A Keratinocyte-specific Epoxygenase, CYP2B12, Metabolizes Arachidonic Acid with Unusual Selectivity, Producing a Single Major Epoxyeicosatrienoic Acid*

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The CYP monooxygenase, CYP2B12, is the first identified skin-specific cytochrome P450 enzyme. It is characterized by high, constitutive expression in an extrahepatic tissue, the sebaceous glands of cutaneous tissues. It is expressed exclusively in a subset of differentiated keratinocytes called sebocytes, as demonstrated by Northern blot analysis, in situ hybridization, and polymerase chain reaction. The onset of its expression coincides with the morphological appearance of sebaceous glands in the neonatal rat. Recombinant CYP2B12 produced in Escherichia coli epoxidizes arachidonic acid to 11,12- and 8,9-epoxyeicosatrienoic acids (80 and 20% of total metabolites, respectively). The identification of arachidonic acid as a substrate for this skin-specific CYP monooxygenase suggests an endogenous function in keratinocytes in the generation of bioactive lipids and intracellular signaling.

Mammalian CYP monooxygenases (CYP gene superfamily) oxidatively metabolize small, hydrophobic compounds, including steroids, sterols, fatty acids, fat-soluble vitamins, drugs, and toxins (1). These enzymes are expressed in most mammalian tissues, where they function in the biosynthesis or catalysis of endogenous and exogenous substrates. Members of the CYP2B gene subfamily are often called phenobarbital-inducible cytochrome P450 enzymes because phenobarbital treatment results in transcriptional activation of certain hepatic CYP2B genes (2). A typical CYP2B monooxygenase has 16-hydroxylase activity with androgenic steroids, such as testosterone, androstenedione, benzphetamine, and ethoxy-, pentoxy-, and benzoylescrofin (2, 4). Our preliminary investigations of a novel CYP2B in mouse skin have investigated homology to rat CYP2B12 revealed interest in this cutaneous P450 enzyme. We report here that CYP2B12 is a gene product unique to sebocytes, a subset of differentiated keratinocytes, and that arachidonate is a substrate for CYP2B12. It is hypothesized that the epoxyeicosatrienoic acids (EETs) produced by this enzyme may function in signal transduction pathways essential for establishing or maintaining the differentiated phenotype of keratinocytes in cutaneous tissues.

MATERIALS AND METHODS

Analysis of CYP2B12 mRNA—Total RNA was isolated by a modification of the method of Chomczynski and Sacchi as described (5), with the following changes: cutaneous tissues were frozen in liquid nitrogen, pulsed with a mortar and pestle, dissolved in guanidinium thiocyanate solution, and sheared with a 25-gauge needle. For Northern analysis, total RNA was size-fractionated on formaldehyde-containing agarose gels and transferred by capillarity to GeneScreen Plus membranes (DuPont). Membranes were baked at 80 °C and stained with methylene blue to assess RNA integrity. Hybridization was carried out overnight with a 32P-labeled cDNA encoding 3′-untranslated sequences of CYP2B12 (570 bp; GenBank accession no. X63545) or CYP2B15 (260 bp; GenBank accession no. D17349). These fragments were generated by reverse transcription-polymerase chain reaction (RT-PCR) from rat skin RNA using oligonucleotides 1 and 2 for CYP2B12 or oligonucleotides 3 and 4 for CYP2B15 (Table I). Hybridized membranes were washed stringently and exposed to autoradiographic film. For RT-PCR, an RNA PCR kit (Perkin-Elmer) was used according to the manufacturer’s instructions, with total RNA as template. Oligonucleotides contained in exons 4 and 9 (Table I, oligonucleotides 5 and 6) were used to determine the tissue distribution of CYP2B12 transcripts. Thus, products amplified from RNA (920 bp) or genomic DNA could be distinguished. The PCR products were size-fractionated on 2% agarose gels. Results were quantified by densitometry and expressed as a percentage of the product size-fractionated on a blank gel, with an equal number of cycles and loaded RNA.

* This work was supported in part by United States Public Health Service Grants P30 AR41943, P30 ES00267, P30 DK26657, P01 DK38226, and R01 DK43849. ** Supported by United States Public Health Service Grant DK38226.
aglass gels and visualized with ethidium bromide. To survey expression of putative CYP epoxigenases in sebaceous tissues by RT-PCR, the following primers (Table I) were used: 1) CYP1A subfamily-specific (oligonucleotides 7 and 8); 2) CYP2B subfamily-specific (oligonucleotides 11 and 12). Two primer pairs were used in the same reaction to achieve consensus for mammalian CYP2B subfamily members (oligonucleotides 13–16). Finally, two pairs of oligonucleotides were used to detect expression of CYP2E1 (oligonucleotides 17 and 18) and CYP2J3 (oligonucleotides 19 and 20).

In Situ Hybridization—Sprague-Dawley rats were obtained from Harlan Sprague-Dawley and handled according to protocols approved by Vanderbilt University. Whole skin samples were immersion-fixed for 24 h in 4% buffered paraformaldehyde. Tissue preparation and in situ hybridization were done as described (5). [35S]-UTP transcripts were produced by in vitro transcription from a 570-bp fragment of CYP2B12 (described above). Specific hybridization was distinguished by comparing silver grain development produced by sense and antisense cRNAs applied to consecutive sections on the same slide.

Expression of CYP2B12 in Escherichia coli—A CYP2B12 cDNA (4) (pBS2B12) was modified and subcloned into pKK233–2 (Amersham Pharmacia Biotech) to make the expression construct ppKK233–2 (2-kDa strain: Stratagene) was transformed with pKK233–2. Media and conditions for expression were as described (6). The cultures were harvested after 48–72 h at 30 °C at 200 rpm. Typical yields were 5,600 nmol of P450/liter of culture by whole cell assay (Table I). The PCR product containing this N-terminal modification was restricted with NcoI, producing the 83-bp fragment, and ligated into ppKK233–2. By DNA sequence analysis, this final pKK2B12 expression construct contained the entire open reading frame of CYP2B12, the MALLAV-NcoI fragment, and 40 bp of untranslated sequence beyond the stop codon. Competent E. coli cells (Top10 strain) were transformed with ppKK233–2. Media and conditions for expression were as described (6). The cultures were harvested after 48–72 h at 30 °C at 200 rpm. Typical yields were 5,600 nmol of P450/liter of culture by whole cell assay. Whole cell or harvested after 48–72 h at 30 °C at 200 rpm. Typical yields were

TABLE I

| Oligonucleotide | Sense Sequence | Antisense Sequence |
|-----------------|----------------|-------------------|
| 1               | Sense: GGCAAGCAGATCAAAGAGG | Antisense: GGCAAGCAGATCAGAAAG |
| 2               | Sense: CACAGTCACACACCTTTCAG | Antisense: CACAGTCACACACCTTTTCAG |
| 3               | Sense: AGTGGCTCAAGACCAAG | Antisense: GGATCGGGTTAGACCAGGACCATGGCTCTGTTATTAGCAGTTCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 4               | Sense: GGATCGGGTTAGACCAGGACCATGGCTCTGTTATTAGCAGTTCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 5               | Sense: GACACGTCATTCCATTGACTCTT | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 6               | Sense: AGTGAGCTCAAGACCA(G/A)CCAG | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 7               | Sense: GACAGTCAGTTTCAGGAGATGCTG | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 8               | Sense: AAGTAGTC(T/A)(G/C)TCTTCTT(G/A)AAG | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 9               | Sense: ATCTC(T/C)TCCTG(C/G)ACTTTA(G/T)C | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 10              | Sense: TGTGCTCCI(G/T)GCAAAGC(T/C)AT | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 11              | Sense: CATGCACCAAGACGACATCGTGAG | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 12              | Sense: CACCTCTGAGTCATGGGACATCAC | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 13              | Sense: GCACCGCATTAAAGATCATGTCG | Antisense: GGATCTAGTCTATAAGACATCTGT |
| 14              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 15              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 16              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 17              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 18              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 19              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 20              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 21              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |
| 22              | Sense: GGTTAGRCCAGCCAGCTGCTGCTGTTATTAGCAGTTCTCCTCCTCACTCTCACTGTGGGCTTCTTGCTA |

**Results**

**Skin-specific Expression of CYP2B12**—CYP2B12 is a cutaneous monoxygenase expressed constitutively in rat preputial gland (the tissue source from which a full-length CYP2B12 cDNA was isolated) (4). Among cutaneous tissues, the single most enriched source of CYP2B12 mRNA is the pair of preputial (male) or clitoral (female) (Fig. 1) glands in the rat. These large, paired sebaceous glands in the genital skin are readily dissected free of surrounding cutaneous tissue and subcutaneous fat. As such, they are composed nearly exclusively of keratinocytes at different stages of differentiation. Although CYP2B12 transcripts were detectable in all cutaneous tissues examined, transcript abundance varied. Among samples of full-thickness skin, tail and anogenital skin showed a relatively high level of expression. Lower levels were found in the abdominal skin, ear, and foot pad. For these latter samples, visible bands of the expected molecular size appeared after longer exposure times than that shown in Fig. 1. The differences in hybridization signal intensity among cutaneous tissues appeared to be proportional to the relative size and abundance of sebaceous glands, observed morphologically.

By Northern blot analysis, CYP2B12 mRNA was undetectable in the liver, lung, kidney, testis, adrenal gland, and intestinal mucosa (4) (Fig. 1). The tissue-specificity of CYP2B12 gene expression was investigated further by the highly sensitive RT-PCR technique. Using total RNA as template, no prod-
cRNA is represented by silver grains, which appear white. The in situ hybridization was used to identify the cutaneous cell type(s) expressing CYP2B12. CYP2B12 antisense cRNA hybridized specifically to preputial gland acini containing sebaceous keratinocytes. Acini are shown in Fig. 2, A and B, surrounding a central collecting duct, which directs the flow of sebum to the skin surface. At higher magnification (not shown), silver grains localized specifically to sebocytes, the terminally differentiated keratinocytes in this gland, but not to undifferentiated keratinocytes at the periphery of individual acini or any other cutaneous tissue. Reproducible epidermal expression was not observed in situ, although a very low level of expression in epidermal keratinocytes cannot be ruled out. CYP2B12 transcripts also localized to sebocytes in other types of sebaceous glands, for example, the Meibomian glands in the eyelid (not shown), anal glands in anogenital skin (not shown), and sebaceous glands associated with hair follicles. In hair follicles (Fig. 2, C and D), CYP2B12 antisense cRNA localized to sebocytes but not to keratinocytes making up the hair follicle root sheath, epidermis, or other cutaneous structures. During postnatal development, the onset of expression of CYP2B12 coincides with the morphological appearance of well-developed sebaceous glands in both sexes (not shown). These data demonstrate that expression of this cutaneous cytochrome P450 enzyme is restricted to a subset of differentiated keratinocytes found in sebaceous glands.

Arachidonic Acid Is a Substrate for CYP2B12—Differentiated keratinocytes are responsible for producing the unusual lipids found in the epidermal water permeability barrier and sebum (13). Because CYP2B12 is expressed constitutively in lipid-laden sebocytes, it seemed plausible that its endogenous substrate(s) would be lipids. To test this hypothesis, recombinant CYP2B12 was produced in E. coli after its first seven amino acids were replaced with the sequence MALLLAV (7). The DNA sequence of this bacterial expression construct, pKK2B12, was compared with the CYP2B12 cDNA in GenBank (4). Except for the deliberate N-terminal modification, only one difference was found in the nucleotide sequence (ATT → AGT) that would lead to an amino acid change at residue 476 (Ile → Ser). At this residue, the sequence of pKK2B12 agreed with that obtained by sequencing RT-PCR products amplified from rat preputial gland RNA.

Reconstituted, recombinant CYP2B12 was active with [14C]arachidonic acid as substrate. Shown in Fig. 3, A, metabolism generated, in a NADPH-dependent manner, a major radioactive fraction eluting at 24 min. This fraction had a reten-
tion time similar to that of authentic 11,12-EET; radioactivity eluting at 34 min represents unmetabolized substrate. Production of the metabolites contained in this fraction was specific for pKK2B12 transformants because no metabolites were observed with membranes isolated from untransformed E. coli cells (Fig. 3B). CYP2B12 substrate specificity was also demonstrated by its lack of catalytic activity toward the short chain fatty acid laurate (C12:0) (not shown). Metabolites eluting at 24 min (Fig. 3A) were pooled and resolved by normal phase HPLC into two radioactive fractions having the same retention times as authentic 11,12- and 8,9-EET (Fig. 4). These two EETs were the sole reaction products observed, with 11,12- and 8,9-EET accounting for 80 and 20% of total metabolites, respectively. Table II shows the chiral analysis of these products. Both EETs were produced in an enantioselective fashion. The predominant enantiomers generated by CYP2B12 were 11(S),12(R)- and 8(R),9(S)-EET. The stereochemistry of 11,12-EET in Table II is similar to that found endogenously in rat liver (14), further evidence that the function of CYP2B12 is metabolism of arachidonic acid.

Structures of the 11,12- and 8,9-EET metabolites were confirmed by NICI/GC/MS. A mass spectral fragmentation pattern for the 11,12-EET-PFB is shown (Fig. 5). Major negative ions were as follows: m/z 319 (loss of the PFB from the molecule), m/z 321 (loss the PFB from the isotopically labeled molecule), and m/z 301 and 303 (loss of PFB, H2O, and oxygen). Similar results were obtained for the purified 8,9-EET produced by CYP2B12 (not shown). The fragmentation patterns are consistent with those of authentic EETs (11). These data demonstrate for the first time that arachidonic acid is a substrate for CYP2B12 and that the regiospecific metabolism by CYP2B12 is unlike most arachidone (CYP) epoxygenases, which typically produce several EET regioisomers as major products (14).

Production of Endogenous EET in Sebaceous Tissue—Endogenous EETs were measured in freshly isolated rat preputial and clitoral glands using a quantitative mass spectral assay (11). From 5.7 g of sebaceous tissue, 202 ng of EET were recovered; 49% (98 ng) was 14,15-EET, 25% (51 ng) was 11,12-EET, and 26% (52 ng) was 8,9-EET. These results indicate the presence of another epoxygenase(s) because recombinant CYP2B12 produced 11,12-EET nearly exclusively. Although the fraction of EETs generated by CYP2B12 in sebaceous tissue cannot be determined, one possibility is that CYP2B12 contributes a major fraction of 11,12-EET. However, another epoxygenase(s) must be responsible for generating a major fraction of total EETs.

The identity of other CYP epoxygenases in rat preputial/clitoral glands was explored using RT-PCR and DNA sequencing, whereas the relative abundance of specific transcripts was evaluated by Northern blot analysis or in situ hybridization. CYP1A and CYP3A subfamily-specific oligonucleotides yielded no PCR product. CYP2C subfamily-specific oligonucleotides yielded very faint bands of predicted size, negligible compared with the large amount of product from CYP2B subfamily-specific primers (not shown). CYP2C PCR products were not sequenced because of their low abundance. Sequencing of CYP2B PCR products indicated the presence of CYP2B1/2B2 mRNAs, but the levels in rat skin were too low to be detected by in situ hybridization. In situ hybridization is more sensitive than Northern analysis. In addition to CYP2B12, which is a major transcript (Fig. 1), and low levels of CYP2B1/2B2, CYP2B15 (15) was the third transcript detected in rat sebaceous RNA. CYP2B15 is a major transcript (Fig. 6) that appears to be expressed at levels comparable to CYP2B12, in the same tis-

![Fig. 4. Resolution of EET regioisomers generated by recombinant CYP2B12. The metabolites eluting at 24 min in Fig. 3A were resolved by normal phase HPLC into two EET regioisomers that eluted with retention times identical to authentic 11,12- and 8,9-EET standards.](image)

![Fig. 5. Mass spectral fragmentation pattern for 11,12-EET generated by recombinant CYP2B12. For product structural analysis, CYP2B12 was reconstituted in the presence of NADPH and 70 μM [1-14C]arachidonic acid (0.2–0.4 μCi/μmol). Purified 11,12-EET-PFB was analyzed by NICI/GC/MS. The major anion m/z 319 is diagnostic for loss of PFB from the molecule; that at m/z 321 is derived by loss of PFB from the isotopically labeled molecule.](image)

**TABLE II**

| Regioisomer | Total EET metabolites | Enantioselectivity |
|-------------|-----------------------|-------------------|
|             |                       | R, S              |
|             |                       | S, R              |
| 14,15-EET   | ND                    |                   |
| 11,12-EET   | 80                    | 30                |
| 8,9-EET     | 20                    | 61                |
| 5,6-EET     | ND                    |                   |

* ND, not detected.*
A role in some aspect of cutaneous lipid metabolism seemed plausible because an important function of differentiated keratinocytes in the epidermis and sebaceous glands is the biosynthesis of specialized lipids (13). Cutaneous lipids are essential to prevent excessive transepithelial water loss and are important for thermoregulation in response to stress (13, 18, 19).

The successful expression of CYP2B12 in E. coli allowed the identification of the first known substrate for this enzyme. Recombinant CYP2B12 catalyzes NADPH-dependent epoxidation of arachidonic acid, an important precursor of many lipid mediators (14). Other CYP2B arachidonic acid epoxygenases are known: rabbit lung CYP2B4 (20), a putative CYP2B4 orthologue in guinea pig lung (21), and rat liver CYP2B1 and CYP2B2 (22). Unlike CYP2B12, these CYP2B epoxygenases are expressed in multiple tissues, and their activities are less regioselective. CYP2B12 produces a single major EET regiosomer, mainly 11,12-EET, from arachidonic acid. This degree of regioselectivity during arachidonic acid epoxidation is quite

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unusual (23) (Table III). An exception is rabbit lung CYP2B4, which generates 5,6-EET as the predominant EET (20). Another distinction is that CYP2B12 catalyzes only epoxidation of arachidonic acid, whereas CYP2B4 (20), CYP2E1 (24), CYP2J3 (16), and CYP2J4 (17), for example, generate ω-6 or ω-1 alcohols or other eicosanoids as major products. Importantly, lauric acid is not a CYP2B12 substrate.

The catalysis of eicosanoid oxidation by CYP2B12 suggests that this enzyme may participate in signal transduction mechanisms in keratinocytes. Although CYP2B12 exhibits a relatively high level of constitutive expression, EET generation may be regulated by cellular phospholipases and arachidonic acid release (14, 23). Another source of EETs might include EETs esterified into cellular glycerophospholipids (25, 26). Though capsaicin (14, 23, 27). In epidermis, EETs or their hydration products, dihydroxyeicosatrienoic acids, involve changes in membrane permeability to Ca$^{2+}$, Na$^+$, K$^+$, and H$^+$, as well as stimulation of peptide hormone secretion and vasomodulation (14, 23, 27). In epidermal keratinocyte cultures, increases in extracellular Ca$^{2+}$ concentrations lead to increases in intracellular Ca$^{2+}$ concentration and result in keratinocyte differentiation (28, 29). The correlation also exists in mouse epidermis in situ. The greatest concentrations of inter- and intracellular Ca$^{2+}$ localize cyto-chemically to the upper epidermal cell layer (30), the stratum granulosum, which contains the most terminally differentiated, nucleated keratinocytes. By analogy, sebocytes are the most terminally differentiated keratinocytes in sebaceous glands. We hypothesize that the CYP2B12-dependent production of 11,12-EET may be involved in establishing the differentiated phenotype or maintaining a differentiated function of keratinocytes in sebaceous tissues.

Acknowledgments—We thank Drs. Yasuna Kobayashi and James Halpert for assistance in expression and purification of CYP2B12 and Dr. Jorge Capdevila for assistance in arachidonic acid metabolism studies and critical interpretation of the data. Support and technical assistance from the Skin Diseases Research Center at Vanderbilt are greatly appreciated, especially the contributions of Drs. Lillian Nanney and Lloyd E. King, Jr., in data interpretation and consultations in cutaneous biology.

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