Cloning of monkey RALDH1 and characterization of retinoid metabolism in monkey kidney proximal tubule cells

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Abstract **All-*trans* and 9-*cis* retinoic acids function as ligands for retinoic acid receptors (RARs and RXRs), which are ligand-dependent transcription factors and play important roles in development and cellular differentiation. Several retinal dehydrogenases are likely to contribute to the production of all-*trans* and 9-*cis* RAs in vivo, but their respective roles in different tissues are still poorly characterized. We have previously characterized and cloned from kidney tissues the rat retinal dehydrogenase type 1 (RALDH1), which oxidizes all-*trans* and 9-*cis* retinal with high efficiency but is inactive with 13-*cis* retinal. Here we have characterized the retinal-oxidizing activity in monkey JTC12 cells, which are derived from kidney proximal tubules. In vitro assay of cell lysates revealed the presence of a NAD^+-dependent dehydrogenase that catalyzed the oxidation of all-*trans*, 9-*cis*, and 13-*cis* retinal. Northern blot analysis of JTC12 RNAs and cloning by reverse transcription-polymerase chain reaction demonstrated expression of a monkey homolog of RALDH1. Bacterially expressed JTC12 RALDH1 catalyzed conversion of all three retinal isomers, with a higher catalytic efficiency for 9-*cis* retinal than for all-*trans* and 13-*cis* retinal. Accordingly, live JTC12 produced 9-*cis* retinoic acid more efficiently than all-*trans* retinoic acid from their respective retinal precursors. Only metabolites corresponding to the same steric conformation were formed from 9-*cis* or all-*trans* retinal, indicating a lack of detectable isomerizing activity in JTC12 cells.**—Brodeur, H., I. Gagnon, S. Mader, and P. V. Bhat. **Cloning of monkey RALDH1 and characterization of retinoid metabolism in monkey kidney proximal tubule cells. *J. Lipid Res.* 2003.** 44: **303–313.**

Supplementary key words retinoic acid • retinol • isomers • retinal dehydrogenase type I • retinoid metabolism

Retinoids are important regulators of cell growth, differentiation, and embryonic development (1–3). They are also needed for normal vision, reproduction, and immunity (4–6). The biological actions of retinoids are mediated through binding and modulation of retinoic acid receptors (RARs) or retinoid X receptors (RXRs), which function as ligand-dependent transcription factors (7). All-*trans* retinoic acid (RA) is a natural ligand for RARs, and 9-*cis* RA binds to both RARs and RXRs (8). The influence of vitamin A (retinol) in the control of gene expression is made possible by enzymes regulating RA synthesis. RA is formed from retinol via a 2-step metabolic pathway that involves oxidation of retinol to retinal and then of retinal to RA (9, 10). Although the metabolic pathways leading to the formation of all-*trans* and 9-*cis* RAs are beginning to be elucidated, the enzymes controlling production of these compounds within specific cells and tissues are still poorly characterized. In particular, it is not firmly established whether both all-*trans* and 9-*cis* RAs can be produced from the precursor all-*trans* retinol. Several studies have shown that externally supplied all-*trans,* 9-*cis*, and 13-*cis* RAs are isomerized into *cis* or *trans* RAs in cells and tissues and appear to reach equilibrium (11–16). Since no isomerase(s) involved in these processes have been identified, it is generally believed that the interconversion of *cis-trans* RA occurs in cells through nonenzymatic mechanism(s), although it is not clear whether this can happen at the level of retinol, retinal, and/or retinoic acid (16, 17).

We have previously reported the purification, cloning, and characterization of a retinal dehydrogenase type 1 (RALDH1) from rat kidney that oxidized all-*trans* and 9-*cis* retinal, but not 13-*cis* retinal to the corresponding acids (18–20). We have also demonstrated that rat RALDH1 is highly expressed in the proximal tubules of the developing kidney,

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Abbreviations: ALDH, aldehyde dehydrogenase; GST, glutathione-*S*-transferase; HPLC, high-pressure liquid chromatography; RA, retinoic acid; RALDH1, 2, and 3, retinal dehydrogenase type 1, 2, 3; RAR, retinoic acid receptor; RXR, retinoid X receptor; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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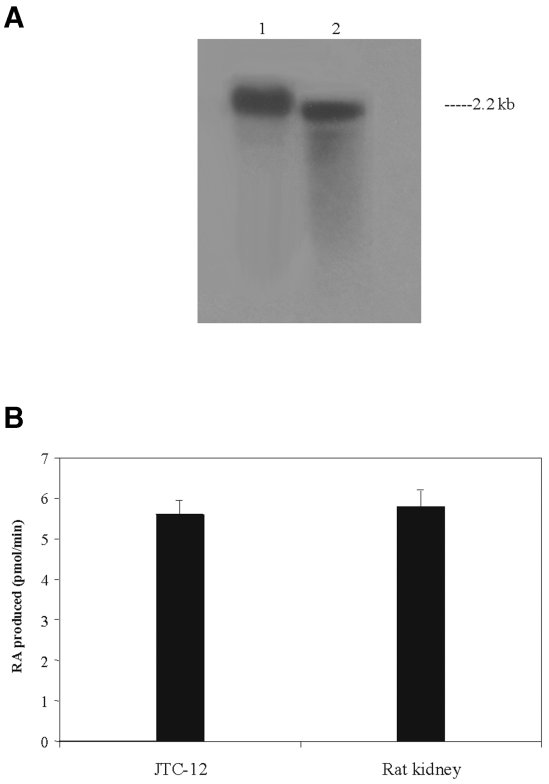

Fig. 1. A: Northern analysis of total RNA (10 μg) from JTC12 cells (lane 1) and adult rat kidney (lane 2). The blot was hybridized under low stringent conditions as described in Materials and Methods. B: Retinal-oxidizing activity in extracts from JTC12 cells and adult rat kidney. Seventy-five micrograms of crude protein extract and 10 μM of all-*trans* retinal were used in the assay.

suggesting a role in RA production during tubulogenesis (21). In addition, rat RALDH1 is expressed in other tissues such as trachea, intestine, and stomach epithelia, indicating its role in RA production needed for epithelial cell differentiation (22, 23). In humans, RALDH1 is highly expressed in kidney and liver tissues (24). On the other hand, in the mouse, RALDH1 is detected in mesonephros of 10-day-old embryo but is not expressed in the adult kidney (25).

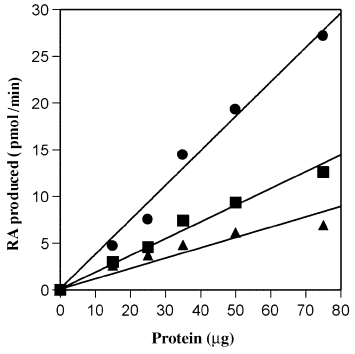

Fig. 2. Rates of retinoic acid (RA) synthesis from 9-*cis* (circle), all-*trans* (square), or 13-*cis* (triangle) retinal with increasing protein concentrations. Various concentrations of crude protein extract from JTC12 cells were incubated with 10 μM of retinal isomers at 25°C for 1 h. Each point in the curve represents the average of triplicate values.

In the present report, we have characterized the RALDH expressed in a monkey kidney cell line (JTC12) and investigated the production of isomers of RA by these cells. The enzyme cloned was found to be the most homologous to human RALDH1. Its expression in kidney JTC12 cells is consistent with the reported high expression of rat RALDH1 in kidney. In addition, monkey RALDH catalyzed formation of all-*trans*, 9-*cis*, or 13-*cis* isomers of RA from the corresponding retinal isomers in vitro, with a 2-fold higher catalytic efficiency for 9-*cis* retinal. Similarly, 9-*cis* RA was formed more efficiently than all-*trans* RA when JTC12 cells were incubated with all-*trans* or 9-*cis* retinal, respectively, without detectable equilibrium between RA isomers.

MATERIALS AND METHODS

Chemicals and reagents

All-*trans*, 9-*cis*, and 13-*cis* retinal, and all-*trans* and 13-*cis* RAs were purchased from Sigma Chemical Co. (St. Louis, MO). Standard all-*trans*, 9-*cis*, and 13-*cis* retinol were synthesized from corresponding retinal by $NaBH_4$ reduction as described earlier (26). 9-*cis* RA was obtained from Hoffmann-La Roche (Basel, Switzerland). The purity of the retinal substrates was assessed by reverse and normal phase high-pressure liquid chromatography (HPLC) (27) and was found to be at least 99% pure. All HPLC-grade solvents were purchased from the Fisher Scientific Co. (Toronto, Ontario).

Cell culture

JTC12 (monkey kidney proximal tubule) cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum, high glucose, and antibiotics penicillin and streptomycin (Sigma) at 37°C in an incubator containing 5% CO_2.

Northern blot analysis

Expression of RALDH transcripts in JTC12 cells was analyzed by Northern blot essentially as described earlier (22). Total RNAs from cells were isolated with Trizol pure reagent (Gibco BRL) according to the manufacturer's protocol. Ten to fifteen micrograms of total RNA were separated in 1.1% agarose gels and transferred to a Nytran membrane. The blots were prehybridized and hybridized at 68°C using Quickhyb reagent (Stratagene, La Jolla, CA). They were then washed twice with 2× SSC, 0.1% SDS at room temperature for 15 min, and then with 0.1× SSC, 0.1% SDS at 50°C for 30 min (low-stringent condition). Under high-stringent conditions, the blots were washed with 0.1× SSC, 0.1% SDS at 65°C for 30 min. Full-length rat kidney RALDH cDNA that was labeled with [^{32}P]dCTP served as a probe.

Immunoblot analysis

For immunoblot analysis, cells and tissues were homogenized in 1.0 ml of 100 mM ice-cold Tris-HCl buffer, pH 8.0, containing 3 mM EDTA and a mixture of protease inhibitors. The homogenate was centrifuged at 10,000 *g* to remove debris and unhomogenized materials. The protein present in the supernatant was boiled in sample buffer, separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Hybond nitrocellulose (Amersham). Blots were blocked with 0.05% Tween-20 in 5% BSA before incubation with an antibody. Immunoreactive protein was detected in an ECL Western blotting system. An antibody raised against rat RALDH1 (peptide

Fig. 3. Nucleotide and deduced amino acid sequences of the monkey retinal dehydrogenase type 1 (RALDH1) open reading frame. The amino acids that are different from human aldehyde dehydrogenase (ALDH) 1 are indicated in bold. Sequence data have been deposited with the GenBank accession number AF542418.

corresponding to residues 5–16) (28) (kindly supplied by Dr. James Lipsky, Mayo Clinic Foundation, Rochester, MN) was used in immunoblot analysis.

Cloning of cDNA

Monkey RALDH1 cDNA was cloned from JTC12 total RNA by reverse transcription-polymerase chain reaction (RT-PCR). The primers from human RALDH1 sequence were used in RT-PCR (29). The forward and reverse primers consist of the *Bam*HI restriction site and 5′-sequences (oligos 1–20, atgtcatcctcaggcacgcc), and an *Eco*RI restriction site and the reverse complement of 3′-sequences (oligos 1487–1506, ctctcagaagaactcataa) respectively. First strand cDNA was synthesized with 2 μg of RNA from JTC12 cells using M-MLV reverse transcriptase (Gibco BRL) and 3′-primer. This was followed by a PCR that contained both 5′- and 3′-primers and Taq polymerase using Super Script system (Gibco BRL). The PCR product was separated on 1% agarose gel, and a 1.5-kb band was isolated on DE 81 paper. The 1.5-kb PCR product that contained the RALDH open reading frame was subcloned into the *Bam*HI and *Eco*RI sites of the pGEX-2T bacterial expression vector (Pharmacia Biotech). Two clones originating from independent PCR reactions were sequenced.

Recombinant RALDH expression and purification

The RALDH1 clone in pGEX-2T vector was transformed into *Escherichia coli* BL21 (DE3) and expressed with glutathione-*S*-transferase (GST) fusion protein at the N terminus. The expressed protein was purified from crude bacterial extract with a GST affinity column followed by excision with thrombin as described previously (30). The purity of the enzyme was assessed by SDS-PAGE.

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moRALDH1    MSSSGTSDLP  VLPTDLKIQY  TKIFINNEWH  DSVSGKKFPV  FNPATEEELC  50
hALDH1      ------P---  --L-------  ----------  ----------  ----------
rRALDH1     ---PAQPAV-  APLAN----H  ----------  ----------  L------VI-
mRALDH1     ---PAQPAV-  APLA-----H  ----------  N---------  L------VI-

moRALDH1    QVEEGDKADV  DKAVKAARQA  FQIGSPWRTM  DASERGRLLY  KLADLIERDR  100
hALDH1      -------E--  ----------  ----------  ----------  ----------
rRALDH1     H---------  ----------  ----------  ---------N  -----M----
mRALDH1     H---------  ----------  ----------  ---------N  -----M----

moRALDH1    LLLATMESMN  GGKLYSNAYL  NDLAGCIKTL  RYCAGWADKI  QGRTIPIDGN  150
hALDH1      ----------  ----------  ----------  ----------  ----------
rRALDH1     -----I-AI-  ---VFA----  S--G-S--A-  K---------  H-Q---S--D
mRALDH1     -------AL-  ---VFA----  S--G----A-  K---------  H-Q---S--D

moRALDH1    FFTYTRHEPI  GVCGQIIPWN  FPLVMLIWKI  GPALSCGNTV  VVKPAEQTPL  200
hALDH1      ----------  ----------  ----------  ----------  ----------
rRALDH1     I--F--R---  ----------  ---L-F----  ----------  ----------
mRALDH1     I-----R---  ----------  --ML-F----  ----------  ----------

moRALDH1    TALHVASLIK  EAGFPPGVVN  IVPGYGPTAG  AAISSHMDID  KVAFTGSTEV  250
hALDH1      ----------  ----------  ----------  ----------  ----------
rRALDH1     ----M-----  ----------  ----------  --------V-  --------Q-
mRALDH1     ----L-----  ----------  ----------  --------V-  --------Q-

moRALDH1    GKLIKEAAGK  SNLKRVTLEL  GGKSPCIVLA  DADLDNAVEF  AHHGVFYHQG  300
hALDH1      ----------  ----------  ----------  ----------  ----------
rRALDH1     ----------  ----------  --------F-  -----I----  ----------
mRALDH1     ----------  ----------  --------F-  -----I----  ----------

moRALDH1    QCCIAASRIF  VEESIYDEFV  RRSVERAKKY  ILGNPLTPGA  TQGPQIDKEQ  350
hALDH1      ----------  ----------  ----------  ---------V  ----------
rRALDH1     ---V------  ----V-----  -K--------  V------Q-I  N---------
mRALDH1     ---V------  ----V-----  K---------  V--------I  N---------

moRALDH1    YDKILDLIES  GKKEGAKLEC  GGGPWGNKGY  FVQPTVFSNV  TDEMRIAKEE  400
hALDH1      ----------  ----------  ----------  ----------  ----------
rRALDH1     H---------  ----------  ---R-----F  ----------  ----------
mRALDH1     H---------  ----------  ---R-----F  ----------  ----------

moRALDH1    IFGPVQQIMK  FKSLDDVIKR  ANNTFYGLSA  GVFTNDIDKA  VTISSALQAG  450
hALDH1      ----------  ----------  ----------  ----K-----  I---------
rRALDH1     ----------  ---I------  ----T---A-  ----K-L-R-  I-V-------
mRALDH1     ----------  ---V------  ----T---A-  -L--K-L---  I-V-------

moRALDH1    TVWVNCYGVV  TAQCPFGGFK  MSGNGRELGE  YGFHEYTEVK  TVTVKISQKN  500
hALDH1      ----------  S---------  ----------  ----------  ----------
rRALDH1     V------MIL  S---------  ----------  H-LY----L-  --AM------
mRALDH1     V------IML  S---------  ----------  H-LY----L-  --AM------

moRALDH1    S
hALDH1      -
rRALDH1     -
mRALDH1     -
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Fig. 4. Comparison of amino acid sequences of monkey RALDH1 (moRALDH1) with other ALDH family members. Sequence identity between ALDHs is indicated by dashes. hALDH1, rRALDH1, and mRALDH1 represent human, rat, and mouse RALDHs, respectively. Sequences were obtained from GenBank accession numbers: hALDH1 NM000689, rRALDH1 L42009, and mRALDH1 NM 013467.

RALDH activity assays

Assays for monkey RALDH1 activity were carried out essentially as described previously for rat kidney RALDH1 (31). Cell lysates or tissue extracts were incubated for 60 min at 25°C with an assay mixture (250 μl) containing 10 μM each of all-*trans*, 9-*cis*, or 13-*cis* retinal (added in 2.5 μl dimethyl sulfoxide), and 603 μM NAD^+ in 100 mM phosphate buffer, pH 7.5, containing 0.02% Tween-80 and 161 mM dithiothreitol. To determine the kinetic constants of purified recombinant RALDH, various concentrations of retinal substrates (1–20 μM) were incubated with a fixed amount of enzyme. Immediately after the reactions, the assay mixture was extracted with 400 μl of butanol-acetonitrile (1:1, v/v), and the reaction product was analyzed by HPLC. Assays were performed under yellow light or in minimal light to minimize the photoisomerization of retinoids.

Retinoid metabolism by JTC12 cells

Cells at least 70% confluent were used in retinoid metabolism studies. In cytotoxic assays and titration experiments, 2.5 million cells in 10 cm dishes were incubated with 1 μM, 5 μM, or 10 μM all-*trans* retinal for 24 h. In later experiments, cells were incubated for 3 h with 10 μM all-*trans*, 9-*cis*, or 13-*cis* retinal. Retinoids were freshly prepared in ethanol, and their purity was checked by UV spectrum and HPLC before their addition to the medium. Formalin-fixed cells served for control incubations with retinoids. After incubations with retinoids, the medium was removed and the cells were washed twice with cold phosphate-buffered saline (PBS). They were then collected from the dish by scraping, and suspended in 0.5 ml PBS. Cell lysates were prepared by freezing and thawing three times. After gently vortexing for a few seconds, the samples were centrifuged at 2,000 rpm for 15 min at

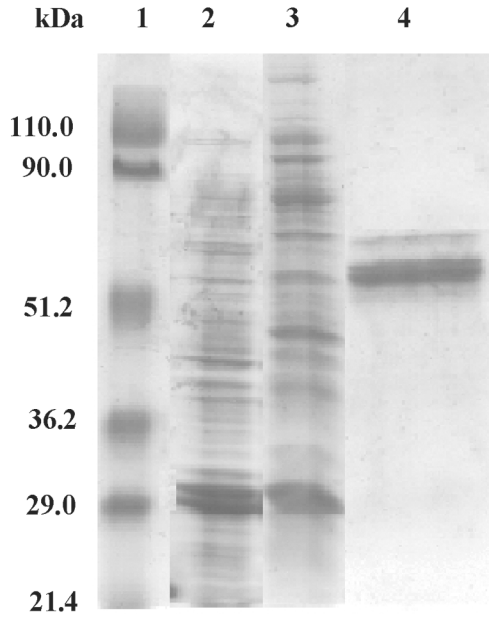

Fig. 5. Expression of monkey RALDH1 in *E. coli*. Lane 1, molecular weight markers; lane 2, bacterial lysate transfected with the empty vector; lanes 3 and 4, whole bacterial extracts from BL21 (DE3) cells transformed with the pGEX-2T-RALDH1 expression vector and purified enzyme by glutathion-Speharose followed by excision with thrombin respectively. Proteins (6 μg) were loaded on an SDS-polyacrylamide gel (10%) and visualized by 0.05% Coomassie Brilliant blue.

4°C to remove cell debris, and supernatants were collected. Retinal, retinol, and RA were extracted from the cell lysates (250 μl) with butanol-acetonitrile (400 μl, 1:1). An aliquot was directly injected onto HPLC for the separation and quantification of retinoids. To detect the formation of retinyl esters, the lysates were extracted twice with 3 ml of petroleum ether. Retinyl esters were separated by alumina column chromatography as described earlier (32), and their identities were verified by spectrophotometry and HPLC (33).

HPLC analysis

RA, retinol, and retinal were separated on a 4.6 × 250 mm Partisil 10 μ ODS (irregular shaped, 10% carbon loading from Phenomenex, Inc., Torrance, CA) with acetonitrile-water (40:60, v/v) containing 10 mM ammonium acetate as the mobile phase and at a flow rate of 1.2 ml/min. In this HPLC system, *cis*-retinoids are not separated from each other but are separated from their respective *trans*-retinoids. The identity of each *cis*-retinoid isomer (9-*cis* and 13-*cis*) and the formation of retinyl esters were studied in other HPLC systems described earlier (33, 34). Retinoids were detected by absorbance at 330 nm on a Shimatzu LC-7A liquid chromatography system. Retinoid peaks were identified and quantified by comparing their retention times and integrated areas under the peak against those of known amounts of pure standard retinoids.

RESULTS

Characterization of the RALDH expressed in JTC12 cells

Northern blot analysis of RNA extracted from JTC12 cells under low-stringency conditions using full-length rat kidney RALDH1 cDNA as a probe revealed an abundant ~2.2 kb transcript (**Fig. 1A**). The size of the transcript was slightly higher than that of rat kidney RALDH1 (Fig. 1A, lane 2). However, under high-stringency conditions, the probe hybridized with rat RALDH1 but not with the transcript from JTC12 cells, suggesting differences in primary sequences between the two mRNAs. Western blot analysis using anti-rat RALDH1 showed no signal (data not presented), indicating that the peptide sequence of JTC12 ALDH differs from that of rat RALDH1 between amino acids 5–16, the epitope recognized by the antibody. Note, however, that this antibody does not recognize human RALDH1, due to sequence divergence in this epitope. We next examined whether the JTC12 ALDH transcript hybridizes with other cytosolic ALDHs, such as RALDH2 and RALDH3, which also catalyze retinal oxidation (30, 35). A cDNA probe corresponding to mouse RALDH2 did not

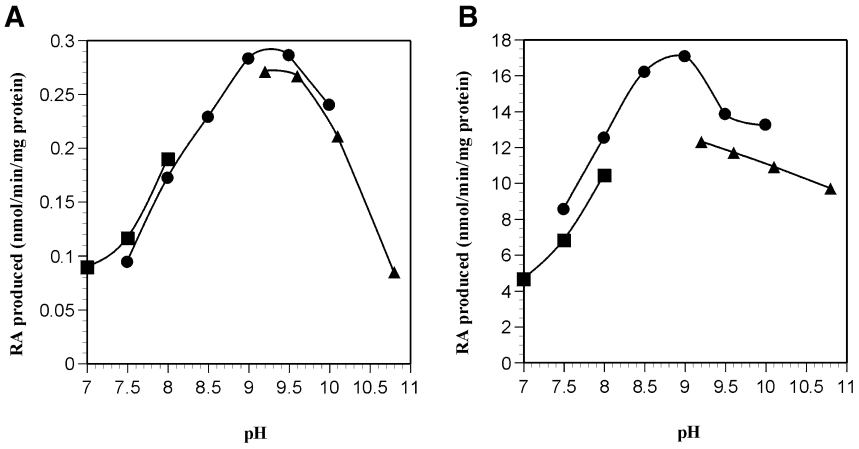

Fig. 6. The pH optimum for all-*trans* RA synthesis is similar for native and recombinant monkey RALDH1. Thirty-five micrograms and 0.6 μg respectively of crude extract (A) and pure recombinant proteins (B) were used in the assay. The reaction was performed with 10 μM of all-*trans* retinal. Each point is the average of triplicate values. Square, phosphate buffer 0.1 mM, pH 7–8; circle, Tris-HCl buffer 0.1 mM, pH 7.5–9.0; triangle, bicarbonate buffer 0.1 mM, pH 9–11.

hybridize, and a mouse RALDH3 probe recognized only a faint doublet around 3 kb (data not presented), suggesting that the main ALDH found in JTC12 cells is the monkey homolog of the RALDH1.

To test whether the JTC12 ALDH has activity with retinal substrates, we performed retinal dehydrogenase assays in crude cell extracts, using initially all-*trans* retinal as substrate. Similar to rat kidney extracts, the JTC12 cell extracts showed high NAD-dependent RA-synthesizing activity (Fig. 1B). The ability of JTC12 cell extracts to catalyze the oxidation of all-*trans*, 9-*cis*, and 13-*cis* retinal was examined to compare the substrate selectivity of the RALDH expressed in these cells with those reported for human, mouse, or rat RALDH1. While all three enzymes catalyze oxidation of all-*trans* and 9-*cis* retinal, they differ in their properties with 13-*cis* retinal, which is a substrate only for human RALDH1. The rates of RA production using JTC12 cell extracts were linear to protein concentrations up to 75 μg. The RALDH present in these extracts had high activity with 9-*cis* retinal, and slightly lower activity with all-*trans* and 13-*cis* retinal (**Fig. 2**). Thus, the substrate selectivity of the JTC12 RALDH with 13-*cis* retinal is closer to that of the human enzyme than to those of the rodent enzymes.

Together, the results of the Northern/Western experiments and of the enzymatic assays suggest that the RALDH expressed in JTC12 cells is most closely related to human RALDH1 (19, 36).

Kinetic properties of the recombinant JTC12 RALDH

Since the initial characterization of the JTC12 RALDH indicated high similarity with human RALDH1, we used 5′-and 3′-primers derived from human ALDH1 in a RT-PCR assay to clone the cDNA corresponding to the monkey enzyme. The cDNA obtained encoded a deduced protein of 500 amino acids (**Fig. 3**) with closest amino acid identity with human ALDH1 (98.6%) (29). Monkey RALDH1 also shared 85.6% and 86.2% amino acid identity with rat and mouse RALDH1, respectively (**Fig. 4**). Note that 9 amino acids out of 12 differ between monkey RALDH1 and rat RALDH1 in the N-terminal epitope recognized by the antibody used in Western blotting, explaining its lack of cross-reactivity with JTC12 extracts.

To investigate whether the cloned cDNA expresses a protein with enzymatic properties for retinal oxidation similar to those observed with crude extracts, we expressed it in an *E. coli* system. The purified recombinant enzyme showed an expected molecular mass of 55 kDa (**Fig. 5**). The pH optimum of the expressed enzyme for all-*trans* retinal oxidation was nine (**Fig. 6B**), similar to the pH optimum of the enzyme characterized in crude extracts (Fig. 6A). Next, we tested the activity of recombinant RALDH for retinal isomer substrates. Similarly to the activity observed in crude cell extracts (Fig. 2), the *E. coli*-expressed enzyme also catalyzed oxidation of the three retinal isomer substrates to the respective RAs (data not presented).

To test whether the kinetic properties of recombinant JTC12 RALDH for retinal substrates are similar to those of

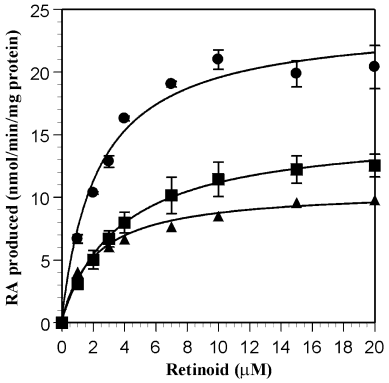

Fig. 7. Saturation kinetics of recombinant monkey RALDH1 for retinal isomers. Assays were performed with 0.4 μg of recombinant protein. Circle, 9-*cis*; square, all-*trans*; and triangle, 13-*cis* retinal. Each point in the curves represents the average of three replicates.

human ALDH1 (36), we assessed its saturation kinetics with all-*trans*, 9-*cis*, and 13-*cis* retinal. The recombinant enzyme exhibited the highest activity for 9-*cis* retinal followed by all-*trans* and 13-*cis* retinal oxidation (**Fig. 7**). The K_m for 13-*cis* retinal was 2-fold lower than for all-*trans* retinal. However, no significant difference was observed in the catalytic efficiency (V_{max}/K_m) of 13-*cis* and all-*trans* retinal conversion to the respective RAs (**Table 1**). On the other hand, 9-*cis* retinal showed ~2-fold higher catalytic efficiency when compared with all-*trans* retinal oxidation. Overall, the kinetic properties were similar to those of purified human ALDH1 (36).

Metabolism of retinal isomers by JTC12 cells

The presence of trace amounts of retinal, the intermediate in the synthesis of RA from retinol, has been demonstrated in tissues (37). However, little is known about whether stereochemical integrity is maintained in cells during RA metabolism. Since JTC12 cells express high levels of an RALDH that is capable of oxidizing all-*trans*, 9-*cis*, and 13-*cis* retinal, metabolic studies performed with these cells should provide valuable information as to the possibility of intracellular conversion between retinal isomers.

In initial experiments, JTC12 cells were incubated for 24 h with various concentrations of all-*trans* retinal (1–10 μM) to assess the sensitivity of the HPLC assay for detection of RA produced, and the potential toxicity of the retinal substrate. Cell viability was examined by trypan blue

TABLE 1. Substrate specificities of recombinant RALDH of JTC12 cells for retinal isomers

Retinal isomers	K_m	V_{max}	V_{max}/K_m
	μM	*nmol/min/mg*	
All-*trans*	3.21	14.08	4.49
9-*cis*	2.63	24.15	9.19
13-*cis*	1.51	9.35	6.22

cDNA for RALDH was isolated from JTC12 cells and expressed in *E. coli*. The recombinant protein was purified as described in Materials and Methods. The kinetic constants represent the average of two or more independent determinations where each point in the curve of each experiment is the average of three replicates.

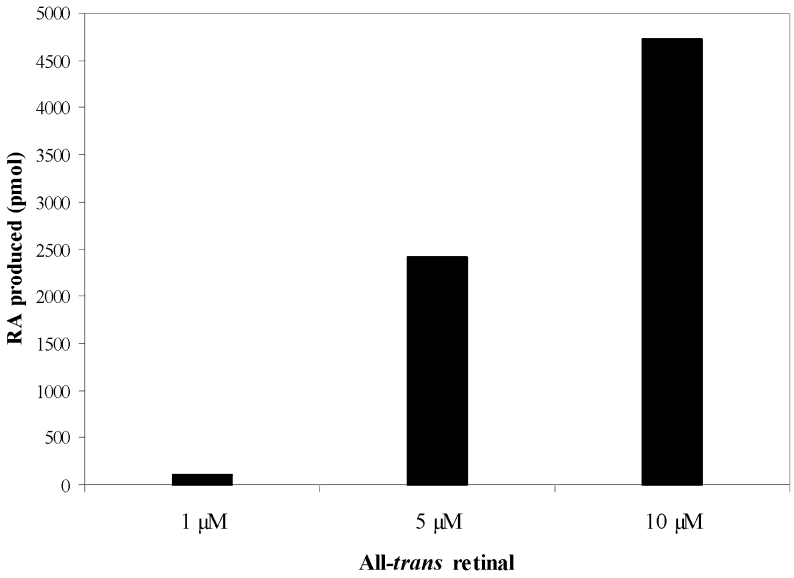

Fig. 8. Levels of RA synthesized in JTC12 cells incubated with various concentrations of all-*trans* retinal. All-*trans* retinal was added to the culture medium in 5 μl (0.1% final concentration in medium) ethanol and incubated for 24 h. RA was extracted from the cells and analyzed by high-pressure liquid chromatography (HPLC) as described in Materials and Methods.

exclusion and indicated that the retinal concentrations used were not toxic. Since a dose-dependent increase in RA levels was observed in this range of concentrations (**Fig. 8**), more detailed metabolic studies were then performed in JTC12 cells using 10 μM of retinal isomers. The purity of all-*trans*, 9-*cis*, and 13-*cis* retinal substrates before incubation was found to be greater than 99% by HPLC analysis (**Fig. 9A**, **B**, **C**, respectively). All retinoid-containing solutions were prepared in the dark just before incubation. The typical HPLC profiles of the lipid extracts from cells incubated with all-*trans*, 9-*cis*, or 13-*cis* retinal for 3 h are presented in Fig. 9D, E, and F, respectively. The cells took up all-*trans* retinal from the medium and converted it into either all-*trans* retinol or all-*trans* RA (Fig. 9D, peaks 2 and 4), while no *cis* isomers of retinol or RA were detected. Interestingly, we did not detect retinal in cell extracts, indicative of its complete metabolism. Similarly, 3 h of incubation with 9-*cis* retinal resulted in the formation of 9-*cis* RA by the cells (Fig. 9E, peak 1), and neither all-*trans* RA nor all-*trans* retinal were produced. Note that 9-*cis* retinol was not detected either. In parallel experiments, cells pre-treated with formaldehyde for 1 min (dead cells) did not produce any metabolites of all-*trans* or 9-*cis* retinal, indicating that metabolites were formed in the cells. On the other hand, the metabolic profile of 13-*cis* retinal showed formation of all-*trans* RA, all-*trans* retinol, and all-*trans* retinal with no 13-*cis* RA or 13-*cis* retinol (Fig. 9F), while trace amounts of 13-*cis* retinal were still present in the cells (Fig. 9F, peak 7). Since mainly all-*trans* retinoid metabolites were present in cells incubated with 13-*cis* retinal, one possibility is that most of the 13-*cis* retinal might have been isomerized nonspecifically in the medium to all-*trans* retinal, and that all-*trans* retinal might have been preferentially taken up by the cells and metabolized to the respective RA or retinol. To explore this possibility, we incubated formalin-fixed cells with 13-*cis* retinal and analyzed the retinoids extracted from the medium. Indeed, more than 45% of 13-*cis* retinal was isomerized to all-*trans* retinal in the medium at 3 h of incubation (data not shown). At present, we have not succeeded in keeping the integrity of the steric configuration of 13-*cis* retinal in the incubation medium, thus preventing assessment of the metabolism of 13-*cis* retinal in JTC12.

Although the presence of retinyl esters was not routinely examined because of the need to use relatively tedious chromatographic systems to separate and quantify the isomers of retinyl esters, alumina chromatography and HPLC analysis demonstrated the presence of retinyl esters in cells incubated with all three retinal isomers (data not shown), indicating that JTC12 cells express retinyl esterases and store retinol in the form of retinyl esters.

The levels of all-*trans* or 9-*cis* RA or retinol formed in cells incubated for 3 h with all-*trans* or 9-*cis* retinal, respectively, is illustrated in **Fig. 10**. At 3 h of incubation, the cellular levels of 9-*cis* RA were 5-fold higher than of all-*trans* RA, while all-*trans* retinol accumulated more than 9-*cis* retinol over the same time period. This higher efficiency in the conversion of 9-*cis* retinal to RA than of the all-*trans* isomer is consistent with the in vitro properties of the monkey RALDH1.

DISCUSSION

Although a wealth of information is available on the role of retinoid receptors in retinoid signaling, less is known about the metabolic pathways involved in the biosynthesis of RA isomers in target tissues. To analyze the

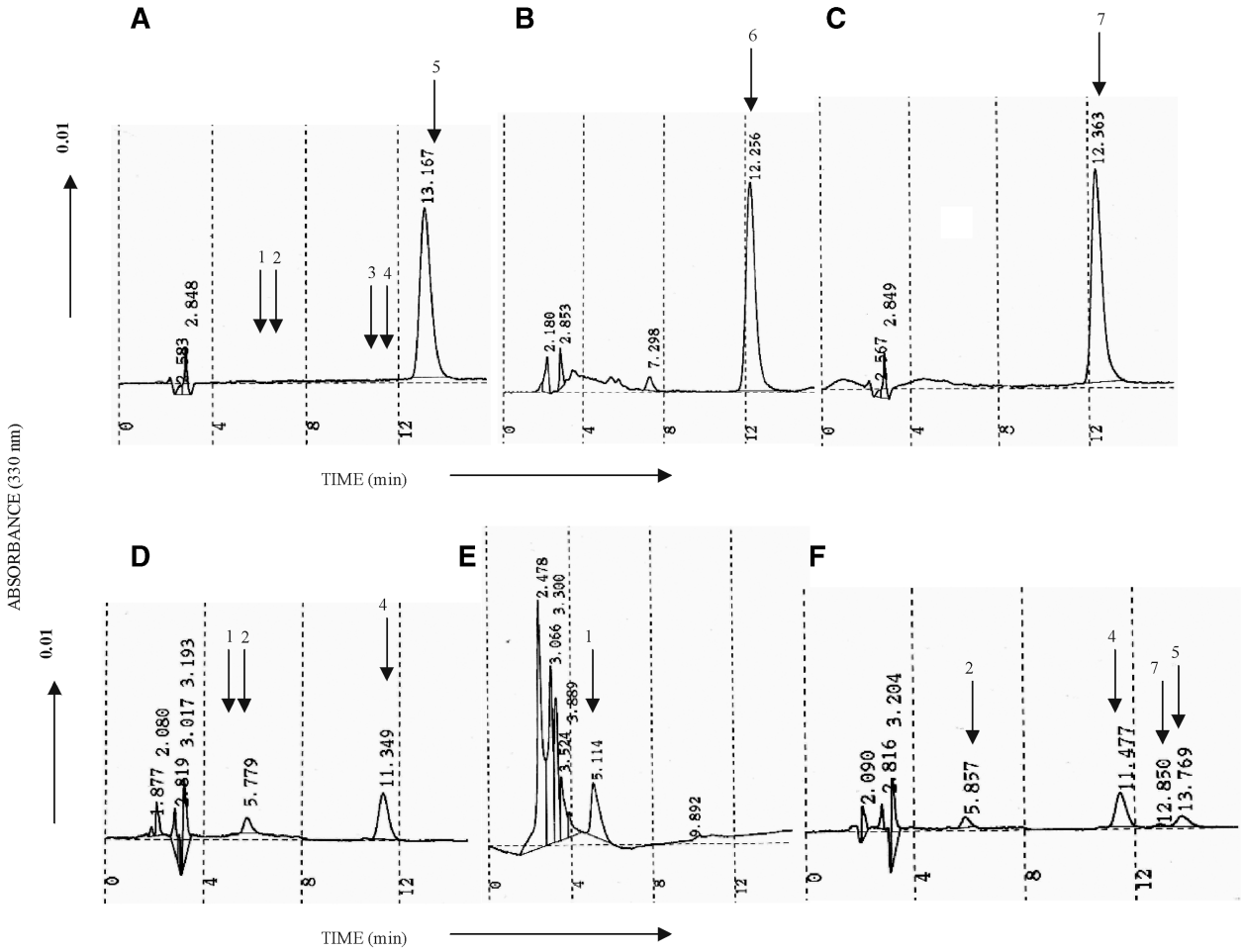

Fig. 9. Representative HPLC chromatograms of standard retinal isomers (upper panel) and their metabolites generated in JTC12 cells (lower panel). Arrows 5, 6, and 7 indicate the elution positions as well as the purity of the all-*trans* (A), 9-*cis* (B), and 13-*cis* (C) retinal standards that were used in the metabolic studies. D–F: Represent the metabolic profiles of cells incubated with all-*trans*, 9-*cis*, and 13-*cis* retinal for 3 h, respectively. Arrow 1 represents the elution position of 9-*cis* and 13-*cis* RA; 2, all-*trans* RA; 3, 9-*cis* and 13-*cis* retinol; and 4, all-*trans* retinol.

biosynthetic pathways of retinoid isomers in an intact cell system, we characterized the RA synthetic enzyme expressed in a kidney-derived cell line, JTC12. The expression levels and activity for the three retinal isomers of the RALDH enzyme expressed in JTC12 cells suggested that JTC12 is a good model to explore the biosynthesis of RA isomers in a cellular environment.

We have previously reported the kinetic properties of several RALDHs for retinal isomer substrates (19, 30, 36), and characterized the amino acid regions that are important for all-*trans* and 9-*cis* retinal oxidation (38). The observation that the JTC12 RALDH activity catalyzed the oxidation of the three retinal isomer substrates (Fig. 2), and the lack of hybridization with a probe for RALDH2, which also oxidizes the three retinal isomers (30), indicated that this activity is most similar to human ALDH1 (36). This homology was confirmed by sequencing of the cloned cDNA (Fig. 3), and the properties of the recombinant enzyme, such as molecular mass (Fig. 5), pH optimum (Fig. 6), and kinetic constants (Fig. 7, Table 1), were found to be very similar to those of human ALDH1 (36). Furthermore, the transcript (~2.2 kb) that hybridized with rat kidney RALDH1 cDNA at low stringency (Fig. 1) hybridized strongly with the cloned cDNA under high-stringency conditions (data not shown), confirming that the cloned monkey RALDH1 is indeed the retinal dehydrogenase expressed to high levels in JTC12 cells.

One aspect of retinoid signaling that is not yet clear is the metabolic route of all-*trans*, 9-*cis*, and 13-*cis* RA formation in vivo. It is generally accepted that all-*trans* retinol is the main precursor retinoid for the formation of RAs in vivo (39). Note in addition that, while it is generally believed that all-*trans* and 13-*cis* RA are naturally occurring retinoid forms (17, 38), the occurrence of 9-*cis* RA in tissues and cells is still disputed (8, 40–43). However, the existence of 9-*cis* and 13-*cis* retinol has been documented (19, 39). Several enzymes involved in the first oxidation step of the RA biosynthetic pathway have been shown to catalyze the oxidation of all-*trans*, 9-*cis*, and 13-*cis* retinol to the respective retinals (9, 10, 44). In the second step of RA synthesis, retinal is oxidized by at least three types of NAD^+-dependent RALDHs differing in tissue expression and catalytic efficiencies for retinal isomers (9). Enzymatic assays have clearly demonstrated that the retinoids

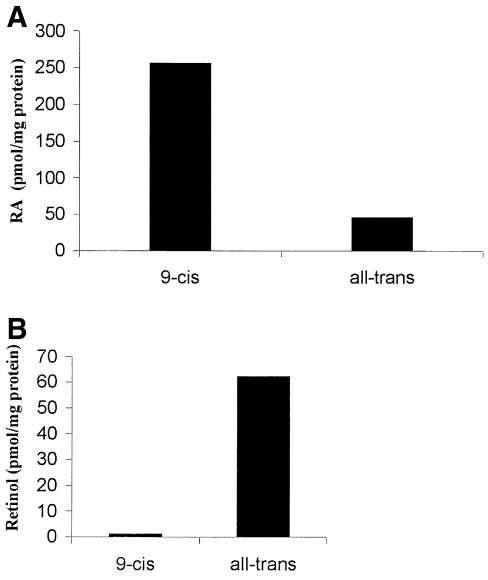

Fig. 10. Biosynthesis of RA and retinol from retinal precursors in JTC12 cells. JTC12 cells were grown in medium supplemented with 10 μM all-*trans* or 9-*cis* retinal for 3 h. RA (A) or retinol (B) metabolites were measured by HPLC. The results represent the mean value of triplicate experiments.

maintain their steric configuration during the oxidation process of retinol to RA in vitro using either pure or recombinant enzymes (19, 30, 39). However, the metabolism of retinoids within tissues and cells is complex. Retinol taken up by the cells undergoes various metabolic transformations that include esterification to retinyl esters, activation to RA, and conjugation to glucuronic acid (9, 10, 45). Since JTC12 cells constitutively express high levels of an RALDH activity that catalyzes oxidation of all three retinal isomers into the corresponding RA isomers in vitro, we wanted to characterize the metabolites formed from each isoform of retinal and to assess whether isomerization can occur in live JTC12 cells.

HPLC analysis of cell extracts incubated with retinal isomers showed a dose-dependent production of RA produced in JTC12 cells (Fig. 8). Although retinal can form Schiff's bases with groups of proteins and amino acids (46), no cytotoxicity was observed at the concentrations used. In addition to oxidation to RA, all-*trans* retinal was also reduced to retinol (Fig. 9, peak 4) and subsequently esterified (data not included), suggesting the presence of retinol reductase and esterase in JTC12 cells. Since the first metabolic conversion of retinol to RA is a reversible reaction, the reductase may well represent a retinol dehydrogenase (10). Several investigators have reported that reduction to retinol and subsequent esterification to retinyl esters are the main metabolic products of administered retinal isomers in cells and tissues (39, 47). However, no retinol was detected after a 3 h incubation period with 9-*cis* retinal, while high levels of 9-*cis* RA were produced compared with all-*trans* RA (Fig. 10A), consistent with the substrate selectivity observed with recombinant RALDH1 in vitro (Fig. 7, Table 1). It is interesting that neither all-*trans* nor 9-*cis* retinal was detectable in the cells (Fig. 9D, E), indicating rapid exhaustion of the substrate by oxidative and reductive enzymes.

In vivo, retinal is formed either from the oxidation of retinol or cleavage of β-carotene. However, the bulk of retinal generated from β-carotene is converted to retinol and then to retinyl esters, which are stored in peripheral target cells, or in the case of intestinal uptake, are transported by chylomicrons to the liver and subsequently stored in stellate cells (48). The rates of cleavage of 9-*cis* and 13-*cis* β-carotene are slower than that of the all-*trans* isomer (49). This may affect the rates of conversion of retinal isomers, formed from carotenoid precursors, to either retinol or RA. In addition, the expression levels and the substrate specificities of RALDHs and reductases may influence the metabolism of retinal isomers. Our experiments on retinal metabolism by JTC12 cells support the notion that the synthesis of either RA or retinol from retinal depends on the relative activities and specificities of RALDHs and retinal reductases for retinal substrates. Since retinal does not exist in free form in the cells, it is likely that retinal taken up by the cells is metabolized rapidly either to RA or retinol.

A recent study demonstrated that all-*trans* RA is a major metabolite of 13-*cis* retinal in pregnant rats supplemented with 13-*cis* retinal (47). We also observed production of all-*trans* metabolites in JTC12 cells incubated with 13-*cis* retinal, but this isomerization could be attributed to the significant conversion of 13-*cis* retinal to all-*trans* retinal in culture media (Fig. 9F). To examine whether 13-*cis* retinal maintains its steric configuration during metabolic conversion in the cells requires methods to stabilize it during incubation. Nevertheless, this problem was not encountered with all-*trans* or 9-*cis* retinal, which were converted to their respective metabolites without detectable isomerization by live JTC12 cells (Fig. 9). Several studies have shown that isomerization of the *trans* and *cis* isomers of externally provided RAs varies between 2% and 15% of total RA in tissues, cells, and microsomal extracts (11–15). Note that the detection limit of RAs in our HPLC systems is 1 pmol, which represents about 7.5% of the all-*trans* RA and 1.5% of the 9-*cis* RA produced in an assay of JTC12 cell extracts after in vivo incubation (Fig. 10A). Thus, if conversion of 9-*cis* to all-*trans* RA takes place in JTC12 cells, it occurs with very low efficiency. These observations are consistent with the findings of Sass et al. (47) and Kraft et al. (50), who reported that all-*trans* and 9-*cis* RA are the predominant in vivo metabolites of administered all-*trans* and 9-*cis* retinal, respectively. Furthermore, Liu et al. (51) demonstrated that parathyroid cells do not convert all-*trans* RA to 9-*cis* RA, but synthesize 9-*cis* RA from all-*trans* retinol. Taken together, these observations suggest that RA isomers are generated in target cells from different precursor retinoids rather than by isomeric exchange. Future studies directed toward the generation and use of selective retinal isomer-oxidizing enzymes (38) in in vitro transfection protocols as well as in an in vivo-expressing transgenic mouse system should provide greater insights into the formation and metabolic conversion of RAs.

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