Identification, Expression, and Substrate Specificity of a Mammalian β-Carotene 15,15′-Dioxygenase*

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We have identified from mouse the first mammalian β-carotene 15,15′-dioxygenase (β-CD), a crucial enzyme in development and metabolism that governs the de novo entry of vitamin A from plant-derived precursors. β-CD is related to the retinal pigment epithelium-expressed protein RPE65 and belongs to a diverse family that includes the plant 9-cis-epoxycarotenoid dioxygenase and bacterial lignostilbene dioxygenases. β-CD expression in Escherichia coli cells engineered to produce β-carotene led to the accumulation of all-trans-retinal at the expense of β-carotene, confirming that β-CD catalyzed the central cleavage of this vitamin A precursor. Purified recombinant β-CD protein cleaves β-carotene in vitro with a V_max of 36 pmol of retinal/mg of enzyme/min and a K_m of 6 μM. Non-vitamin A carotenoids were also cleaved, although with much lower activity. By Northern analysis, a 2.4-kilobase (kb) message was observed in liver, kidney, small intestine, and testis, tissues important in retinoid/carotenoid metabolism. This message encoded a 63-kDa cytosolic protein expressed in these tissues. A shorter transcript of 1.8 kb was found in testis and skin. Developmentally, the 2.4-kb mRNA was abundant at embryonic day 7, with lower expression at embryonic days 11, 13, and 15, suggesting a critical role for this enzyme in gastrulation. Identification of β-CD in an accessible model organism will create new opportunities to study vitamin A metabolism.

In vertebrates, vitamin A in its various oxidative and isomeric forms is essential for embryonic development (1), pattern formation (2, 3), and vision (4). Retinoic acid, through its interaction with the nuclear retinoic acid receptor and retinoid X receptor, profoundly affects cell differentiation and development. Because animals are unable to synthesize vitamin A de novo from endogenous isoprenoid precursors, they must instead derive it from cleavage of β-carotene and certain other carotenoids with an unsubstituted β-ring (e.g. γ- and α-carotenes, β-zeacarotene, and β-cryptoxanthin). It is generally accepted that central cleavage of β-carotene by a putative dioxygenase gives rise to two molecules of all-trans-retinal, whereas eccentric cleavage with subsequent processing leading to a single molecule of retinoic acid from an apocarotenal is quantitatively far less important (5). β-Carotene cleavage activity is reported highest in the intestinal mucosa, but is found at high activity levels in liver, kidney, lung, and fat tissues, among other sites. However, an inability to purify the protein catalyzing this reaction has hindered thorough investigation of this crucial first step in vitamin A metabolism.

Because of a loose similarity between the mammalian protein RPE65 and the neoxanthin cleavage enzymes of plants, our laboratories have considered the hypothesis that the putative β-CD1 would belong to an emerging family of carotenoid-cleaving dioxygenases known mainly from examples in plants (6), but with members also in bacteria and Metazoa. The first described representative was a bacterial lignostilbene α,β-dioxygenase, which cleaves the central carbon–carbon double bond of lignostilbene, a bicyclic lignin model compound, into two molecules of vanillin (7). The initial plant representative of this family is maize VP14, the neoxanthin cleavage enzyme of the abscisic acid pathway (8) that is a 9-cis-epoxycarotenoid dioxygenase (9). The essential similarity of these disparate reactions is shown in Fig. 1.

The original animal representative of the family is RPE65, a protein restricted in its expression to the retinal pigment epithelium (10, 11). Although the specific biochemical function of RPE65 is not yet known, it is required for the all-trans- to 11-cis-isomerization reaction that regenerates the 11-cis-retinal chromophore of rhodopsin in the visual cycle of the retina (12). A thematic feature of the eukaryotic members of the family therefore appears to be an interaction with carotenoids or carotenoid derivatives to cleave or otherwise alter conjugated carbon–carbon double bonds. We used iterative searching of the genomic and EST data bases to find genes encoding polypeptides similar in sequence to RPE65. We describe here the characterization of one such RPE65 homolog from mouse, detail the developmental and tissue specificity of its expression, and define its enzymatic activity and substrate specificity. The protein does indeed cleave β-carotene to produce all-trans-retinal, is related, although not closely, to a β-CD recently identified from Drosophila melanogaster (13), and is of the same family as the recently described chicken β-carotene 15,15′-dioxygenase (14). Identification of the mouse β-CD protein and its gene provides the requisite tools for study of early steps of mammalian carotenoid metabolism in an accessible model system and gives additional insights into an ancient family of retinoid/carotenoid-metabolizing enzymes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s)AF271298.

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1 The abbreviations used are: β-CD, β-carotene 15,15′-dioxygenase; EST, expressed sequence tag; HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; kb, kilobase(s).
Mouse β-Carotene 15,15'-Dioxygenase

δ- and ε-carotenes were constructed by inserting an Arabidopsis mono-γ-cyclase (21) or a lettuce di-ε-cyclase cdNA3 into a unique site in the plasmid that confers the ability to produce lycopene. Culture tubes containing 5 ml of LB broth (with 150 μg/ml ampicillin and 30 μg/ml chloramphenicol) were each inoculated with a single fresh colony and shaken overnight at 28 °C (30). To 2 ml of the overnight cultures was used to inoculate 50 ml of LB broth (with ampicillin and chloramphenicol) in 250-ml flasks. Arabinose was added to a final concentration of 0.2%, and the cultures were grown at 28 °C in darkness for 48 h. Cells were harvested by centrifugation, and the pellets were extracted immediately or frozen at −20 °C for up to 1 week before extraction. Cultures of E. coli that produce and accumulate lycopene (wild type and pBAD/TOPO (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol for the recombinant pBAD/β-CD protein was prepared for assay by dialysis in the presence of B-PER detergent extract of untransformed TOP10 (Invitrogen). E. coli cells (100 μl of a 5 × 107 cells/ml) were used in this buffer (without the protease inhibitor) in a reaction volume of 0.4 ml at 37 °C for 2 h with 10 μg of recombinant enzyme using a substrate concentration range of 0–30 μM. The concentration of retinal was measured by HPLC as described above. Under these conditions, enzymatic activity was linear with enzyme concentration up to 35 μg/ml with time up to 3 h.

Antiserum to Mouse β-CD and Immunoblotting—A multiple antigenic peptide (22) with the sequence NYBKPDPQTLTLEK, corresponding to amino acids 142–157 of mouse β-CD, was synthesized (Princeton Biomolecules, Lawnborgh, PA) and used to immunize rabbits. Serum was harvested and stored at −20 °C. This antibody was used at a final dilution of 1:5,000 in 1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline (TPBS). Immunoreactive bands were detected using the one-component 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphate substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

RNA Gel Blot Analysis of Mouse β-CD mRNA Expression—The open reading frame of mouse β-CD was amplified using the forward primer 5'-GCT CTA GAT TAA AGA CTT GAG CCA CCA TG-3' and reverse primer 5'-GCT CTA GAT TAA AGA CTT GAG CCA CCA TG-3'. An amplification product of the expected length was cloned into the expression vector pBAD/TOPO (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol for the recombinant pBAD/β-CD. The resultant recombinant clones were isolated and sequenced to verify orientation and sequence. Expression of β-CD was induced as follows. Overnight cultures of E. coli containing pBAD/β-CD were grown in LB broth supplemented with 100 μg/ml ampicillin (LB/ampicillin). These cultures were used to inoculate 5-ml LB/ampicillin cultures. At mid-log phase, expression of β-CD was induced with serial dilutions of L-arabinose (16) followed by incubation for an additional 4 h, and aliquots were harvested by centrifugation. Accumulation of the expressed β-CD protein was assayed by SDS-polyacrylamide gel electrophoresis (16) followed by immunoblotting (17) with detection using a specific antisera to a peptide sequence within mouse β-CD (see below).

Assay of Cleavage Activity of Mouse β-CD Expressed in E. coli—The pBAD/β-CD expression construct was transformed into competent cells prepared as described (18) from a variety of carotenoid-accumulating strains of E. coli. Strains of E. coli that produce and accumulate lycopene and β-carotene have been described (19, 20). Strains that produce...
ersburg, MD) and mouse embryonic development stage (CLONTECH, Palo Alto, CA) Northern blots. Hybridization and washing followed the manufacturer's protocols (Ambion Inc.). Processed blots were exposed to Hyperfilm MP autoradiographic film (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom).

Subcellular Fractionation of Mouse Tissues—Freshly dissected kidney, liver, lung, testis, and small intestine were homogenized in 10 volumes of PBS containing one Complete protease inhibitor mixture tablet per 25 ml. The homogenate was centrifuged for 10 min at 1000 g, and the low speed supernatant was recentrifuged for 15 min at 30,000 g. The resulting supernatant was then centrifuged at 55,000 rpm for 30 min in a Beckman TL-100 tabletop ultracentrifuge using a TLA-100.2 fixed angle rotor. The supernatant was reserved, and the pellet was resuspended in 0.2 volume of homogenizing buffer. These samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using the conditions described above.

RESULTS

Identification and Characterization of a cDNA Encoding Mouse β-CD—Iterative tBLASTn searches of the dbEST division of the GenBank™/EBI Data Bank using bovine RPE65 (accession number L11356) as the query sequence identified an EST (AW044715) showing 39% similarity to RPE65, the maize epoxycarotenoid cleavage enzyme VP14, and the bacterial lignostilbene a,b-dioxygenase. The 2120-base pair cDNA (deposited in the GenBank™/EBI Data Bank under accession number AF271298) contains an open reading frame that is 1698 base pairs in length, encoding a protein 566 amino acids in length with a calculated molecular mass of 63,859 Da. The deduced polypeptide is without an obvious signal peptide, predicted transmembrane domains, or potential sites for N-linked glycosylation. Alignment of the deduced mouse polypeptide with chicken β-CD (GenBank™/EBI accession number AJ271386), Drosophila b-CD (accession number AB041507), and the predicted product of a human cDNA of unknown function (accession number AK001592) showed 70, 30, and 85% identities, respectively (Fig. 2). The human cDNA thus appears to be an ortholog of mouse β-CD, and both are related to chicken β-CD. The mouse polypeptide has only 37% amino acid identity to mouse RPE65. Ten residues, including 4 histidine residues and 6 acidic residues, are absolutely conserved in these sequences, in all RPE65 proteins (8). In addition, there is a particularly well conserved region (consensus sequence EDDGVVLSXVVS) at residues 469–480 of mouse β-CD that may be considered a family signature sequence. Besides the mouse sequence characterized herein, its presumptive human ortholog, and the chicken and Drosophila β-CD proteins enumerated above, ESTs encoding presumptive orthologs from zebrafish and pig were noted as well as more distant family members from a variety of species. Because of the accessibility of the mouse as a model organism for further studies, we chose to specifically characterize the mouse gene.

Expression of Mouse β-CD in E. coli—Agar plate cultures of

![Fig. 2. Alignment of metazoan polyene chain dioxygenase family members.](http://www.jbc.org/content/6562/15/6562/F2.large.jpg)
In this report, we have described the catalytic activity and tissue-specific expression pattern of a mouse β-CD. The protein clearly belongs to an extended family of dioxygenases that interact with carotenoids and other polyenes and that include the plant neoxanthin cleavage enzymes, the bacterial lignostilbene dioxygenases, and the vertebrate protein RPE65. The activity of mouse β-CD seen with alternative carotenoid substrates (Fig. 3) indicates that the specificity of the catalytic activity is centered more in the polyene chain of the substrate than at the end groups. This observation is in accord with the activities previously reported for crude tissue preparations (23–25). Therefore, a more accurate and inclusive designation for the family as a whole may be the polyene chain dioxygenase family.

The conserved histidines and acidic residues of the various polyene dioxygenase family members are likely to be involved in coordinating the iron required for activity in these enzymes (9, 26). The signature sequence, a run of acidic residues connected through a glycine to a hydrophobic tail, could be involved in electron transfer processes during catalysis. Although the precise enzymatic activity of RPE65 has not yet been determined, RPE65 is capable of binding ferrous iron and thus shares this characteristic as well as the signature sequence with other family members. The resemblance of RPE65 to the other dioxygenase family members and the fact that its absence prevents the retinal pigment epithelium-specific isomerization of all-trans- to 11-cis-retinol in the visual cycle (12) suggest a direct catalytic participation in the visual cycle.

Although β-CD activity in partially purified preparations from various sources has been localized both to cytosolic and membrane fractions, the overwhelming consensus is that it is a cytosolic protein (23–25, 27), as we also have found. However, an important aspect of the carotenoids and their derivatives as substrates is their lipophilicity (including a tendency to partition in membranes) and generally insoluble nature in aqueous solution. Interestingly, none of the polyene dioxygenase family members, including mouse β-CD, are predicted to be integral membrane proteins. However, the plant 9-cis-epoxycarotenoid cleavage enzymes are associated with chloroplast membranes (9). The only metazoan member of the family whose biochemical properties have been examined in this respect is RPE65, which is a non-integral, microsomal membrane-associated protein.

DISCUSSION

In Vivo and in Vitro Activity of Mouse β-CD—HPLC analysis of hexane extracts of β-carotene-accumulating E. coli yielded the expected β-carotene peak (Fig. 4A). Expression of mouse β-CD in such cultures resulted in the appearance of all-trans-retinal with a concomitant loss of β-carotene (Fig. 4B). The spectra of both the β-carotene and all-trans-retinal peaks were analyzed (see insets in Fig. 4, A and B) and exhibited the expected shapes and absorbance maxima. β-Carotene, δ-carotene, ε-carotene, and lycopene-accumulating cultures, quantitated by HPLC analysis, showed 2.14, 1.87, 1.23, and 1.50 pmol/mg of bacterial protein, respectively, in cultures containing the empty vector. In contrast, cultures containing the mouse β-CD insert had no detectable β-carotene. However, δ-carotene, ε-carotene, and lycopene were present in such cultures at levels of 3.2, 57.9 and 65.6 fmol/mg of bacterial protein, respectively.

Purified recombinant β-CD protein cleaves β-carotene in vitro with a $V_{\text{max}}$ of 36 pmol of retinal/mg of enzyme/min and a $K_m$ of 6 μM. At levels of lycopene concentrations comparable to the $K_m$ for β-carotene, no acyclic retinal was detected. However, trace amounts were seen at 2.5–3 times the β-carotene $K_m$.

Expression of Mouse β-CD in Tissues—Multiple tissue RNA gel blots, analyzed with riboprobe, showed substantial steady-state mRNA levels in several of the tissues known to be active in vitamin A metabolism (Fig. 5A). The major message, seen in liver, kidney, testis, and small intestine, was 2.4 kb. Although the 2.4-kb message was in low abundance in the small intestine, a tissue known to contain strong β-CD activity, it is likely that this was due to dilution of the mRNA contribution of the mucosa by that of the outer muscular layers of the gut. Smaller messages were also seen in testis and skin; in particular, a 1.8-kb message was quite abundant in testis. Analysis of a mouse developmental blot showed a strong signal for the 2.4-kb message at 7 days post-conception, which declined as the developmental age increased (Fig. 5B). In immunoblots, a band of immunoreactivity of the expected size of 63 kDa was detected in 30,000 × g and 100,000 × g supernatant fractions of liver, kidney, testis, and small intestine, but not in the 100,000 × g pellet fractions (data not shown).

$^3$ S. Gentleman, unpublished data.
tein, but can be separated from retinal pigment epithelium membranes by mild detergent or high salt extraction (11). Furthermore, RPE65 will specifically associate with phospholipid liposomes (28). It is possible that mouse \( \beta \)-CD, despite its cytosolic nature, will also interact with membranes in the context of its physiological mechanism.

The tissue distribution of mouse \( \beta \)-CD was, in general, as expected, although the level in the small intestine was somewhat weak. However, the \( \beta \)-CD activity of this tissue is concentrated in mature enterocytes (and is highest in the jejunum) (29), which constitute a relatively small proportion of the total tissue used to provide the RNA for this blot. Thus, one possibility is that the weak signal in this tissue is due to dilution of the RNA with that from other cell types. Another possibility is that there is a tissue-specific \( \beta \)-CD in small intestine, in addition to the one described here. The testis also is active in the uptake and metabolism of carotenoids, as \( \beta \)-carotene and lycopene have been repeatedly demonstrated in testis (30–32). Cleavage of \( \beta \)-carotene has been shown to be an important source of retinoic acid in vitro in testis as well as in small intestine, liver, kidney, and lung (33), and the high expression of \( \beta \)-CD in testis noted here is consistent with this finding.

**FIG. 4. Detection of all-trans-retinal by HPLC of \( E. \) coli extracts.** Organic extracts of \( E. \) coli cultures transformed with pBAD/\( \beta \)-CD and induced with 0.2% arabinose were analyzed as described under “Experimental Procedures.” A, cultures containing only the lacZ (control) construct accumulated \( \beta \)-carotene. B, Cultures expressing mouse \( \beta \)-CD produced all-trans-retinal. Solid lines, absorbance at 370 nm; dashed lines, absorbance at 450 nm. Insets, spectra of peaks indicated by asterisks. A \( \beta \)-carotene (BC) standard eluted at 2.2 min; an all-trans-retinal (RAL) standard eluted at 7.4 min (not shown). AU, absorbance units.

**FIG. 5. Tissue and developmental expression of mRNA for mouse \( \beta \)-CD.** A, tissue distribution in adult mouse tissues. The blot was hybridized with a 3'-antisense riboprobe to mouse \( \beta \)-CD. A transcript of 2.4 kb was noted in kidney, liver, small intestine (Sm. Intest.), and testis, whereas a transcript of 1.8 kb was seen in skin and testis. B, RNA gel blot of total mouse embryos from embryonic days 7–15. This blot was hybridized with the same probe as described for A. The 2.4-kb transcript was highly expressed at embryonic day 7 and less so at later embryonic stages.
Napoli and Race (33) have suggested that cleavage of β-carotene may be an important source of retinoic acid in target tissues such as testis and may play an under-appreciated role in retinoid homeostasis.

Mouse β-CD has a low level of activity toward carotenoids other than β-carotene, including lycopene, observable in in vivo assays. As to its distribution, lycopene is accumulated primarily in liver, but also in intestine, prostate, and testis (34), and appears to have several biological activities (35). For example, in a recent study, lycopene, but not its acycloretinoid deriva-
tives, was effective in stimulating gap junction communication (36). Therefore, in this case, the function of the dioxygenase with respect to lycopene might be to terminate its activity.

The developmental expression of mouse β-CD is particularly intriguing, given its early elevated expression at embryonic day 7 and much reduced expression thereafter. At embryonic day 7, with the embryo proceeding into gastrulation, a number of retinoids and retinoid-interacting proteins are being expressed (37). Analysis of endogenous retinoids in the mouse embryo (37) shows that all-trans-retinal is the first retinoid seen (at embryonic day 6.5), whereas both all-trans-retinoic acid and all-trans-retinol do not appear until embryonic day 7.5. In the mouse embryo, mRNAs of two dehydrogenases involved in retinoic acid production, alcohol dehydrogenase IV and aldehyde dehydrogenase I, are up-regulated between embryonic days 6.5 and 7.5 (37, 38). Although the early accumulation of all-trans-retinal may be due to low level activity of alcohol dehydrogenase IV on maternally supplied all-trans-retinol, there may also be a contribution from the cleavage of β-carotene. Ulven et al. (37) suggested this possibility, an interpretation supported by our finding of high β-CD mRNA expression at embryonic day 7. Thus, the activity of β-CD could serve to produce all-trans-retinal from β-carotene, a nontoxic provitamin source of retinoids in the early embryo.

The identification of mouse β-CD establishes the existence in mammals of an ancient family of carotenoid-metabolizing enzymes. These enzymes, giving a means by which animals can cleave or modify these plant-derived compounds as required, provide access to retinoids for animals and facilitate their pan-oply of developmental and physiological effects. We can surmise that the adoption by animals of hormonally and otherwise physiologically active carotenoid metabolites (retinoids) was initially facilitated by such enzymes evolved from an ancient common ancestor.

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