cDNA Cloning, Tissue Distribution, and Substrate Characteristics of a cis-Retinol/3α-Hydroxysterol Short-chain Dehydrogenase Isozyme*

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We report here a mouse cDNA that encodes a 316-amino acid short-chain dehydrogenase that prefers NAD+ as its cofactor and recognizes as substrates androgens and retinols, i.e. has steroid 3α- and 17β-dehydrogenase and cis/trans-retinol catalytic activities. This cis-retinol/androgen dehydrogenase type 2 (CRAD2) shares close amino acid similarity with mouse retinol dehydrogenase isozyme types 1 and 2 and CRAD1 (86, 84, and 87%, respectively). CRAD2 exhibits cooperative kinetics with 3α-adipoly (3α-hydroxysteroid dehydrogenase activity) and testosterone (17β-hydroxysteroid dehydrogenase activity), but Michaelis-Menten kinetics with androsterone (3α-hydroxysteroid dehydrogenase activity), 11-cis-retinol, all-trans-retinol, and 9-cis-retinol, with V/K0.5 values of 1.6, 0.2, 0.1, 0.04, 0.005, and not saturated, respectively. Carbenoxolone (IC50 = 2 μM) and 4-methylpyrazole (IC50 = 5 μM) inhibited CRAD2, but neither ethanol nor phosphatidylcholine had marked effects on its activity. Liver expressed CRAD2 mRNA intensely, with expression in lung, eye, kidney, and brain (2.9, 2, 1.6, and 0.6% of liver mRNA, respectively). CRAD2 represents the fifth isozyme in a group of short-chain dehydrogenase/reductase isozymes (retinol dehydrogenases 1-3 and CRAD1), closely related in primary amino acid sequence (~85%), that are expressed in different quantities in various tissues, have different substrate specificities, and may serve different physiological functions. CRAD2 may alter the amounts of active and inactive androgens and/or convert retinols into retinoids. These data expand insight into the multifunctional nature of short-chain dehydrogenases/reductases and into the enzymology of steroid and retinoid metabolism.

The SDR1 superfamily consists of ~100 bacterial, plant, and animal enzymes ranging in size from ~25 to 38 kDa that are related in terms of tertiary structure, including conserved cofactor-binding sites and catalytic residues (1-3). But the members of the SDR superfamily have relatively few strictly conserved residues, and indeed, different members do not always share substantial amino acid identity. SDRs tend to have a multifunctional nature, i.e. they catalyze dehydrogenations/reductions of seemingly disparate substrates. In animals and bacteria, members of this superfamily catalyze the activation or inactivation of prostaglandins and many steroids. An apparent subgroup of the SDR superfamily, consisting of enzymes closely related to each other, catalyzes the metabolism of all-trans-retinol, cis-retinols, and androgens. This subfamily includes RoDH isozymes 1–3 and CRAD1 (4–7). A related SDR also catalyzes 11-cis- and 9-cis-retinol dehydrogenations (8–10).

Vertebrates require retinoid hormones, derived from the pro-hormone retinol (vitamin A), for vision, reproduction, embryogenesis, and maintenance of normal epithelial, bone, nerve, and immune system function (11). The retinol metabolite all-trans-retinoic acid satisfies all known retinoid functions in retinol-deficient animals, except for light transduction during vision, because it cannot undergo reduction into the opsin cofactor retinal, and spermatogenesis, because it cannot cross the mammalian blood-testis barrier in low concentrations. All-trans-retinoic acid functions through the three ligand-dependent transcription factors known as RARα, RARβ, and RARγ (12, 13). An all-trans-retinoic acid isomer, 9-cis-retinoic acid, controls the in vitro activity of a distinct group of receptors known as RARβ, RARγ, and RXRα and RXRγ. RXRs affect the function of several receptors in the steroid/retinoid/thyroid/vitamin D superfamily of ligand-activated transcription factors, including RARs, through heterodimerization. Because receptor function depends on ligand concentrations, pathways of all-trans-retinoic acid and 9-cis-retinoic acid biosynthesis require understanding. A pathway of all-trans-retinol conversion first into all-trans-retinal and then into all-trans-retinoic acid has been established, but pathways of 9-cis-retinoid acid biosynthesis have not been determined (14). One problem with the latter has been identifying processes for enzymatically generating the putative hormone. Thus, enzymes that produce 9-cis-retinoids incur much interest.

Androgens virilize males through supporting formation, growth, and maturation of reproductive organs and secondary sex characteristics (15). Endocrine glands, including the testes, produce testosterone (4-androsten-17β-ol-3-one), whereas the irreversible steroid 5α-reductases of the prostate and other androgen target tissues produce the testosterone metabolite dihydrotestosterone (5α-androstan-17β-ol-3-one) (16). Testosterone directs internal male genital formation; dihydrotestosterone directs embryonic external sex organ development and the phenotypic changes associated with male puberty (17). Both testosterone and dihydrotestosterone function through the androgen receptor. As with the other receptors mentioned above, androgen receptor function depends on ligand concentrations. Dihydrotestosterone undergoes inactivation via reduction into 3α-andiol (5α-androstan-3α,17β-diol), catalyzed by
members of the aldo-keto reductase superfamily. Dehydrogenation of 3α-adiol by the SDR 17β-steroid dehydrogenase generates the impotent androgen, androsterone (5α-androstan-3α-ol-17-one), cleared as its glucuronide (18). Pathways that regenerate dihydrotestosterone from 3α-adiol occur in vivo and presumably contribute to regulating androgen receptor function (19). Until recently, however, the specific enzymes responsible for regeneration of dihydrotestosterone were not known. The reports of 3α-adiol dehydrogenase activities of RoDH1 and CRAD1 provided candidates for such enzymatic activity (7, 20).

Here, we report the isolation of a cDNA that encodes a heretofore unknown SDR, CRAD2. Many tissues express CRAD2 mRNA, but liver is the quantitatively major site of expression. CRAD2 shows 3α- and 17β-hydroxyisor steroids dehydrogenase activities and catalyzes the dehydrogenation of retinols, including 9-cis-retinol. Expression of CRAD2 provides a means of altering the concentrations of active versus inactive androgens and of generating retinals from retinols.

**MATERIALS AND METHODS**

**Production of a CRAD2-specific Probe**—An 11-d.p.c. mouse embryonic agedagm (CLONTECH) was screened with a probe consisting of nucleotides 683–1089 of CRAD1 (7). The positive plaques were then screened through two more rounds with the 367-base pair probe isolated from pBSK/E6. Phage DNA from three of five plaques isolated was digested with EcoRI and cloned into pBlueScript II SK−/−. One of the six clones, pBSK/E6, was completely sequenced by nested deletion. Despite the use of a cDNA library, the 2.2-kb insert represented a genomic clone that included exon 1 of a gene in the SDR family. A 367-base pair probe containing 55 base pairs of 5′-end untranslated region and 312 base pairs of the coding region in exon 1 of E6 was generated by polymerase chain reaction with the primers 5′-TTACTCTCTGAAAACGGGGC (sense) and 5′-GCTC (antisense).

**CRAD2 cDNA Isolation**—A mouse liver agt10 library (CLONTECH) was screened with a probe consisting of nucleotides 683–1089 of CRAD1 (7). The positive plaques were then screened through two more rounds with the 367-base pair probe isolated from pBSK/E6. Phage DNA from three of five plaques isolated was digested with EcoRI. The inserts were ligated into pBlueScript II SK−/− to produce pBSK/CRAD-39, pBSK/ CRAD-188, and pBSK/CRAD-218 and sequenced in both directions by dideoxy chain termination.

**Expression of CRAD2**—The coding region of an SDR was digested from agt10 plasmid DNA containing the insert of pBSK/CRAD-218 with EcoRI and ligated into pcDNA3 to produce pcDNA3/CRAD2. CHO-K1 cells were cultured and transfected using LipofectAMINE with pcDNA3/CRAD2 or with pcDNA3 (mock) as described (7). Cell pellets were suspended in 10 mM HEPES and 150 mM KCl, 1 mM EDTA, and 1 mM Na+; pH 8.0, with the 900 × g supernatant of mock- or pcDNA3/CRAD2-transfected CHO cells, unless noted otherwise. Retinoid dehydrogenase assays were quenched with 0.1 M O-ethylhydroxylamine and 0.35 mL of mehtanol, incubated at room temperature for 10 min, and extracted with 2.5 mL of hexane. The retinoids in the hexane extract were quantified by normal-phase high-performance liquid chromatography, with a detection limit ~1 pmol.

**Enzyme Assays**—Incubations and analysis of products have been described in detail (7). Briefly, retinoid and steroid dehydrogenase assays were done at 37 °C in 0.25 mL of 50 mM HEPES, 150 mM KC1, 1 mM EDTA, and 1 mM Na+; pH 8.0, with the 900 × g supernatant of mock- or pcDNA3/CRAD2-transfected CHO cells, unless noted otherwise. Retinoid dehydrogenase assays were quenched with 0.1 M O-ethylhydroxylamine and 0.35 mL of methanol, incubated at room temperature for 10 min, and extracted with 2.5 mL of hexane. The retinoids in the hexane extract were quantified by normal-phase high-performance liquid chromatography, with a detection limit ~1 pmol. Sodium 3H-labeled steroids were detected by thin-layer chromatography. 

**Northern Blotting**—Northern blots were done with the mouse multiple tissue Northern blot (CLONTECH), which provides 1 μg of poly(A)+ RNA/lane on a nylon membrane. The probe was a chemically synthesized 75-base-long oligonucleotide of nucleotides 1–75 of CRAD2.

The probe was labeled with 32P by random priming. Prehybridization was done in 10 ml of hybridization solution (50% formamide, 5 × Denhardt’s solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5 × saline/sodium phosphate/EDTA) at 40 °C for 4 h. Hybridization was overnight in the same solution containing 2 × 106 cpm of probe. The final wash was done at 55 °C with 1 × SSC and 0.1% SDS. Signals were visualized with a Bio-Rad GS-505 Molecular Imager system.

**RNase Protection Assay**—A CRAD2-specific probe was amplified by polymerase chain reaction from pBSK/CRAD-39 with the sense primer 5′-CAAGATTCTATCCCGAC (nucleotides 1135–1152) and the antisense primer 5′-CAAGATTCTATCCCGAC (nucleotides 1463–1480). The polymerase chain reaction product was subcloned into pGEM-T (Promega) and linearized with SpeI. A 32P-labeled antisense probe was transcribed with T7 RNA polymerase (Promega) for 1 h at 37 °C in 10 mM dithiothreitol; 0.5 mM each ATP, CTP, GTP, and TTP; and 50 μCi of UTP (800 Ci/mmol). The 308-nucleotide antisense β-actin mRNA probe (nucleotides 51–358) was used as an internal standard was transcribed from pT7 mouse β-actin (Ambion Inc.) under the same conditions. DNA templates were removed by DNase I digestion. Transcripts were purified with 5% polyacrylamide and 8 × urea gels. RNase protection assays were done with the HybSpeedTM RPA kit (Ambion Inc.) following the manufacturer’s instructions. Total RNA (50 μg) was extracted from mouse tissues with guanidium thiocyanate/phenol/chloroform and coprecipitated with κRNA probes (1 × 106 cpm for CRAD2 and 5 × 104 cpm for mouse β-actin) by 0.5 M ammonium acetate and 70% ethanol. Pellets were resuspended in 10 μl of hybridization buffer (Ambion Inc.) for four alternating 15-s periods of vigorous vortexing and incubation at 95 °C for 3 min. Samples were hybridized at 68 °C for 10 min. A 100-μl aliquot of RNase A/T1 mixture diluted 1:100 was allowed to digest the unhybridized probes and RNA for 30 min at 37 °C. Inactivation/precipitation mixture (150 μl) was added, and the samples were kept at −20 °C for 30 min. After centrifugation, the supernatants were removed, and the pellets were dissolved in 8 μl of gel loading buffer for denaturing gels by heating at 95 °C for 4 min. The samples were loaded onto 5% polyacrylamide and 8 × urea gels and run at ~180 V for 2 h in 1 × Tris borate/EDTA. Quantitative analysis was performed with a Bio-Rad Molecular Imager FC.

**RESULTS**

**cDNA and Amino Acid Sequences**—A mouse embryonic cDNA library was screened with a probe that encoded amino acids 2–126 of RoDH1, i.e. sequence highly conserved among the three known RoDH isozymes and CRAD1 (4–7). A partial genomic clone was isolated, most likely from contamination during preparation of the commercial cDNA library. Because we have isolated genomic clones for RoDH/CRAD, it was recognized as exon 1 of an unknown SDR with high sequence similarity to CRAD1. To obtain the complete coding region of the novel cDNA, a mouse liver cDNA library was first screened with a probe from CRAD1; the positive plaques were re-screened with a probe generated by polymerase chain reaction from the exon of the novel SDR. Two of the three positive plaques identified included complete coding regions, and all three had identical deduced amino acid sequences in areas of overlap. The amino acid sequences, however, were distinct from those of RoDH isozymes 1–3 and CRAD1. The new SDR was named CRAD2 because its substrate specificity resembled that of CRAD1 (see below).

The deduced amino acid sequence of CRAD2 contained 20 of the 23 amino acids conserved in ~70% of SDRs, including the cofactor-binding residues G90(X)G92, the L109XNNAG sequence (unknown function), and the catalytic residues Y175(X)K (Fig. 1). All three RoDH isozymes, CRAD1, and CRAD2 show high sequence conservation in their first 115 N-terminal amino acids. The largest difference occurs between CRAD2 and RoDH2, which differ in six amino acid residues, four of which represent nonconservative changes (T13N, Q22K, N71S, and R104T). CRAD2 differs from CRAD1 by only five

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2 X. Chai and J. L. Napoli, unpublished results.
amino acids of the first 115, four of which are nonconservative (E20V, N43T, Q68E, and A92T). RoDH1, identical to RoDH3 in this area, and CRAD1 also differ by only five amino acids of the first 115 N-terminal residues: three changes are nonconservative (T13N, Q22K, and N71S). This region contains the cofactor-binding site, the conserved SDR sequence LVNNAG, and a putative membrane-anchoring sequence.

Outside of this group of five SDRs (CRAD1, CRAD2, and RoDH1–3), rat 17β-HSD6 has the closest amino acid similarity and identity to CRAD2 (Table I). The SDR that catalyzes 11-cis-retinol dehydrogenation has less sequence similarity to CRAD2 than does 17β-HSD6, suggesting that the CRADs and RoDHs belong to a distinct subgroup of SDRs that may not include the 11-cis-retinol dehydrogenase (8, 9). The bovine 11-cis-retinol dehydrogenase, for example, differs from CRAD2 by 50 amino acid residues in the first 115 residues.

Most SDRs have two or fewer cysteine residues, but at least four have four cysteine residues: rat 11β-hydroxysteroid dehydrogenase (24), human (R)-3-hydroxybutyrate dehydrogenase (25), and human 15-hydroxyprostaglandin dehydrogenase (27). The retinoid-associated SDRs (RoDH1–3, CRAD1, and 11-cis-retinol dehydrogenase), in contrast, are cysteine-rich, with six to seven cysteine residues each. CRAD2 represents the most cysteine-rich one, with eight cysteine residues. Five of these are conserved (Cys-37, Cys-60, Cys-176, Cys-256, and Cys-266) in all known retinoid-associated SDRs, and a sixth (Cys-274) is conserved in all but the 11-cis-retinol dehydrogenase (8, 9).

**Enzymatic Activity of CRAD2**—CRAD2 expressed transiently in CHO cells had 10-fold higher rates of activity with NAD versus NADP (Table II). CRAD2 showed cooperative kinetics with 3α-adiol (3α-hydroxydehydrogenase activity) and testosterone (17β-hydroxydehydrogenase activity), with Hill coefficients of 1.9 ± 0.3 (mean ± S.D., n = 4) and 1.5 ± 0.3 (n = 3), respectively (Fig. 2). The V/K₀.₅ value for testosterone was 8-fold lower than that for 3α-adiol, indicating much more potent androgen 3α-hydroxydehydrogenase than 17β-hydroxydehydrogenase activity (Table III). Michaelis-Menten kinetics

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**Table I**

Comparison of amino acid sequences of CRAD2 with related SDRs

| Species   | SDR              | Amino acid homology |
|-----------|------------------|---------------------|
|           |                  | Identity | Similarity |
| Mouse     | CRAD2            | 100       | 100        |
| Rat       | RoDH1            | 83        | 87         |
| Mouse     | CRAD1            | 82        | 87         |
| Rat       | RoDH3            | 83        | 87         |
| Mouse     | RoDH1            | 82        | 86         |
| Mouse     | RoDH2            | 79        | 84         |
| Rat       | RoDH2            | 79        | 84         |
| Rat       | 17β-HSD6         | 67        | 75         |
| Human     | RoDH             | 63        | 70         |
| Bovine    | 11-cis-RoDH      | 53        | 61         |
| Human     | 11-cis-RoDH      | 52        | 61         |
TABLE II
Coactivator use of CRAD2
Reactions were run with 5 μM substrate and 100 μg of protein for 30 min. Data are the means ± S.D. of triplicates.

| Retinol          | NAD pmol assay | NADP pmol assay |
|------------------|----------------|-----------------|
| 11-cis           | 266 ± 27       | 27 ± 3          |
| All-trans        | 70 ± 9         | ND*             |
| 13-cis           | 54 ± 7         | 7 ± 2           |
| 9-cis            | 20 ± 5         | ND              |

* ND, product not detected in amounts greater than those produced by mock-transfected cells.

DISCUSSION

cDNA cloning of CRAD2 reveals a fifth member of a subgroup of SDRs (CRAD1, CRAD2, and RoDH1–3) whose members share amino acid sequence identities of 80–97%. Nearly 95% conservation of the first 115 N-terminal amino acid residues represents a distinguishing feature of these five members. Rat 17β-HSD6, the SDR with the nearest amino acid identity to the group of five, shares only 86% identity in this area with the group of five. Human RoDH has only 83% identity in this region, and the SDR associated with 11-cis-retinol dehydrogenase has only 57% amino acid identity in this area, not markedly different from its overall identity to the group of five. Most likely, this conservation of the N terminus serves a unique, but as yet undetermined function. Similarly, amino acid residues 260–304 shared 84–98% identity from amino acid residues 260 to 304.

It appears that at least two distinct subgroups of SDRs occur with activity toward retinoids. The “group of five” enzymes possibly compose a subgroup distinct from the other enzymes most closely related in sequence, namely 17β-HSD6, human RoDH, and 11-cis-RoDH. For example, both the overall difference in identity and the difference in identity in the conserved N terminus indicate that the SDR candidate for a human homologue of an RoDH isozyme represents an SDR of its own. The recent cloning of a cDNA that encodes a mouse SDR (RDH4) supports this supposition. RDH4, which has 9-cis-retinol dehydrogenase activity, has ~87% amino acid identity to the 11-cis-retinol dehydrogenase, 4-Methylpyrazole inhibits the medium-chain alcohol dehydrogenase class I isozymes potently (μM Ki values), the class II isozymes modestly (Ki ~ 2 μM), and the class IV isozymes variably (mouse Ki ~ 1.5 mM and rat Ki ~ 0.2 mM). 4-Methylpyrazole inhibited CRAD2 activity with an IC50 value of 5 mM. Ethanol enhanced RoDH1 and RoDH2 activities by ~30%, but 140 mM ethanol had little impact on CRAD2 activity (data not shown). Phosphatidylcholine stimulates RoDH1 activity 7-fold and RoDH2 activity 3-fold (4, 5), but 2 μM phosphatidylcholine did not enhance CRAD1 activity.

CRAD2 mRNA Tissue Expression—Northern blot hybridization revealed intense expression of CRAD2 mRNA in mouse liver, with the most intense signal at 1.7 kb and two less intense signals at 3.5 and 2.9 kb (Fig. 5). Lung also showed the 1.7-kb mRNA, albeit at ~3% of the 1.7-kb liver signal. In comparison, CRAD1 was expressed very intensely in both liver and kidney, with major mRNA species at 3.5 and 2.7 kb in both tissues, a major 3.0-kb band in liver only, and a weaker 4.4-kb band in liver and kidney. RoDH isozymes 1–3 showed a single 1.7-kb band in liver by Northern blot analysis (4–7). No signals for CRAD2 were observed by Northern blotting in heart, brain, spleen, skeletal muscle, or testis, as was the case for CRAD1 and RoDH isozymes 1–3. The more sensitive RNase protection assays detected CRAD2 mRNA expression in the following (relative intensity normalized to the β-actin signal: liver (100) > lung (3) > eye (2) > kidney (1.6) > brain (0.6) (Table IV). No signals were detected in testis or heart. RNase protection assays also revealed low levels of CRAD1, RoDH1, and RoDH2 in multiple tissues, but did not reveal expression of RoDH3 outside of the liver (4–7).

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Graded concentrations of carbenoxolone (°a dehydrogenases (Fig. 5). CRAD1 had a consistent activity for several transfections and therefore likely reflect transfections, these rates observed for each CRAD were consistent. CRAD2 also differs from CRAD1 and RoDH1 in its relatively high inherent differences between the two. CRAD2 also differs from CRAD1, which shows similar activity (V/K<sub>0.5</sub> values) for these two cis-retinols (7). Recombinant RoDH isoforms have not been tested with cis-retinols. The activities with androgens also differed among the three tested. CRAD2 was most efficient as a 3α-hydroxysteroid dehydrogenase, but had less efficient 3α-hydroxysteroid dehydrogenase activity than either RoDH1 or CRAD1, which had K<sub>m</sub> values ~0.1–0.2 μM for 3α-adiol. CRAD1 had a V<sub>m</sub> value (27 nmol/min/mg of protein) with 3α-adiol that was 8-fold faster than that of CRAD2. Even though the experiments were done at different times and care must be exercised when comparing V<sub>m</sub> values produced from different transfections, these rates observed for each CRAD were consistent for several transfections and therefore likely reflect inherent differences between the two. CRAD2 also differs from CRAD1 and RoDH1 in its relatively high 17β-hydroxysteroid dehydrogenase activity. Neither CRAD1 nor RoDH1 had activity with dihydrotestosterone, and both had negligible activity with testosterone.

The prostate epithelial cell steroid 5α-reductase reduces the C<sub>4</sub>-ene of testosterone to produce the major biologically active androgen of prostate, dihydrotestosterone (15). The effects of dihydrotestosterone are, in turn, limited by 3α-hydroxysteroid dehydrogenases (Fig. 5). 3α-Hydroxysteroid dehydrogenases are members of the aldo-keto reductase superfamily (expressed in prostate, liver, kidney and several other tissues) that reduce the 3-oxo function of dihydrotestosterone to produce 3α-adiol. 3α- and 17β-Hydroxyoesteroid dehydrogenases constitute a pathway of dihydrotestosterone inactivation. 3α-Adiol binds to the androgen receptor with 5 orders of magnitude less affinity than dihydrotestosterone (31–33). 17β-Hydroxyoesteroid dehydrogenases, members of the SDR superfamily, convert 3α-adiol into androsterone, a steroid with even less androgen activity in vivo than its precursor (20). On the other hand, 3α-hydroxysteroid dehydrogenases that function oxidatively would convert the relatively inactive 3α-adiol and androsterone into dihydrotestosterone and androstanedione, respectively. The latter could then undergo activation into dihydrotestosterone through reduction of its 17-oxo function (Fig. 5). The three SDRs tested so far with androgens (RoDH1, CRAD1, and

### TABLE III
CRAD2 activity with steroid and retinoid substrates

| Substrate                      | Activity | Product               | K<sub>m</sub> | Rate* | V/K<sub>0.5</sub> |
|-------------------------------|----------|-----------------------|---------------|-------|------------------|
| 3α-Adiol<sup>a</sup>          | 3α-HSD   | Dihydrotestosterone   | 2.2           | 3.4   | 1.6              |
| Testosterone<sup>b</sup>      | 17β-HSD  | Androstenedione       | 1.9           | 0.3   | 0.2              |
| Androsterone                  | 3α-HSD   | Androstanedione       | 0.6           | 0.6   | 1.0              |
| 11-cis-Retinol                | RoDH     | 11-cis-Retinal        | 5             | 0.2   | 0.04             |
| All-trans-retinol             | RoDH     | All-trans-retinal     | 6.9           | 0.34  | 0.005            |
| 9-cis-Retinol                 | RoDH     | 9-cis-Retinal         | >28           |       |                  |
| Dihydrotestosterone           | 17β-HSD  | Androstanedione       | >25           |       |                  |
| Estradiol                     | 17β-HSD  | Estrone               | ND            |       |                  |
| Corticosterone                | 11β-HSD  | Dehydrocorticosterone | ND            |       |                  |

<sup>a</sup> nmol/min/mg of protein.

<sup>b</sup> These two substrates showed cooperative kinetics with CRAD2; other substrates showed typical Michaelis-Menten kinetics.

<sup>c</sup> ND, no activity detected (activity < 0.02 nmol/min/mg of protein). Reactions with estradiol and corticosterone were done with 5 μM substrate for 30 min with 100 μg of protein.

![Fig. 3. Inhibition of CRAD2 by carbenoxolone and 4-methylpyrazole. Graded concentrations of carbenoxolone (open circles; μM) or 4-methylpyrazole (open diamonds; mM) were assayed for impact on CRAD2-catalyzed metabolism. Assays were done for 30 min with 5 μM 3α-adiol and 2 μg of protein from the 800 x g supernatant of transfected CHO cells. Data are the means ± S.D. of triplicates.](image1)

![Fig. 4. Distribution of CRAD2 in mouse tissues. Top panel, RNase protection assays were done as described under “Materials and Methods” on RNA prepared from the tissues of 2-month-old male mice. Lane 1, probe; lane 2, yeast RNA; lane 3, DNA markers; lane 4, eye; lane 5, brain; lane 6, heart; lane 7, kidney; lane 8, liver; lane 9, lung; lane 10, testis. These data were normalized to the signals produced by the mouse β-actin probe. Bottom panel, Northern blot hybridization was carried out as detailed under “Material and Methods” with a commercially available blot from a gel of mouse poly(A<sup>+</sup>) RNA. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. bp, base pairs.](image2)

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<sup>3</sup> A. Romert and E. Eriksson, personal communication.
CRAD2) have the most efficient activity as 3α-hydroxysteroid dehydrogenases of any enzymes known so far. They may contribute to androgen action by producing dihydrotestosterone by reduction into 3α-adiol. 17β-HSD converts 3α-adiol into androsterone, which undergoes glucuronidation and elimination. RoDH/CRAD may regenerate dihydrotestosterone by oxidizing 3α-adiol. Secondarily, RoDH/CRAD may oxidize androsterone into androstanedione, which may undergo reduction into dihydrotestosterone by an as yet unknown 17β-HSD.

3α-Adiol may not function solely as an androgen inactivation product. Pregnant mice with a null allele in the type 1 5α-reductase gene failed to deliver pups on time, but instead entered prolonged labor on days −21–22 (36). About half resorbed their fetuses or expelled dead fetuses, and the other half either died during labor or suffered massive sepsis. The defect was seemingly related solely to parturition because the birth canal suffered no apparent developmental defect, and apparently normal pups were delivered on day 19.5 by cesarean section. Dosing with 3α-adiol increased the incidence of normal parturition from 27 to 93%. An equivalent dose of dihydrotestosterone was less effective, raising the incidence to 57%. These data prompted the suggestion that 3α-adiol may serve as a hormone required for parturition in mice. If so, then a second function of RoDH/CRAD could involve affecting the onset of parturition through altering the 3α-adiol concentration.

Observance of dual androgen/retinoid substrate SDRs provides opportunity for providing insight into the physiological interactions between retinoids and steroids. Spermatogenesis requires functional steroid and retinoid receptors (37, 38). Retinoic acids inhibit prostate epithelial cell growth (39, 40), and inhibition of all-trans-retinoic acid metabolism in the rat Dunning prostate cancer model inhibits carcinoma relapse after castration by raising all-trans-retinoic acid plasma levels (41). All-trans-retinoic acid decreases concentrations of dihydrotestosterone, 3α-adiol, and androsterone in serum and seems to cause a metabolic deviation away from the 5α-path in liver (39, 42). Other than causing a 3-fold decrease in androgen receptor binding (39, 43), very little is known about the mechanisms of retinoid effects on androgen activity. Indeed, very little is known in general about the extent of the retinoid/androgen interaction. Conversely, androgens affect the actions of retinoids by decreasing the mRNA of RARα ~5-fold in prostate epithelia and 15–20-fold in seminal vesicles, while increasing it 2-fold in kidney (44). The dual androgen and retinoid activities of RoDH1 and CRAD isozymes could position them as mediators of retinoid/androgen interactions. Mechanisms of such potential interactions might include direct competitive and/or allosteric effects or indirect effects through gene expression via RARs, RXRs, and/or the androgen receptor.

The 11-cis-retinol dehydrogenase activity of CRAD2 and its expression in the eye are consistent with CRAD2 contributing 11-cis-retinal for use as a rhodopsin chromophore. 11-cis-Retinoids, however, have not been demonstrated outside of the eye. Therefore, extracellular and perhaps intraocular CRAD2 may support 9-cis-retinoic acid biosynthesis by converting 9-cis-retinol, available from diet or from 9-cis-β-carotene metabolism (45–53), 9-cis-retinoic acid metabolism in the rat Dunning prostate cancer model (45, 248–258), and 9-cis-retinol as substrate (43, 45, 59, 6003–6013) into 9-cis-retinal. Although CRAD2 was not saturated kinetically with physiological levels of 9-cis-retinol, it showed sufficient activity with low 9-cis-retinol concentrations to contribute to the pool of 9-cis-retinal, which is in the low μM range. Two other SDRs have been reported with 9-cis-retinol dehydrogenase activity. CRAD1 showed a K_M value of ~5 μM and a V_max of ~10 nmol/min/mg for 9-cis-retinol (7). The second, originally reported as 11-cis-retinol dehydrogenase (8, 9), was shown subsequently also to recognize 10 μM 9-cis-retinol as substrate in a one-point assay (10). With the data currently available, it is not possible to assess the relative contributions of these three enzymes to the production of 9-cis-retinoic acid in vivo.

The exact function of CRAD2, and CRAD1 as well, may depend on loci of expression, substrate availability, hormonal influences, and other as yet unappreciated factors. Further investigations will address these issues. Finally, the variable effects of agents such as carbonyolozone and 4-methylpyrazole on different SDRs and alcohol dehydrogenases suggest that caution should be exercised in interpreting experiments in vivo using such reagents.

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