Identification and Characterization of a Stereospecific Human Enzyme That Catalyzes 9-cis-Retinol Oxidation

A POSSIBLE ROLE IN 9-cis-RETINOIC ACID FORMATION*

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All-trans- and 9-cis-retinoic acid are active retinoids for regulating expression of retinoid responsive genes, serving as ligands for two classes of ligand-dependent transcription factors, the retinoic acid receptors and retinoid X receptors. Little is known, however, regarding 9-cis-retinoic acid formation. We have obtained a 1.4-kilobase cDNA clone from a normalized human breast tissue library, which when expressed in CHO cells encodes a protein that avidly catalyzes oxidation of 9-cis-retinol to 9-cis-retinaldehyde. This protein also catalyzes oxidation of 13-cis-retinol at a rate approximately 10% of that of the 9-cis isomer but does not catalyze all-trans-retinol oxidation. NAD* was the preferred electron acceptor for oxidation of 9-cis-retinol, although NADP+ supported low rates of 9-cis-retinol oxidation. The rate of 9-cis-retinol oxidation was optimal at pHs between 7.5 and 8. Sequence analysis indicates that the cDNA encodes a protein of 319 amino acids that resembles members of the short chain alcohol dehydrogenase protein family. mRNA for the protein is most abundant in human mammary tissue followed by kidney and testis, with lower levels of expression in liver, adrenals, lung, pancreas, and skeletal muscle. We propose that this cDNA encodes a previously unknown stereospecific enzyme, 9-cis-retinol dehydrogenase, which probably plays a role in 9-cis-retinol acid formation.

Retinoids (vitamin A and its analogs) are essential dietary substances that are needed by mammals for reproduction, normal embryogenesis, growth, vision, and maintaining normal cellular differentiation and the integrity of the immune system (1–5). Within cells, retinoids regulate gene transcription acting through ligand-dependent transcription factors, the retinoic acid receptors (RARs)1, and the retinoid X receptors (RXRs) (6, 7). All-trans-retinoid acid binds only to RARs with high affinity, whereas its 9-cis isomer binds with high affinity to both RARs and RXRs. The actions of all-trans- and 9-cis-retinoic acid in regulating cellular responses are distinct and not interchangeable.

In contrast to the great explosion of information regarding the actions of retinoid receptors in regulating gene transcription, information regarding how the abundant precursor retinol is physiologically activated to form the ligands needed to activate retinoid receptors is only slowly emerging (see Refs. 8 and 9 for recent reviews). It is clear that the pathway for conversion of retinol to retinoic acid involves first the oxidation of retinol to retinaldehyde and then the oxidation of retinaldehyde to retinoic acid. Numerous enzymes that are able to catalyze either retinol or retinaldehyde oxidation have been identified, purified, and/or characterized (8–10). These enzymes are members of four distinct families: the alcohol dehydrogenases, the short chain alcohol dehydrogenases, the aldehyde dehydrogenases, and cytochrome P-450s (8–10). At present, the most attention has focused on enzymes responsible for the oxidation of all-trans-retinol to all-trans-retinaldehyde (11–15). Several recent reports have indicated that both alcohol dehydrogenases and short chain alcohol dehydrogenases may be responsible for catalyzing all-trans-retinol oxidation (11–15), but the exact in vivo roles of each of these dehydrogenases in all-trans-retinoic acid formation remains controversial (8).

9-cis-Retinoic acid has been reported to be present in mammalian tissues and cells (16–18), but it has not been convincingly established how 9-cis-retinoic acid is formed within tissues and cells. Urbach and Rando have reported that liver microsomes can nonenzymatically catalyze the isomerization of all-trans-retinoic acid to the 9-cis isomer (19). Others have demonstrated that 9-cis-β-carotene can be converted to 9-cis-retinoic acid within rat tissues (20). However, this latter pathway cannot be an essential one for 9-cis-retinoic acid formation because rats maintained on a β-carotene-free purified diet containing only retinol as a precursor for retinoic acid formation are normal. In this communication, we report the characterization of a cDNA clone for a novel human enzyme that we have designated 9-cis-retinol dehydrogenase (9cRDH) and that catalyzes in a stereospecific manner the oxidation of 9-cis-retinol to 9-cis-retinaldehyde, a first enzymatic step needed for 9-cis-

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1 The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoic acid receptor X; RAR, retinoid X receptors; 9cRDH, 9-cis-retinol dehydrogenase; PBS, phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4); HPLC, high performance liquid chromatography; bp, base pair(s); kb, kilobase(s); CHO, Chinese hamster ovary.

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retinoic acid formation. Because it has been established that 9-cis-retinaldehyde can be further oxidized to 9-cis-retinoic acid by abundant tissue retinaldehyde dehydrogenases (21–23), it is possible that 9cRDH catalyzes a key oxidation step in the formation of 9-cis-retinoic acid.

**EXPERIMENTAL PROCEDURES**

**cDNA Characterization and Sequence Analysis**—Using a primer homology strategy based on sequence information provided by Napoli and colleagues (11–14), we screened a mouse testis library for a retinol dehydrogenase cDNA clone. We obtained a 550-bp cDNA that we submitted for automated DNA sequence analysis (ABI Applied Biosystems, model 373A, Columbia, MD) through the Columbia University Comprehensive Cancer Center Core DNA Sequencing Facility. When this partial cDNA sequence was compared with sequences that had been deposited in GenBank (National Center for Biotechnology Information, Bethesda, MD), a very high degree of sequence homology was observed with an unidentified cDNA sequence that had been partially sequenced (330 of approximately 1400 bp) and deposited with GenBank by the IMAGE Consortium (accession number R50456). We obtained the cDNA through Research Genetics, Inc. (Huntsville, AL), and its complete nucleotide sequence was determined. The 1.4-kb cDNA was subcloned so that overlapping sequences were obtained for all regions of the cDNA and was used for all studies reported below.

**Expression of 9-cis-Retinol Dehydrogenase**—The 1.4-kb human cDNA was directionally cloned as a BamHI-EcoRI insert into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) where expression is driven by the strong promoter from the immediate early gene of the human cytomegalovirus. Both the expression vector containing the cDNA insert and vector alone were transfected using calcium phosphate into CHO cells according to standard procedures (24). Routinely, 20 μg of plasmid DNA (with or without the cDNA insert) was transfected into 2 × 10^6 CHO cells maintained on 100-mm tissue culture plates. At the time of transfection, the CHO cells were approximately 80% confluent. 27 h after transfection, the transfection medium was removed from the CHO cells, and they were washed with 5 ml of ice-cold 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). Washed CHO cells were scraped from the plate and collected by centrifugation at 500 × g at 4 °C for 10 min. The pelleted cells were washed with 5 ml of ice-cold PBS two additional times and stored as a cell pellet at −20 °C for up to 2 weeks prior to assay for retinol dehydrogenase activity.

**Retinoids**—All-trans-retinol was obtained as a gift from Dr. Christian Eckhoff of Hoffmann-LaRoche, Inc. (Nutley, NJ), and 13-cis-retinol was purchased from Sigma. 9-cis-retinol was synthesized by NaBH4 reduction of authentic 9-cis-retinaldehyde (Sigma) and subsequently purified by normal phase HPLC essentially as we have described for 11-cis-retinol synthesis and purification (25). All-trans-, 13-cis-, and 9-cis-retinaldehydes were purchased from Sigma.

**Enzymatic Assay of Retinol Dehydrogenase Activity**—Transfected CHO cell pellets were homogenized in PBS at 4 °C using 50 strokes of a Dounce homogenizer. For assay of retinol dehydrogenase activity, aliquots of unfractionated CHO cell homogenate were incubated with 10 μM retinol (either as the all-trans, 13-cis, or 9-cis isomer added in ethanol) and either 2 mM NAD⁺ or 2 mM NADP⁺ in 10 mM Hepes
tubes that had been flushed with N2 to shield the retinoids from both light and O2. All subsequent extractions and procedures were carried out in a manner that minimized exposure of the retinoids to light and O2. The elution positions of 13-cis-retinaldehyde (1), 9-cis-retinaldehyde (2), all-trans-retinaldehyde (3), 13-cis-retinol (d), 9-cis-retinol (5), and all-trans-retinol (6) are indicated with arrows.

FIG.2. Representative HPLC profiles for extracts of incubation mixtures consisting of 10 μM 9-cis-retinol (A), 10 μM 13-cis-retinol (B), or 10 μM all-trans-retinol (C) and 32 μg of CHO cell homogenate protein obtained from cells transfected with vector (pcDNA3, Invitrogen) containing the cDNA insert and 2 mM NAD+ containing the cDNA insert and 2 mM NAD+. In D, 10 μM 9-cis-retinol was incubated with 38 μg of CHO cell homogenate protein obtained from cells transfected with vector alone and 2 mM NAD+. The elution positions of 13-cis-retinaldehyde (1), 9-cis-retinaldehyde (2), all-trans-retinaldehyde (3), 13-cis-retinol (d), 9-cis-retinol (5), and all-trans-retinol (6) are indicated with arrows.

For assay of 9cRDH activity in rat tissues, 300-g male Sprague-Dawley rats were sacrificed in a CO2-saturated atmosphere and liver, kidney, spleen, testis, and epididymis were quickly removed and placed on ice. Immediately after dissection, each tissue was finely minced with razor blades, placed into a Dounce homogenizer containing 4 volumes of ice-cold PBS and homogenized with 50 strokes of the homogenizer. The resulting homogenate was centrifuged at 500 g for 10 min to remove debris and was maintained on ice prior to its use for 9cRDH assay. Assays of 9cRDH activity were carried out exactly as described above for CHO cell homogenates. For each assay, a blank containing substrate and homogenate was maintained on ice for 1 h and was subsequently extracted to correct for possible endogeneous 9-cis-retinaldehyde presence in the enzyme sources; however, in no homogenate was any 9-cis-retinaldehyde observed.

The protein concentrations of CHO cell homogenates and of each homogenate from the rat tissues were determined using the Bradford reagent (Pierce) according to the manufacturer’s instructions.

HPLC Procedures—Stereoisomers of both retinol and retinaldehyde were separated on a 4.6 × 250-mm Vydac 101HS54 silica column using hexane:α-propanol:1-octanol (98:1.5:0.1 v/v) flowing at 1.5 ml/min as the mobile phase. The running column was preceded by a silica guard column. Retinols and retinaldehydes were detected by UV absorbance at 350 nm. Retention times for all-trans-, 13-cis-, and 9-cis-retinols were established using purified compounds obtained as described above. Retention times for all-trans-, 13-cis-, and 9-cis-retinaldehyde were determined using commercial standards (Sigma). Quantities of each retinol and retinaldehyde isomer present in extracts were determined by comparisons of the integrated areas under the HPLC peaks with a standard curve constructed relating integrated peak area with known masses of each retinoid isomer. The concentrations of each retinoid isomer were determined by UV-visible spectrophotometry using published extinction coefficients for each retinol and retinaldehyde isomer (26).

Northern Blot Analysis for 9cRDH Expression in Human Tissues—Northern blot analysis was used to explore 9cRDH expression in human testis, kidney, lung, liver, heart, adrenals, pancreas, thyroid, skeletal muscle, placenta, mammary gland, and a mammary tumor. All tissues, with the exception of mammary gland and mammary tumor, were obtained at autopsy. The mammary gland and mammary tumor were obtained as frozen blocks embedded for diagnosis. Total RNA was isolated from each tissue sample using standard procedures (24). Total RNA samples were electrophoresed on 0.8% agarose containing 2.2 M formaldehyde at 0.5 V/cm for 14 h. After electrophoresis the gel was soaked in 20 × SSC for 1 h and blotted overnight onto a nitrocellulose membrane using 10 × SSC. The total RNA transferred to the nitrocellulose membrane was baked at 80 °C in a vacuum oven for 2 h. The blot was probed with a cRNA probe generated from the full-length human 9cRDH cDNA clone in pcDNA3 (as used for CHO cell expression studies). The cRNA probe was labeled using SP6 polymerase and [32P]UTP. Hybridization was carried out at 65 °C in 5 × SSC, 60% formamide, 1% SDS, 5 × Denhardt’s solution, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, and 7% dextran sulfate. After hybridization, the final wash of the RNA-RNA blot was at 80 °C in 0.2 × SSC and 0.1% SDS for 1 h.
RESULTS

We were interested in obtaining a cDNA clone for a retinol dehydrogenase from a mouse testis library for use in study of the cellular sites of retinoic acid formation within the testis. Using a primer homology strategy similar to strategies described by Napoli and colleagues (11–14), we obtained a partial length product (550 bp) which, upon search of known sequences present in GenBank, was found to have a very high sequence homology to a previously unidentified cDNA having a length of 1.4 kb and for which a partial sequence had been obtained as part of the Human Genome Project. Because this cDNA was much larger than the one we obtained from the screen of the mouse testis library, we obtained the cDNA for preliminary study. Our preliminary characterizations of this human cDNA suggested to us that the cDNA encoded a protein that could catalyze the reduction of NAD⁺ when a mixture of retinol isomers was incubated with expressed protein encoded by the cDNA; consequently, we set out to characterize more extensively this human cDNA and the protein that it encodes.

Because only 330 bp of the approximately 1400 bp present in the human cDNA had been sequenced, we completed the sequencing of this cDNA. The complete nucleotide sequence for the cDNA is provided in Fig. 1 along with the deduced amino acid sequence for the protein that it encodes. Sequence analysis of the cDNA revealed the presence of a putative translation start site approximately 80 bp downstream from its 5‘-end and continuing for approximately 170 bp after the occurrence of a translation stop codon. The cDNA consisted of 1239 bp and could encode a protein of 319 amino acids. A search of the GenBank for homologous sequences indicated that bovine retinal pigment epithelial 11-cis-retinol dehydrogenase and rat liver all-trans-retinol dehydrogenases, types I, II, and III, were highly homologous to that of the 1.4-kb cDNA. For comparison, the deduced amino acid sequences for bovine 11-cis-retinol dehydrogenase and rat all-trans-retinol dehydrogenases, types I and II, are also provided in Fig. 1. No other sequences present in the GenBank data base were similarly homologous to the sequence that we obtained. Computer analysis of the predicted amino acid sequence indicated that the protein contained no membrane spanning domains. Furthermore, computer analysis of the amino acid sequences indicated that the protein encoded by the cDNA clone is most probably a member of the family of short chain alcohol dehydrogenases, like the bovine 11-cis-retinol dehydrogenase (27, 28) and the rat liver retinol dehydrogenases, types I, II, and III (11–14).

Based on these sequence homologies and the results from our preliminary studies, it seemed likely to us that we had obtained a human cDNA clone for a retinol dehydrogenase. However, based on our preliminary studies, it was not fully clear whether this cDNA was a human homolog of one of the rat liver all-trans-retinol dehydrogenases (11–14) or whether we had cloned a new and previously undescribed retinol dehydrogenase. Because 11-cis-retinol dehydrogenase is expressed only in the retinal pigment epithelium (27, 28) and 11-cis-retinol is found only in the eye (4), we could not have cloned the human homolog for this enzyme. To determine the substrate specificity of the enzyme encoded by our cDNA, we expressed the cDNA in CHO cells and incubated homogenate from these CHO cells with all-trans-, 9-cis-, or 13-cis-retinol. As is seen in Fig. 2, in the presence of 2 mM NAD⁺, homogenate from the transfected CHO cells avidly oxidized 9-cis-retinol to 9-cis-retinaldehyde but was unable to catalyze the oxidation of all-trans-retinol to all-trans-retinaldehyde. The CHO cell homogenate catalyzed the oxidation of 13-cis-retinol to its corresponding aldehyde, but at a rate that was only 10% of that observed for the oxidation of 9-cis-retinol. Over 60% of the 9-cis-retinol added (at an in assay concentration of 10 μM) could be oxidized to 9-cis-retinaldehyde by the CHO cell homogenate. Oxidation of 9-cis-retinol was both protein- and time-dependent, and NADP⁺ was a poor co-factor for the reaction (8% of the activity of NAD⁺ when both are provided at concentrations of 2 mM). The CHO cell homogenates were unable to catalyze any detectable oxidation of all-trans-retinol, regardless of whether this retinoid was added to the CHO homogenate in organic solvents, bound to rat testis cellular retinol-binding protein, type I, bound to albumin, or in detergent emulsions. We conclude from these experiments that the 1.4-kb cDNA encodes a stereospecific 9-cis-retinol dehydrogenase (9cRDH) that has not been previously identified.

Further characterization of 9cRDH activity expressed in CHO cells indicated that the enzyme has a pH optimum in the range of 7.5–8.0 and that 9cRDH activity is not inhibited by ethanol or zinc chelators like EDTA or o-phenanthroline, unlike cytosolic alcohol dehydrogenases that can oxidize retinol to retinaldehyde (15, 29). Neither exposure to high NaCl concentrations (up to 1 M) nor exposure to reducing reagents (1 mM β-mercaptoethanol and 1 mM dithiothreitol) influenced 9cRDH activity. CHO cell expressed 9cRDH activity, however, is sensitive to detergents and is rapidly inactivated by exposure to 1% (w/v) Triton X-100 or to 1% (w/v) sodium cholate. In addition, the 9cRDH activity present in CHO cell homogenates is rapidly lost upon storage at −20 °C, although the activity does not appear to be lost when CHO cells are frozen intact.

The distribution of 9cRDH expression in human tissues was assessed by Northern blot analysis (Fig. 3). The transcript identified by Northern blot analysis is approximately 1.5 kb, a

| Tissue             | 9-cis-Retinaldehyde formed (nmol/h/mg protein) |
|--------------------|-----------------------------------------------|
| Liver              | 2.4 ± 0.4*                                    |
| Kidney             | 4.9 ± 0.1                                     |
| Spleen             | 1.6 ± 0.3                                     |
| Epididymis         | 2.8 ± 1.0                                     |

*Values are expressed as the means ± 1 S.D. for activity determinations for homogenates prepared from tissues from three animals.
size that agrees well with that predicted by the full-length human 9cRDH cDNA clone. 9cRDH mRNA is most abundant in normal mammary tissue and is relatively abundant in kidney and the testis. Liver, heart, and adrenals each express 9cRDH mRNA at nearly equal levels, but these are lower than those of mammary tissue, kidney, and testis. 9cRDH mRNA is present at low levels in lung, pancreas, and skeletal muscle. Interestingly, 9cRDH is only very weakly expressed in total RNA prepared from a human mammary tumor. We also asked whether 9cRDH activity could be detected in whole tissue homogenates prepared from rat liver, spleen, kidney, epididymis, and testis. As shown in Table I, 9cRDH specific activity was highest in the kidney followed by the testis, epididymis, liver, and spleen.

**DISCUSSION**

It is generally accepted that 9-cis-retinoid acid is a physiologically important molecule for mediating retinoid actions in regulating gene expression, but only limited information has been available regarding how 9-cis-retinoic acid or any 9-cis-retinoid is formed within tissues and cells. This is unlike the visual process where it is now well established that isomerization of all-trans-retinoids to 11-cis-retinoids is catalyzed by a specific enzyme and that the isomerization takes place at the level of the retinol and not the retinaldehyde (4). Because of the first reports in 1992 that 9-cis-retinoic acid is a ligand for the RXRs, several studies have explored possible pathways for 9-cis-retinoic acid formation. Urbach and Rando have reported that membranes prepared from bovine liver will catalyze non-enzymatically the isomerization of all-trans-retinoids to 9-cis-retinoic acid (19). This isomerization was shown to depend on free sulfhydryl groups present in the microsomes and not to involve the participation of an enzyme (19). Krimys, Russell, and colleagues have reported that 9-cis-β-carotene serves as a precursor for 9-cis-retinoic acid in *in vivo* in the rat (20). However, because rats maintained on carotenoid-free diets display normal health, the conversion of 9-cis-β-carotene to 9-cis-retinoic acid cannot be an essential pathway for formation of this retinoid acid isomer. In studies of retinaldehyde dehydrogenases purified from rat kidney (21, 22) and rat liver (23), the ability of these enzymes to catalyze the oxidation of 9-cis-retinol to 9-cis-retinoic acid was taken to suggest that a pathway starting with 9-cis-retinol may be important for 9-cis-retinoic acid formation (21, 22). To further substantiate this possibility, Bhat, Lacroix, and colleagues demonstrated the presence of 9-cis-retinol in rat kidney at levels that were approximately 10% of that of all-trans-retinol (22). Our work characterizing a sterosepecific 9cRDH activity and demonstration of the broad tissue distribution of this enzyme adds additional support to the hypothesis that 9-cis-retinoic acid is formed within tissues via a pathway that involves both 9-cis-retinol and 9-cis-retinaldehyde.

A search of the GenBank® for DNA sequences homologous to that of 9cRDH revealed that the sequence of the 9cRDH cDNA is approximately 87% homologous to that of the full-length cDNA for bovine retinal pigment epithelium 11-cis-retinol dehydrogenase (27) and approximately 48% homologous to the coding region of the cDNA sequence for rat liver all-trans-retinol dehydrogenase, type II (15). At the amino acid level, the deduced amino acid sequence for 9cRDH is 89% identical to that of bovine 11-cis-retinol dehydrogenase (27) and 53% identical to that of rat liver all-trans-retinol dehydrogenase, type II (13). Like the bovine 11-cis-retinol dehydrogenase and the rat liver all-trans-retinol dehydrogenases, type I, II, and III, 9cRDH is a member of the family of short chain alcohol dehydrogenases. Moreover, 9cRDH shares many properties including pH optimum, insensitivity to inhibition by ethanol, and sensitivity to detergent inactivation with the other members of this protein family. Most importantly though, like these other short chain alcohol dehydrogenases, 9cRDH shows a marked stereospecificity for retinol substrates.

Based on work showing that a retinaldehyde dehydrogenase purified from rat kidney can catalyze the oxidation of both all-trans- and 9-cis-retinaldehyde and that 9-cis-retinol is present at relatively high levels in the rat kidney (21, 22), it has been hypothesized that 9-cis-retinoic acid is formed in the kidney through a two-step oxidation starting with 9-cis-retinol. Our demonstration of 9cRDH in the kidney provides strong support for this hypothesis. Moreover, identification of this short chain alcohol dehydrogenase, 9cRDH, raises many additional interesting questions regarding the biochemical processes that are important for providing 9-cis-retinoids to tissue and cell. One such question concerns how 9-cis-retinoids are formed. Whether or not isomerization of all-trans-retinoic acid actually occurs nonenzymatically in living cells has not been addressed experimentally, although it is clear that some cell types do have the capacity to isomerize all-trans-retinoic acid to the 9-cis isomer (30). Another important question regarding 9-cis-retinoid formation concerns whether there are other short chain alcohol dehydrogenases present in tissues and cells distinct from 9cRDH that catalyze 9-cis-retinol oxidation. There are at least three short chain alcohol dehydrogenases that catalyze all-trans-retinol oxidation (11–14), and it would not seem unreasonable that multiple forms of 9cRDH may also exist. Perhaps most importantly, though, it is essential that we gain understanding of the specific physiologic role or roles played by each of these short chain alcohol dehydrogenases and by each of the alcohol dehydrogenases that catalyze oxidation of retinol to retinaldehyde. Are these enzymes redundant or do they all play significant roles in retinoic acid formation but in specific and defined cellular and metabolic contexts? Although it is clear that many enzymes are able to catalyze retinol oxidation, convincing physiologic functions for these enzymes within living organisms remain elusive.

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