesis, but retinoid metabolism is an unlikely physiological function for the nematode dhs-22 and dhs-24. As a species belonging to the Ecdyzoa phylum, *C. elegans* lacks the RAR-subfamily of nuclear receptors and, unlike *Drosophila*, it also lacks opsins [51], utilizing a different recently discovered mechanism for light detection [52]. Thus nematodes have no need for production of retinoic acid or retinoid chromophores and, therefore, their RDH12 homologs probably possess different functions. However, nematode SDRs may represent the ancestral forms that gave rise to the family of enzymes capable of utilizing retinaldehyde. Characterization of enzymatic properties of *C. elegans* proteins might provide additional clues to whether the ancestral enzymes in

Urbilaterian already possessed the ability to reduce retinaldehyde, or whether such ability was acquired during further diversification of this family of proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

SDR, short chain dehydrogenase/reductase; RDH, retinol dehydrogenase.

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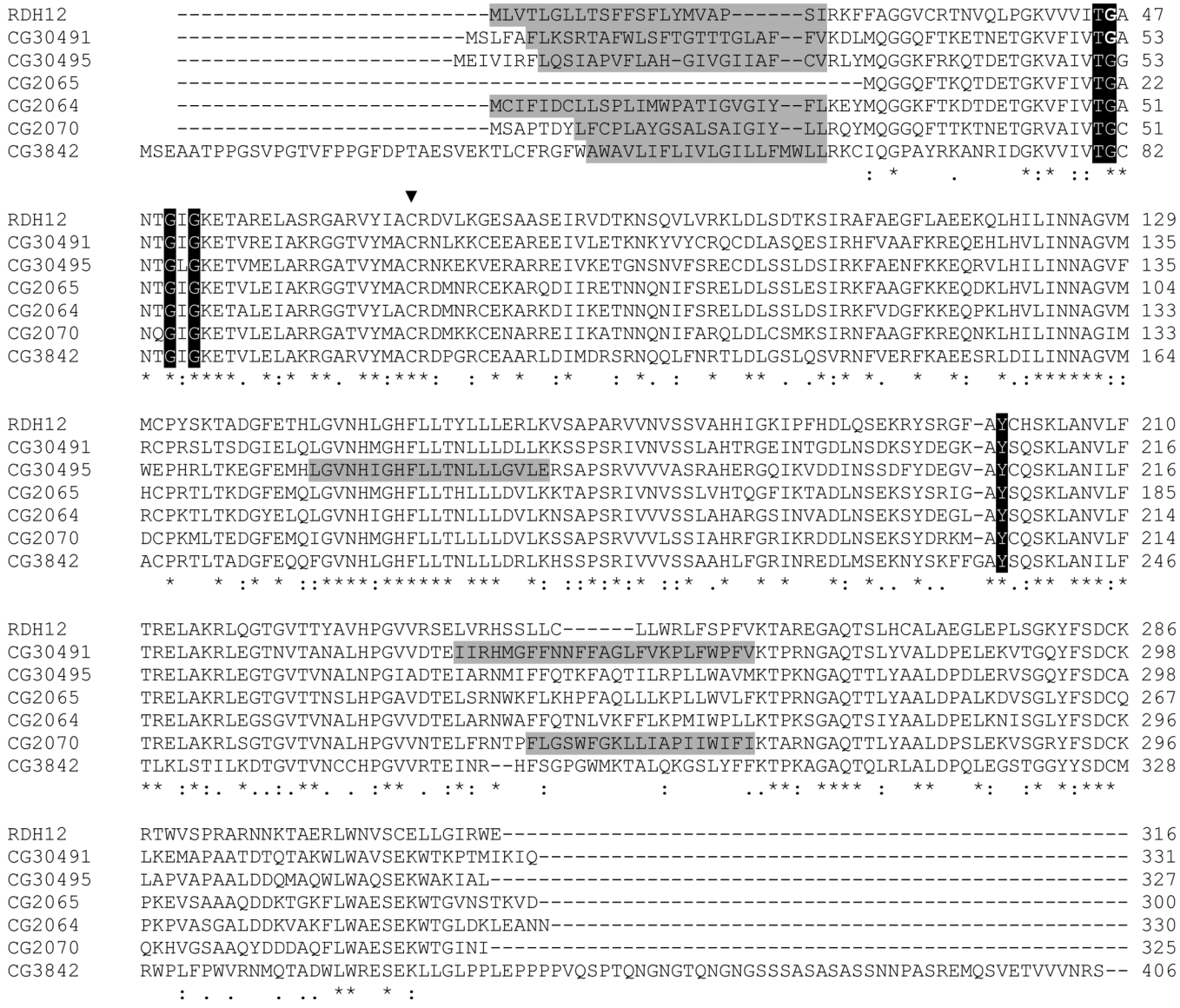

Figure 1. Protein alignment of human RDH12 and its fruit fly homologs

The putative transmembrane hydrophobic segments are highlighted in grey. The residues of the cofactor-binding tetrad TGxxxGxG and the active site Cys are shown in white on black background. The arrow indicates the amino acid residue position that in NAD^+-preferring SDRs is normally occupied by aspartate.

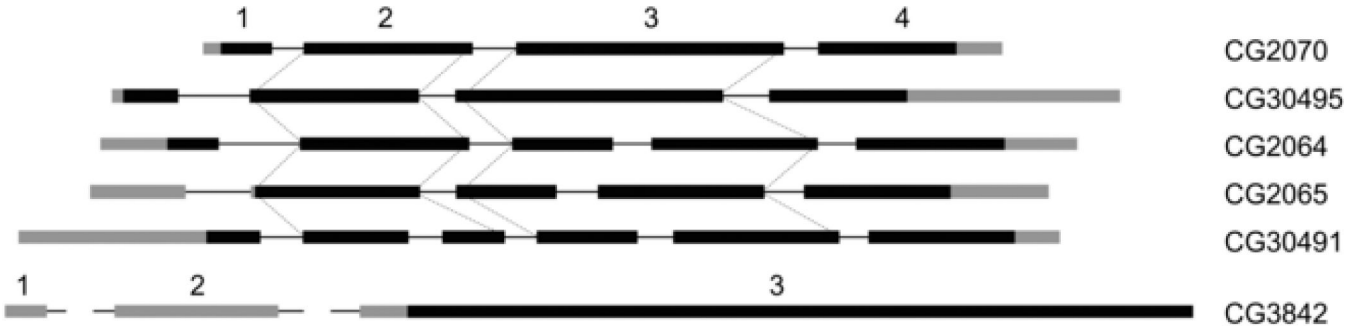

Figure 2. Exon-intron structure of *Drosophila* RDH-related genes

Five of the homologs located on chromosome 2 share similar genomic organization with a conserved size of coding exons. The second (2) and third (3) exons in CG2070 and CG30495 are split into two exons in other homologs. CG3842 located separately on chromosome X contains only one coding exon.

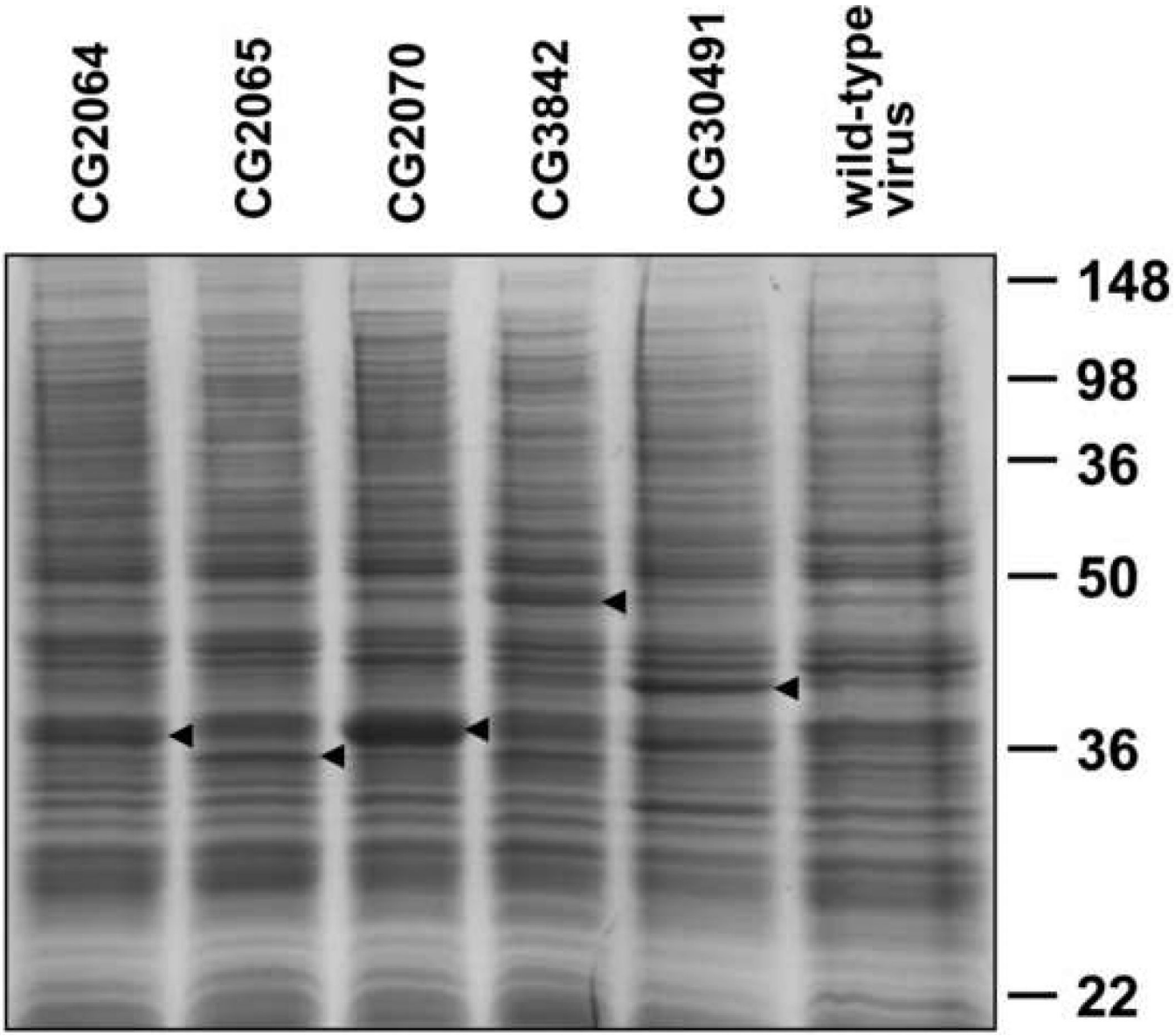

Figure 3. Visualization of recombinant *Drosophila* proteins in microsomal fraction of Sf9 cells

Coomassie Blue staining of Sf9 microsomal proteins following the separation in 12% SDS-PAGE. Bands corresponding to overexpressed recombinant *Drosophila* proteins are indicated by arrows. Molecular mass standards are shown on the right.

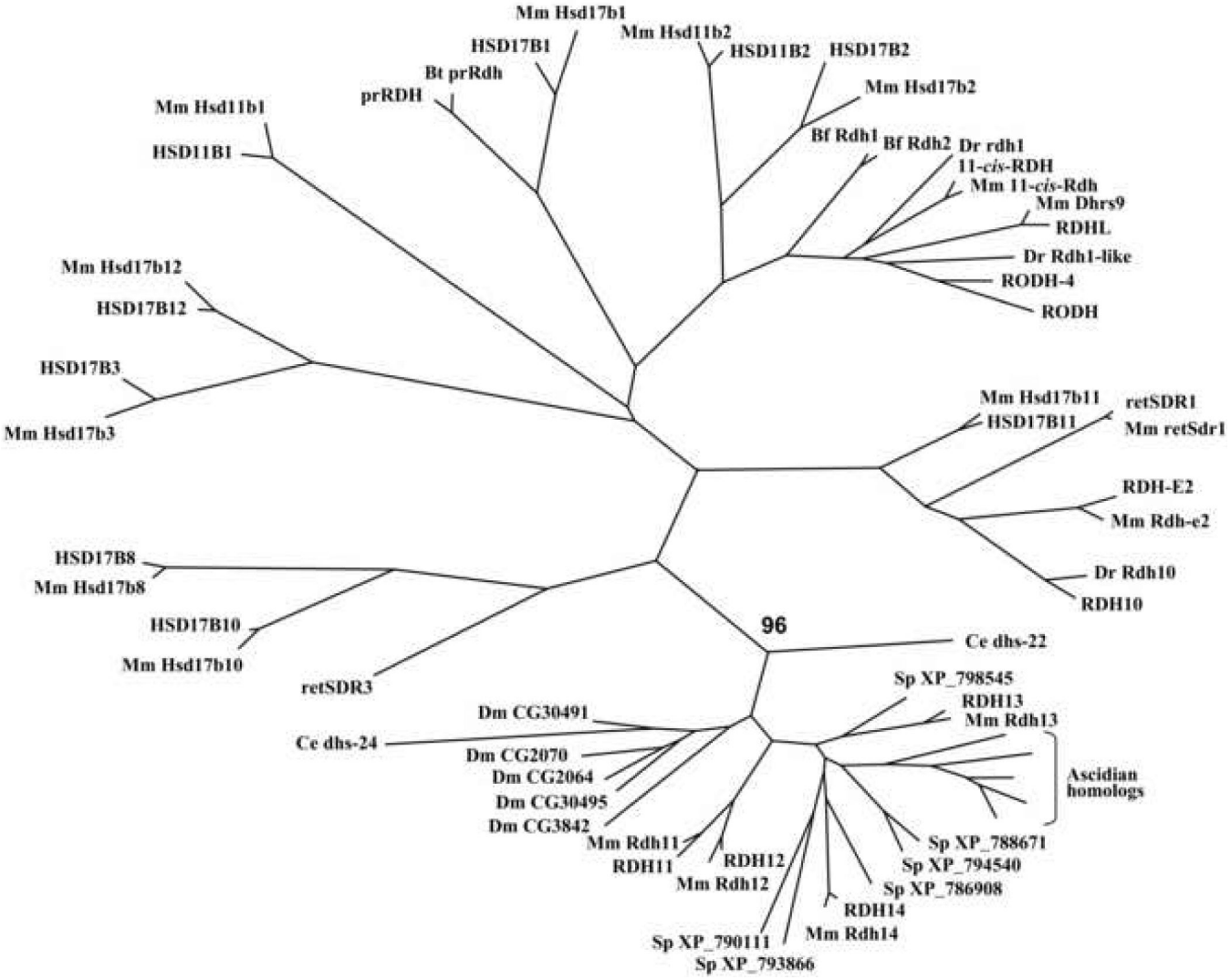

Figure 4. Phylogenetic position of RDH12-related proteins among other SDRs

Maximum likelihood phylogenetic tree was obtained using full-length protein sequences only. *Drosophila* CG2065 was omitted because it appears to have a truncated N-terminus in relation to other fruit fly homologs. The number at the base of the RDH12-related cluster indicates the bootstrap support for this group obtained with 100 replications. Prefixes indicate species as follows: *Dm – Drosophila melanogaster* (fruit fly); *Sp – Strongylocentrotus purpuratus* (sea urchin); *Ce – Caenorhabditis elegans* (roundworm); *Ci – Ciona intestinalis* (sea squirt); *Mm – Mus musculus* (house mouse); *Bt – Bos Taurus* (domestic cow), *Dr – Danio rerio* (zebrafish); *Bf – Branchiostoma floridae* (Florida lancelet). Human proteins do not have prefixes.

Table 1

Distribution of retinaldehyde reductase activity in subcellular fractions of Sf9 cells expressing *Drosophila* SDRs.

Fraction	Retinaldehyde reductase activity, relative units		α-Glucosidase activity, relative units
	CG2070	CG3842	
Mitochondria	3	5	2
Microsomes	14	27	5
Cytosol	1	1	1

The retinaldehyde reductase activity of fractions obtained by differential centrifugation was determined by measuring the formation of retinol from 5 μM retinaldehyde in the presence of 1 mM NADPH. The reaction rates were normalized per protein concentration and the lowest rate was taken as 1. The α-glucosidase activity was measured by the formation *p*-nitrophenol based on a molar extinction coefficient 18300 $M^{-1}cm^{-1}$, and normalized per protein concentration in the assay. The lowest activity was also taken as 1.

Table 2

Kinetic Characterization of *Drosophila* SDRs' Activities.

SDRs	all-*trans*-retinaldehyde			all-*trans*-3-hydroxyretinaldehyde		
	K_m, *μM*	V_{max}, *nmol/min·per mg microsomes*	*Specific activity, relative units*	K_m, *μM*	V_{max}, *nmol/min·per mg microsomes*	*Specific activity, relative units*
CG2064	4.2 ± 1.2	1.6 ± 0.2	1.0 ± 0.13	2.7 ± 0.9	0.45 ± 0.07	1.0 ± 0.2
CG2065	0.7 ± 0.1	1.6 ± 0.1	1.8± 0.1	4.4 ± 0.6	1.0 ± 0.1	4.0 ± 0.4
CG30491	0.6 ± 0.1	12.6 ± 0.7	6.2 ± 0.4	3.2 ± 0.3	29 ± 1	51.0 ± 1.5
CG2070	0.4 ± 0.1	21 ± 2	5.8 ± 0.6	unsaturable[a]	—	—
CG3842	0.27 ± 0.05	7.0 ± 0.4	2.1 ± 0.1	4.1 ± 0.1	25.0 ± 0.3	47.6 ± 0.6

[a] CG2070-expessing microsomes did not show saturation with up to 8 μM all-*trans*-3-hydroxyretinaldehyde. V_{max} values were determined for microsomal preparations expressing recombinant *Drosophila* proteins and are expressed in nmol/min·per mg microsomal protein. Specific activities are expressed in relative units, because they were estimated by obtaining the band areas of recombinant proteins in SDS-PAGE gel using densitometric analysis and normalizing the V_{max} values per band area of the corresponding protein. The lowest specific activity (CG2064) was assumed as 1 unit.