Identification of the Retinoic Acid-inducible All-trans-retinoic Acid 4-Hydroxylase*

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Vitamin A metabolism gives rise to several active forms of retinoic acid (RA)† which are involved in regulating gene expression during development, regeneration, and the growth and differentiation of adult epithelial tissues (1–3). Until recently, studies have focused mainly on the activities of all-trans- and 9-cis-RA metabolites but little is known about the mechanisms regulating their formation. The normal balance of these metabolites is dependent upon the rate of formation from metabolic precursors, retinol and retinaldehyde (4), and the rate of catabolism. The mechanism of interconversion between all-trans-RA and 9-cis-RA in vivo is unclear; the asymmetric distribution of these metabolites in developing embryos suggests that they may be preferentially sequestered or generated by tissue-specific isomerases (5).

RA catabolism is thought to proceed through the formation of polar intermediates including 4-hydroxy-retinoic acid (4-OH-RA) and 4-oxo-retinoic acid (4-oxo-RA) (6). Whether the 4-oxo-RA and 4-OH-RA metabolites are simply intermediates in the RA catabolic pathway or whether they have specific activities which differ from those of all-trans-RA and 9-cis-RA remains to be established. Studies in Xenopus by Pijnappel and co-workers (7) provide support for the latter possibility by showing that 4-oxo-RA can efficiently modulate positional specification in early embryos and exhibits a more potent ability to regulate Hoxb-9 and Hoxb-4 gene expression than all-trans-RA. Furthermore, 4-oxo-RA binds to retinoic acid receptor-β (RAR-β) with affinity comparable to all-trans-RA (7) but poorly to RAR-γ (8), suggesting that this metabolite exhibits some receptor selectivity. 4-oxo-RA also binds to cellular retinoid acid-binding protein but with an affinity slightly lower than that of all-trans-RA (9). Recently, Takatsuka et al. (10) have shown that growth inhibitory effects of RA correlate with RA metabolic activity. Whether there is a causal relationship between production of RA metabolites and growth inhibition is unknown.

The generation of 4-oxo-RA and 4-OH-RA metabolites is believed to be a cytochrome P450 dependent process. This notion is largely supported by the effectiveness of general P450 inhibitors such as ketoconazole and liarozole to inhibit the production of these metabolites from RA (11–14). In certain tissues (testis, skin, and lung) and cell lines (NIH 3T3, HL 60, F9, and MCF-7) RA metabolism can be induced by RA pretreatment (6, 10, 15-17). In F9 cells, the targeted disruption of RAR genes suggests that RAR-α and RAR-γ isoforms may play an important role in regulating the enzymes responsible for this increased metabolism (18). Inducible RA metabolic activity has also been implicated in progressive RA resistance in the treatment of acute promyelocytic leukemia (19). Little is presently known about the specific cytochrome P450s involved in the metabolism of RA in extrahepatic tissues during developmental processes. None of the genes encoding these extrahepatic RA metabolic activities have been cloned to date.

Zebrafish fins regenerate through a RA-sensitive process which utilizes many of the gene regulatory pathways involved in early vertebrate development (20–22) and we have used this model for the identification of RA-regulated genes. We have identified a potential RA feedback loop in zebrafish caudal fin regeneration in response to exogenous RA. In COS-1 cells transfected with the P450RAI cDNA, all-trans-RA is rapidly metabolized to more polar metabolites. We have identified 4-oxo-RA and 4-OH-RA as major metabolic products of this enzyme. P450RAI represents the first enzymatic component of RA metabolism to be isolated and characterized at the molecular level and provides key insight into regulation of retinoid homeostasis.

Retinoic acid (RA) metabolites of vitamin A are key regulators of gene expression involved in embryonic development and maintenance of epithelial tissues. The cellular effects of RA are dependent upon the complement of nuclear receptors expressed (RARs and RXRs), which transduce retinoid signals into transcriptional regulation, the presence of cellular retinoid-binding proteins (CRABP and CRBP), which may be involved in RA metabolism, and the activity of RA metabolizing enzymes. We have been using the zebrafish as a model to study these processes. To identify genes regulated by RA during exogenous RA exposure, we utilized mRNA differential display. We describe the isolation and characterization of a cDNA, P450RAI, encoding a novel member of the cytochrome P450 family. mRNA transcripts for P450RAI are expressed normally during gastrulation, and in a defined pattern in epithelial cells of the regenerating caudal fin in response to exogenous RA. In COS-1 cells transfected with the P450RAI cDNA, all-trans-RA is rapidly metabolized to more polar metabolites. We have identified 4-oxo-RA and 4-OH-RA as major metabolic products of this enzyme. P450RAI represents the first enzymatic component of RA metabolism to be isolated and characterized at the molecular level and provides key insight into regulation of retinoid homeostasis.

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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) U68234.

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† The abbreviations used are: RA, retinoic acid; 4-OH-RA, 4-hydroxyretinoic acid; 4-oxo-RA, 4-oxo-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid-X-receptor; CRBP, cellular retinoid binding protein; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.
involving the conversion of RA to 4-oxo- and 4-OH-RA. The cloning of the RA-regulated P450RAI identifies a new mechanism whereby RA activities can be controlled in a cell specific manner.

**MATERIALS AND METHODS**

**Danio rerio Stocks**

*D. rerio* were maintained and fin amputations performed as described previously (20).

**Northern blot Analysis**

RA Inducibility—Fish were allowed to regenerate their caudal fins for 72 h. At 48 h all-trans-RA, to reach a final concentration of 10 μM, or MeSO was added directly to the tank water. mRNA was prepared using the Micro Fast-Track mRNA isolation kit (Invitrogen, CA) according to the manufacturer's directions. 3.0–5.0 μg of poly(A)-RNA was electrophoresed, blotted, and probed with the full-length P450RAI cDNA as described previously (20).

Developmental Expression Profile—Zebrafish embryos were collected at different developmental stages. Poly(A)−enriched mRNA (0.75 μg from 8 h embryos, 2.6 μg from 14 h embryos, and 1.5 μg from 20 h embryos) was separated by agarose gel electrophoresis, blotted, and probed as described above with the full-length P450RAI cDNA. Loading of mRNA samples was monitored by ethidium bromide staining as in Fig. 1.

**Differential Display of mRNAs**

Differential mRNA display was performed essentially as described by Liang and Pardee (23) with modifications by White and Petkovich (24). Regenerating tissues were collected 3 days post-amputation (24 h post-RA addition) and quick frozen in liquid nitrogen. Poly(A)−RNA was isolated using the Micro- Fast Track kit. Duplicate independent reverse transcription reactions were performed with isolated poly(A)−RNA from both the RA− and control-treated samples for each specific 3′-poly(T) primer used (5′-T12VN-3′, where V represents nucleotides G, A, or C and N represents nucleotides G, A, T, or C). For each reaction 0.1 μg of poly(A)−RNA was reverse-transcribed in a 20-μL reaction volume containing 300 units of Superscript Reverse Transcriptase (Life Technologies, Inc./BRL), 10 μM of each dNTP, 50 units of Superscript Reverse Transcriptase (Life Technologies, Inc.), 1 × PCR buffer, 2 μM each dGTP, dATP, dCTP, and dTTP, 10 μM dithiothreitol, and 5 pmol of 5′-T12VN-3′ primer. The reactions were mixed and incubated at 35°C for 60 min followed by 5 min at 95°C. PCR amplification was performed in a Perkin Elmer PCR machine as follows: 1 μL of cDNA synthesis reaction, 5 units of Taq DNA polymerase (Life Technologies, Inc.), 1 × PCR buffer, 2 μM each dGTP, dATP, dCTP, and dTTP, 10 μM of primer, 1.5 μl of [α-32P]dATP (Redivue™, Amersham) 1.2 μg MgCl₂, 0.5 μM upstream primer (5′-TGGCGCATGTTA-3′), and 0.5 μM of the corresponding 5′-T12VN-3′ primer. PCR conditions were as follows: 1 cycle, 94°C for 5 min; 40 cycles, 94°C for 30 s, 42°C for 1 min, 72°C for 30 s; followed by a final extension of 5 min at 72°C. Aliquots (4 μL) of the PCR reactions were loaded onto a 6% nondenaturing polyacrylamide gel and electrophoresed at 60 watts, 45°C. The gel was dried and exposed to Kodak X-AR film for 12–24 h at room temperature.

**Screening of a D. rerio cDNA Library**

A random-primed *D. rerio* 6–18 h embryo cDNA library constructed in Uni-ZAP II (Stratagene) was generously donated by P. Chambon. 4.5 × 10⁶ independent plaques were screened using the random-primed, [α-32P]dATP-labeled 337-base pair PCR fragment isolated by mRNA differential display as a probe. Filters were prehybridized for 1–4 h at 42°C in 50% formamide, 5 × SSPE, 1 × Denhardt’s solution, 0.2 μg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 42°C by adding denatured probe to the prehybridization solution. Filters were washed two times for 20 min in 2 × SSC, 0.05% SDS at room temperature and exposed to Kodak X-AR film overnight at −70°C. Positive plaques were picked into 500 μL of SM buffer and rescreened until purified. Phlebucus-containing colonies, generated using the *in vivo* excision protocol, were plated onto LB + ampicillin plates and grown overnight at 37°C. Sequence data was generated using the T7 Sequencing Kit (Pharmacia) and analyzed using the GeneWorks software package (Intelligenetics).

**Whole Mount in Situ Hybridization**

Me_SO− and RA-treated regenerates were isolated 72 h post-amputation (24 h post-RA/Me_SO addition), washed in phosphate-buffered saline and analyzed for P450RAI expression by whole mount in situ hybridization (performed as described previously (20)).

**RESULTS**

Characterization of a Novel Cytochrome P450—Transcripts expressed in fin tissue regenerating in the presence or absence of RA were compared using the differential display PCR technique. One of the differential display products which exhibited a particularly strong dependence on the presence of RA for its expression (Fig. 1A) was isolated, sequenced (Fig. 1B), and its corresponding full-length cDNA characterized. The amino acid sequence of this cDNA, which we have named P450RAI, is shown in Fig. 1C. BLAST search analyses revealed sequence homology between P450RAI and multiple members of the cytochrome P450 superfamily. Alignments indicated that P450RAI exhibited less than 30% amino acid identity with other cytochromes and therefore could not be classified into any of the previously defined subfamilies (26). P450RAI, however, contains many of the structural motifs which are common to all cytochrome P450 family members including the characteristic heme-binding domain located in the C-terminal portion of the protein (Fig. 1D). We have recently isolated, from a fetal brain library, a human cDNA very closely related to P450RAI, suggesting that this novel subfamily of cytochromes is highly conserved between fish and human.

**Cell Specific Induction of P450RAI by All-trans-RA—Northern blot analysis of mRNAs expressed in regenerate tissue isolated from control (dimethyl sulfoxide-treated) and RA-treated fish was performed with full-length P450RAI cDNA probe. P450RAI transcripts were not detectable in regenerate tissue from control fish (Fig. 2A, lane 4) but were very strongly induced in tissues isolated from fish exposed to RA for 24 h (Fig. 2A, lane 5).

Whole mount *in situ* hybridization was used to determine the cellular localization of P450RAI expression in regenerating fin tissue. Fig. 2B shows regenerating fins from control and RA-treated fish. P450RAI transcripts are not detectable in control fin tissue (Fig. 2B, i). In regenerating tissue from RA-treated fish, P450RAI transcripts are most abundant in a specific layer of epithelial cells extending across the distal edge of the wound epithelium (Fig. 2B, ii). Some low level staining is also detectable in inter-ray tissue (Fig. 2B, ii). Histological sections (Fig. 2B, iv) of RA-treated fins show that the cells expressing P450RAI are located deep within the epithelial layer at the distal tip of the blastemas mesenchyme. Interestingly, this level of P450RAI expression is not seen in other epithelial cells which make up the regenerate.

Developmental Expression of P450RAI—Northern blot analysis was performed on RNAs prepared from embryos collected at 8, 14, and 20 h post-fertilization corresponding to early gastrulation, early segmentation (10 somites), and late segmentation (21 somites), respectively (see Fig. 2C). P450RAI is strongly expressed during early gastrulation whereas P450RAI levels appear to be lower in later embryos. Importantly, these results indicate that p450RAI is expressed in response to en-

**HPLC Analysis**

Media from transfected cells incubated with 575 pm [11,12-3H]RA (23.1 Ci/mmol) or 1 μM RA for either 4 or 24 h were acidified with 0.1% acetic acid. Lipid-soluble metabolites were separated from aqueous-soluble metabolites using a total lipid extraction of the medium (20). Conversion of [11,12-3H]RA to total aqueous-soluble metabolites was measured using aliquots of the aqueous-soluble extract subjected to β-scintillation counting. Lipid-soluble extracts were evaporated to dryness under a stream of nitrogen and resuspended in 93.5/5/0.5, hexane/isopropl alcohol/methanol/acetic acid. Metabolites were separated by HPLC using a Zorbax-SIL (5 μm, 8 × 0.62 cm) column eluted with a solvent system of 93.5/5/0.5, hexane/isopropl alcohol/methanol/acetic acid at a flow rate of 1 ml/min.

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dogenous signals and appears to be developmentally regulated.

**Metabolism of All-trans-RA by P450RAI-transfected Cells**—

The apparent RA inducibility of P450RAI prompted us to determine whether or not RA was a substrate for this cytochrome. For these experiments, the full-length P450RAI cDNA was cloned into the eukaryotic expression vector pSG5 (27). COS-1 cells were transiently transfected with either pSG5 or pSG5-P450RAI and then incubated with either picomolar concentrations of [11,12-3H]all-trans-RA or micromolar concentrations of non-radioactive all-trans-RA. P450RAI expression in COS-1 cells promoted the rapid conversion of RA into both lipid- and aqueous-soluble metabolites (Fig. 3, A and B). Fractional total lipid extracts of transfected cells were separated by normal-phase HPLC on Zorbax-SIL. Comparison between extracts from pSG5- and pSG5-P450RAI-transfected cells indicated that P450RAI significantly increased RA metabolism in these cells. Incubation of P450RAI-transfected cells with 575 pM [11,12-3H]all-trans-RA for either 4 or 24 h resulted in accumulation of RA metabolites, one of which co-migrated with synthetic standards 4-OH-RA and 18-OH-RA, and a second slightly less polar metabolite which co-migrated with 4-oxo-RA standard (Fig. 3, A and B). Recromatography of RA metabolites using other HPLC systems confirmed the identity of these two metabolites as 4-OH-RA and 4-oxo-RA (Table I). Although we do not know if the aqueous-soluble radioactivity represents glucuronides of RA metabolites or glucuronides of RA itself, others have reported rapid glucuronidation of 4- and 18-hydroxy-RA in mammalian cell extracts (10, 17).

A similar pattern of P450RAI-dependent metabolism was observed using a much higher RA concentration (1 μM). P450RAI-transfected cells incubated for 4 or 24 h with 1 μM RA generated two closely-running peaks which were discernible in a 350-nm HPLC trace depicted in Fig. 3 (panels C and D) but were essentially undetectable in control pSG5-transfected cells (Fig. 3, panel C and D). These peaks co-migrated with 4-oxo-RA and 4-OH-RA standards. Diode array spectrophotometric detection of the P450RAI-generated peaks allowed us to establish that the spectral properties of the two metabolite peaks matched the standard retinoids (in hexane-based solvents we found: 4-OH-RA, $\lambda_{max} = 350 \text{ nm}$; 4-oxo-RA, $\lambda_{max} = 355 \text{ nm}$; in...
methanol-based solvents we found; 4-OH-RA, \( \lambda_{\text{max}} = 340 \text{ nm} \); 4-oxo-RA, \( \lambda_{\text{max}} = 360 \text{ nm} \) (for review of published values, see Refs. 36 and 37)).

**DISCUSSION**

We have identified a RA metabolizing cytochrome P450 which is induced in zebrafish caudal fin wound epithelium in response to RA treatment. While RA metabolizing activity has previously been detected in epithelial tissues of several species (6, 15-17), until now, none have been characterized at the molecular level. P450RAI is remarkable for its strong up-regulation by RA and its highly localized expression in a specific set of cells in the wound epithelium of regenerating zebrafish caudal fins. It will be interesting to determine whether the RA-regulation of P450RAI represents transcriptional control of the P450RAI promoter and whether this cell-selective induction of P450RAI involves other factors which limit the RA inducibility to a small subset of cells in the wound epithelium.

Interestingly, studies in F9 cells where RARs have been selectively disrupted through homologous recombination indicate that RAR-\( \alpha \) and RAR-\( \gamma \) may have important roles in the regulation of RA metabolism in these cells (18). While we do not presently know whether the RA-inducible RA metabolism in F9 cells represents murine P450RAI activity, we note that both RAR-\( \alpha \) and RAR-\( \gamma \) are expressed in the regenerating caudal fin consistent with the possibility that they may be involved in the direct regulation of P450RAI expression by RA (20).
The expression of P450RAI in 8-h embryos suggests that this enzyme may play an important role in the developmental regulation of endogenous RA levels. Zebrafish have been shown to be highly sensitive to exposure to exogenous retinoids at this stage of development. The localized induction of P450RAI in the fin regenerate suggests it may be involved in a number of important cellular processes. P450RAI may play a morphogenetic role by controlling the biosynthesis of retinoids involved in the regeneration of the caudal fin. It has previously been proposed that metabolites of RA such as 4-oxo- and 3,4-didehydro-RA may have specific roles in morphogenesis (7, 28). Support for this possibility comes from studies in Xenopus embryos indicating that for some RA-regulated activities, 4-oxo-RA appears to be more potent than all-trans-RA (7).

Moreover, there is a strong positive correlation between the ability of cells in vitro to metabolize RA and the effectiveness of RA to inhibit their proliferation (10). This suggests that the metabolites of RA rather than RA itself are mediating the growth inhibitory effects. In this regard, the localized expression of P450RAI suggests that this enzyme may be involved in the production of active metabolites with cell specific functions different from those of all-trans-RA.

It is important to note, however, that we do not detect P450RAI expression in untreated fins by Northern blot analysis or in situ hybridization. This implies that RA levels in the regenerate are not sufficiently high within the first 72 h of regeneration to induce P450RAI expression. While this does not preclude the possibility that this enzyme is involved in the localized generation of RA metabolites which differentially affect morphogenesis, it is more consistent with P450RAI being involved in autoregulated target cell catabolism of RA. Consequently P450RAI may limit the response of target cells to RA, while also restricting the range of the biological effect to a limited number of cells. In accordance with this possibility, the activities of RA metabolizing cytochrome P450s are increased in a number of mammalian cell lines in response to RA (6, 16, 17).

This mode of ligand autoregulation has been proposed for the cytochrome P450, CYP24, which is involved in vitamin D catabolism (29). Notably, the CYP24 substrate, 1α,25-(OH)2D3, regulates the CYP24 promoter through a vitamin D response element (30–32), resulting in the accelerated conversion of 1α,25-(OH)2D3 to multiple catabolic products (33).

The ability of RA to increase its own metabolism in other species and tissues suggests that functional counterparts for P450RAI will be found. Indeed, we have recently cloned a
human cDNA encoding an RA-inducible P450RAI homologue expressed in epithelial tissues. The existence of a human counterpart to P450RAI supports the notion that it plays a highly conserved functional role in RA signaling. In addition, the degree of structural and functional conservation further supports the usefulness of the zebrafish model for studying the developmental role of RA (20, 34).

Little is presently known about the specific enzymes involved in RA metabolism. The cell-specific regulation of developmental role of RA (20, 34).

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