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Identification of CYP2A3 as a Major Cytochrome P450 Enzyme in the Female Peripubertal Rat Breast

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ABSTRACT

On isolation of rat breast cytochrome P450, one of the proteins whose amino terminus was sequenced was CYP2A3. CYP2A3 was detected by Western blotting in cytochrome P450 fractions isolated from breast of 3-, 6-, and 9-week-old rats but was low during pregnancy and lactation. Reverse transcription-polymerase chain reaction analysis and sequencing of the PCR product confirmed the presence and identity of CYP2A3 in the rat breast. Breast microsomal coumarin-7-hydroxylase activity paralleled the developmental pattern observed for CYP2A3 on Western blots. In the lung, coumarin-7-hydroxylase activity was 10-fold higher than that in the breast, but the developmental pattern was similar to that in the breast. Lung microsomes from 9-week-old rats activated the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline to mutagenic metabolites which could be detected with the Ames test. This activation could be inhibited by the CYP2A3 antiserum. With breast microsomes, which contain ≈10% of the cytochrome P450 in the lung, activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline could not be reliably measured. Immunohistochemical localization revealed that CYP2A3 was expressed in a limited number of epithelial cells in the ducts of 6-week-old rat breast. Double staining with smooth muscle actin, a marker for myoepithelial cells, showed no staining of CYP2A3 immunoreactive cells, indicating that these cells were not myoepithelial. The data clearly show that a cytochrome P450 that can activate environmental procarcinogens is developmentally regulated and concentrated in specific cells in the breast. The peripubertal period seems to be a window in time when the breast may be more sensitive to procarcinogens that are substrates for CYP2A3.

Exposure to environmental carcinogens early in life is thought to be one of the first events in the development of breast cancer (Russo *et al*., 1990; Wolff *et al*., 1996). Exposure to hormones and growth factors plays an important role in the subsequent promotion and progression of carcinogenesis (Dickson and Lippman, 1995; Helzlsouer and Couzi, 1995; Feigelson and Henderson, 1996). Because there is an abundance of carcinogens in the environment, the susceptibility to chemical carcinogenesis must be related to the capacity of the individual cell to protect itself. One of the major enzyme systems in the body that influences chemical carcinogenesis is the cytochrome P450 superfamily of enzymes (Nelson *et al*., 1996). Individual members of this superfamily can reduce the risk of chemical carcinogenesis by inactivating procarcinogens and thereby facilitating their excretion from the body. Enzymes in this family also are known to enhance chemical carcinogenesis by converting procarcinogens to ultimate carcinogens (Sipes and Gandolfi, 1986). These carcinogens can bind to DNA and cause errors in DNA replication when the cells divide. Cells can correct most of these errors, so a mutation occurs only when the system is overloaded through exposure to carcinogen, an abundance of cytochrome P450 enzymes that produce the ultimate carcinogen, a lack of cytochrome P450 enzymes that can eliminate the procarcinogen from the cell, or defective repair systems. Activation of chemical carcinogens involved in initiation of breast cancer may occur in tissues like the liver, in which cytochrome P450 is abundant. However, many ultimate carcinogens are unstable and short lived, and it is likely that they are produced within the breast itself. Several studies have revealed that the breast can form reactive metabolites from mammary carcinogens, such as the food mutagen IQ and the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene. The formation of these metabolites can be detected as covalent binding to DNA (Tay and Russo, 1981; Singletary and Mil-

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ABBREVIATIONS: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; BSA, bovine serum albumin; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; RT, reverse transcription; SSC, standard saline citrate; PBS, phosphate-buffered saline; AB, avidin-biotin.

ner, 1986; Moore, 1988; Pfau *et al.*, 1992; Carmichael *et al.*, 1996). To delineate the role of breast cytochrome P450 in breast carcinogenesis, the cytochrome P450 profile of the breast must be understood. The breast does contain multiple forms of cytochrome P450, which are regulated as a function of age and endocrine status.

We have shown that most of the cytochrome P450 in pregnant, lactating, and postlactation rats could be accounted for by CYP2E1, CYP1A1, CYP4A, and CYP3A (Hellmold *et al.*, 1995). In rats <3 weeks old, the predominating forms were CYP3A and CYP2D4. The major forms in the 6–9-week-old rats were not identified. The focus of this study was identification of cytochrome P450 enzymes in the breast of rats around the time of puberty.

Materials and Methods

Chemicals. *Thermus aquaticus* (*Taq*) polymerase, avian myeloblastosis virus reverse transcriptase, RNasin, oligo(dT), and deoxynucleotide triphosphates were purchased from Promega (Madison, WI). HRP-conjugated streptavidin was obtained from Chemicon (Stockholm, Sweden). Enhanced chemiluminescence detection kit was purchased from Amersham (Buckinghamshire, England). Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel, Germany). Coumarin was obtained from ICN Pharmaceuticals (Costa Mesa, CA). 7-Hydroxycoumarin was obtained from EGA Chemie (Steinheim, Germany). Activated Sepharose was obtained from Pharmacia Biotech (Stockholm, Sweden). Molecular mass standards in the range of 14–97 kDa were obtained from Promega. A 123-base-pair DNA ladder was obtained from Life Technologies (Täby, Sweden). QIAquick PCR purification kit was obtained from Qiagen (Santa Clarita, CA). All other chemicals were of analytical grade and obtained from Kebo Lab AB (Stockholm, Sweden), Sigma Chemical (St. Louis, MO), or Merck AG (Darmstadt, Germany).

Animals and tissues. Sprague-Dawley rats and DBA mice were obtained from B&K Universal AB (Sollentuna, Sweden). Lactating rats were used 2–3 weeks postpartum. The animals were allowed to acclimatize for a few days after arrival and were kept on hardwood bedding under standardized conditions of light (6:00 a.m to 8:00 p.m.), temperature (21 ± 1°), and humidity. Food and water were available *ad libitum*. The animals were killed by decapitation after light carbon dioxide anesthesia. Human liver samples taken during cholecystectomies were obtained from the Department of Surgery, Huddinge Hospital.

Preparation of microsomes or total membrane fractions. For microsomal and total membrane preparations, breast tissue from 10–30 rats was pooled to obtain sufficient material. Three independent pools were analyzed for each time point. The abdominal mammary glands were excised and immersed in cold 0.25 M sucrose. The tissue was weighed and transferred to homogenization buffer composed of 100 mM Tris·HCl, pH 7.4, 20% glycerol, 150 mM KCl, 0.2 μM dithiothreitol, and 1 mM EDTA. The tissue was minced with scissors and subsequently homogenized with a Polytron homogenizer (PT 3000; Kinematica, Lucerne, Switzerland). The homogenate was filtered through a piece of gauze. Phenylmethylsulfonyl fluoride (0.2 mM) was added before homogenization. The total membrane fractions were obtained through direct centrifugation of the homogenate at 105,000 × *g* for 1 hr. To prepare microsomes, the homogenate first was centrifuged at 9,000 × *g* for 30 min, and the microsomes were isolated from the supernatant through further centrifugation at 105,000 × *g* for 1 hr. The microsomes were resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol. Liver microsomes were prepared by the same procedure. All centrifugations were performed at 4°. The protein content of the microsomes was determined according to Lowry *et al.* (1951) using BSA as a standard.

Purification of cytochrome P450 by hydrophobic chromatography. Cytochrome P450 fractions were prepared as described previously (Hellmold *et al.*, 1995). Three independent preparations were analyzed for each time point.

Protein sequencing of CYP2A3. Partially purified breast cytochrome P450 was precipitated and loaded onto SDS gels with 10 pmol cytochrome P450/lane. After electrophoresis, proteins were blotted onto polyvinylidene difluoride membrane filter (Perkin-Elmer, Stockholm, Sweden). Protein bands in the molecular mass range of 60–45 kDa were excised from the membrane, and their amino termini were sequenced as described previously (Warner and Gustafsson, 1994) with an Applied Biosystems 473 A protein sequencer (Perkin-Elmer, Stockholm, Sweden).

CYP2A3 antibody. Rabbits were immunized with a BSA-coupled peptide unique for CYP2A3 (Neosystem Laboratoire, Strasbourg, France). The peptide sequence KNPNTEFYMKNC, encompassing amino acids 281–291 in CYP2A3, was checked for cross-reactivity with other proteins in the Swiss and PIR protein databases. The corresponding sequence of CYP2A6, CYP2A1, and CYP2A2 differed by 1, 3, and 5 amino acids, respectively, from the CYP2A3 peptide sequence. The antibody was affinity-purified on a column of antigen peptide coupled to activated Sepharose. The antibody was eluted with 0.1 M glycine, pH 2.5, neutralized with Tris·HCl, pH 8.8, and subsequently used for Western blot analysis. Specificity of the antibody was checked on the basis of adsorption of the antibody on a column of CYP2A6 containing microsomes (Genetest, Woburn, MA) coupled to activated Sepharose. The adsorbed antibody was eluted with 0.1 M glycine, pH 2.5, neutralized with Tris·HCl, pH 8.8, and subsequently used for Western blot analysis.

Western blotting analysis. Proteins were precipitated with chloroform/methanol and separated by gel electrophoresis according to Laemmli (1970) with a 9% separating gel. The proteins were transferred to a nitrocellulose membrane (Towbin *et al.*, 1979). Per well, 5 pmol of cytochrome P450 for breast and 10 μg of protein for liver and lung microsomes were loaded. The filter was blocked in buffer consisting of 20 mM Tris·HCl, pH 7.5, and 150 mM NaCl, containing 0.2% Nonidet P-40 and 10% fat-free milk; rinsed in the buffer; and incubated with primary antibodies and a secondary anti-rabbit IgG coupled to HRP (BioRad, Solna, Sweden). The protein/antibody complex was visualized with the use of enhanced chemiluminescence. Monoclonal anti-rat CYP2B1 antibody was obtained from Oxford Biomedical Research (Oxford, MI). Polyclonal anti-rat CYP2E1 antibody was obtained from Oxygene (Dallas, TX) (Johansson *et al.*, 1988). Microsomes from lymphoblastoid cell lines expressing human CYP2A6 and rat CYP2A1 were obtained from Genetest.

Mutagenicity test. The Ames *Salmonella typhimurium* mutagenicity test was performed with a 30-min preincubation at 37° before plating (Maron and Ames, 1983). *S. typhimurium* TA98 was kindly provided by Dr. B. Ames (Berkeley, CA). A range of 0–2 mg of microsomal protein/plate was used to determine the optimal protein concentration for activation. Briefly, a standard incubation mixture contained 0.5 mg of microsomal protein, 4 μg of IQ dissolved in water, 0.1 M sodium phosphate buffer, pH 7.4, 16 mM glucose-6-phosphate, 4 units/ml glucose-6-phosphate dehydrogenase, 1.6 mM NADP, and 1–2 × 10^8 bacterial cells in a volume of 100 μl. The total incubation volume was 500 μl. Inhibition studies were performed on the mutagenic activation of IQ by lung microsomes using the CYP2A3 antiserum. Microsomes were incubated with antiserum for 20 min on ice before being added to the Ames incubation mixture. As negative controls, the CYP2A3 antiserum was preincubated with 1 mg/ml BSA-coupled CYP2A3 peptide before being added to the microsomes; in addition, microsomes were incubated with preimmune serum for 20 min on ice. Liver microsomes from BNF induced rats were included as a positive control in each experiment. All measurements were performed at least twice on duplicate plates. The number of spontaneous revertants was ≈25/plate.

Oligonucleotide primers and probes. Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer

(Perkin-Elmer), and the internal probe was end-labeled with biotin and purified by high performance liquid chromatography (CyberGene AB, Huddinge, Sweden). Table 1 shows the primers and probes used for PCR and Southern blot analysis. Conserved oligonucleotide primers were designed for CYP2A1, CYP2A2, and CYP2A3. Specific oligonucleotide primers and probe were designed for β-actin, and a specific biotinylated probe was designed for CYP2A3 that corresponded to a portion of the peptide sequence used for antibody production. The specificity of the primers and probes was checked for sequence similarity in the EMBL+Genbank databases. The probe for CYP2A3 was 65% and 53% homologous to CYP2A1 and CYP2A2, respectively.

RT-PCR analysis. Total RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform single-step method (Chomczynski and Sacchi, 1987). For the RT reaction, 5 μg of RNA was denatured at 94° for 2 min. The RNA was added to PCR buffer consisting of 10 mM Tris·HCl, pH 9.0, 50 mM KCl, 7.5 mM $MgCl_2$, and 0.1% Triton X-100 together with 1.0 mM concentration of each deoxynucleotide triphosphate, 2.2 μM oligo(dT), 50 units of RNasin, and 20 units of avian myeloblastosis virus reverse transcriptase in a final volume of 50 μl. The reaction was allowed to proceed for 1 hr at 42°, after which the enzyme was inactivated at 95° for 10 min. The reverse-transcribed RNA was stored at −20°. Two negative controls were included: one without the RNA template and one without the reverse transcriptase.

The PCR was run on a Gene Amp PCR system 2400 (Perkin-Elmer). The reaction consisted of 5 μl of cDNA, PCR buffer, 0.2 mM concentrations of each deoxynucleotide triphosphate, 1 μM concentration of the CYP2A primer pair or 0.12 μM concentration of the primer pair amplifying the β-actin gene, and 1 unit of *Taq* polymerase in a total volume of 100 μl. The conditions used for CYP2A amplification were 30 sec at 94°, 30 sec at 50°, and 1 min at 72° for 60 cycles. For β-actin, 30 cycles were used. The following negative controls were included: without reverse transcriptase in the RT reaction, without RNA in the RT reaction, and PCR mixture without cDNA in the PCR. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. For the breast and the negative controls, the total PCR was precipitated with ethanol and dissolved in 10 μl of water. The PCR products were stored at −20°.

Southern blot analysis of CYP2A3. The PCR products obtained from one PCR of 6-week-old rat lung and three pooled PCRs of 6-week-old breast and all negative controls were purified using QIAquick PCR purification kit. The DNA was eluted in a final volume of 50 μl. Ten microliters of the concentrated PCR products from rat lung and breast was loaded onto a 1.5% agarose gel. The DNA was denatured and then neutralized by soaking the gel first in 0.5 M NaOH containing 1.5 M NaCl for 45 min and then in 1 M Tris·HCl, pH 7.4, containing 1.5 M NaCl for 45 min. The DNA was transferred to nitrocellullose according to the capillary transfer method using SSC transfer buffer (20× SSC containing 150 mM NaCl and 15 mM sodium citrate). The membrane was baked for 2 hr at 80° and prehybridized in 6× SSC, 1% SDS, 1 mg/ml Ficoll, 1 mg/ml polyvinyl pyrrolidone, 1 mg/ml BSA, and 5 μM EDTA for 6 hr at 65°. Hybridization was carried out under the same conditions with 100 ng/ml biotinylated probe for 16 hr at 42°. This was followed by washing for 1–2 hr at 42° three times for 30 min in 6× SSC. Membranes hybridized with biotinylated probes were incubated 1 hr at 37° in PBS (containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4$, 1.4 mM KH_2PO_4, 0.2% Nonidet P-40, 3% BSA). The membranes were incubated for 1 hr at 37° in a solution of 1.0 μg/ml HRP-conjugated streptavidin in PBS containing 1.5% BSA and 0.2% Nonidet P-40. Unbound HRP-conjugated streptavidin was removed by washing three times for 30 min with fresh PBS containing 0.2% Nonidet P-40. The DNA/HRP-conjugated streptavidin complex was visualized with enhanced chemiluminescence.

DNA sequencing of PCR product. The RT-PCR products obtained from one PCR of 6-week-old rat lung cDNA and three pooled PCRs of 6-week-old breast and liver cDNA were purified using QIAquick PCR purification kit. The DNA was eluted in a final volume of 50 μl. Approximately 50 ng of DNA was used for cycle sequencing with TaqFS dye terminator premix (Perkin-Elmer, Stockholm). The sequence was analyzed on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer) (sequencing performed at CyberGene AB, Huddinge, Sweden).

Coumarin-7-hydroxylation. Coumarin-7-hydroxylation was measured essentially as described by Aitio (1978). The reaction mixture contained of 1 mg of microsomal protein, 10 nM coumarin, and an-NADPH generating system (5 mM NADP, 0.6 unit of isocitrate dehydrogenase, 5 mM isocitrate, 15 mM $MgCl_2$) in a total volume of 1 ml of 0.05 mM Tris·HCl buffer, pH 7.5. The incubation was carried out in 1.5 ml Eppendorf tubes. Incubations were started after a 3-min preincubation at 37° by the addition of the NADPH-generating system. After 20 min, the reactions were quenched by the addition of 25 μl of perchloric acid, and the protein was removed through centrifugation at 1500 × *g*. The supernatant was extracted with 3 ml of chloroform through vortexing for 30 sec. The layers were separated by centrifugation at 1500 × *g* for 10 min. Then, 2.5 ml of 30 mM sodium borate, pH 9.3, was added to the organic phase, and the tube was vortexed before centrifugation for 5 min at 1 500 × *g*. The concentration of 7-hydroxycoumarin was measured fluorometrically using a Shimadzu RF 510 (Shimadzu Europe, Duisberg, Germany) spectrofluorometer set at an excitation wavelength of 390 nm and an emission wavelength of 440 nm. Calculated values given in the figures are the mean values of three independent experiment with pools of 5–10 rats each.

Immunocytochemistry. Six-week-old virgin and lactating rats were anesthetized deeply with sodium pentobarbital (60 mg/kg IP) and perfused first with physiological saline followed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (0.1 M, pH.7.3). Subsequently, the breast tissue was dissected and further fixed by immersion in the same fixative for 1 hr. After being rinsed with PBS, pH 7.4, the tissues were cryoprotected with 15% sucrose in PBS for 16 hr. The tissues were frozen with liquid carbon dioxide and sectioned with a Microm 500 HM cryostat at 10 μm. Sections were thawed on chrome/gelatin-treated glass slides. The AB complex method was used for immunocytochemical demonstration of

TABLE 1
Oligonucleotide primers and probes used for PCR and Southern blot analysis

Target	Sense primer[a]	Antisense primer[a]	Internal probe[a]	Size of PCR fragment	Databank accession no.
				bp	
CYP2A	(1) ACAGTCTCCAATGTCATTAGC (508-528)	CATCTTCATTCGGTCCTCATA (1026-1006)		518	
	(2) GGGCAGCTCTATGAGATGTTC (628-648)			398	
CYP2A3			CCCCAATACTGAGTTCT (702-722)		J02852
β-Actin	CTGGCACCACACCTTCTAC (1585-1603)	GGGCACAGTGTGGGTGAC (2287-2269)	GATCATGTTTGAGACCTTCAA (2157-2177)	238	J00691

[a] Numbers in parentheses denote the location in the mRNA sequence of CYP2A3 and in the gene of β-actin, respectively.

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Band 14                                  PTPGPFIGNYLQLNTEKMYSSL
P450 2A3    SVAFLSVLVLMSVWKQRKLSGKLPPGPTPLPFIGNYLQLNTEKMYSSLMKISQRYG
P450 2A1  VVILASLSVMLLVSLWQQKIRGRLPPGPTPLPFIGNYLQLNTKDVYSSITQLSERYG
P450 2A2  VVILASLSVMFLVSLWQQKIRERLPPGPTPLPFIGNYLQLNMKDVYSSITQLSERYG
          10        20        30        40        50        60
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Fig. 1. Sequence comparison of the amino terminus of protein band 14 in the cytochrome P450 fraction from lactating rat breast with CYP2A1, CYP2A2, and CYP2A3.

CYP2A3. The primary antibody, the affinity purified CYP2A3 antibody, was diluted 1:30–60 in PBS containing 0.3% Triton X-100, and the sections were incubated for 48 hr at 4°. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG and AB complex coupled to HRP for 30 min (Vector Elite Kit; Vector Laboratories, Burlingame, CA). The AB complex was visualized with 0.02% diaminobenzidine and 0.02% hydrogen peroxide. To examine the cell types expressing CYP2A3 immunoreactivity, double staining with an indirect immunofluorescence method was used. The sections were incubated with a mixture of CYP2A3 antibody, diluted 1:10, and mouse monoclonal smooth muscle actin antibody, diluted 1:80 (DAKO, Glostrup, Denmark), for 24–48 hr at 4°. Subsequently, the sections were washed with PBS and incubated with a mixture of rhodamine-conjugated goat anti-rabbit IgG, diluted 1:100 (Boehringer-Mannheim Biochemica, Mannheim, Germany), and fluorescein-conjugated goat anti-mouse IgG, diluted 1:20 (Amersham, Buckinghamshire, UK), for 30 min at 37°. The sections were washed with PBS, embedded in a mixture of glycerol and PBS containing 0.1% paraphenylenediamide, and examined under a Nikon FXA microscope equipped with proper filter combinations for epifluorescence. For control purposes, the sections were incubated with normal rabbit IgG or affinity purified CYP2A3 antibody preadsorbed with the peptide used for immunization at a concentration of 1 μM.

Results

Sequencing of CYP2A3. The proteins in the cytochrome P450 fraction from lactating rats were resolved on SDS gels, and the amino termini of several bands in the range of 50 kDa were sequenced. A sequence comparison of the amino terminal of P450s identified one of the bands around 50 kDa to be highly similar to that of CYP2A3, whereas in the same region, CYP2A1 and CYP2A2 differed by five and six amino acids, respectively. The sequence comparison is shown in Fig. 1. As reported earlier (Warner and Gustafsson, 1994), many cytochrome P450 enzymes undergo some proteolytic digestion, probably during sample preparation, and the proline-rich region amino terminal to the membrane insertion is removed.

Characterization of CYP2A3 antibody. The antibody recognized a single band of ≈50 kDa in rat lung microsomes as shown in Fig. 2. The upper bands around 67 kDa are explained by the coupling of the peptide to BSA, and the intensity of these bands depends on the amount of blood retained in the lungs. In liver microsomes, the antibody recognized bands that comigrated with the bands in the lung and an additional band at ≈60 kDa of unknown identity. The CYP2A3 antibody did not recognize CYP2A1 on Western blots using microsomes containing CYP2A1 at a concentration of ≤2 pmol/lane (data not shown).

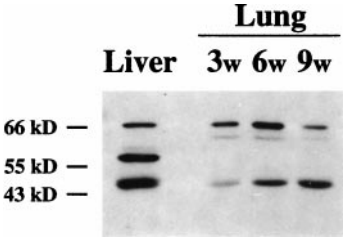

Fig. 2. Western blot analysis of CYP2A3 in rat lung microsomes from 3-, 6-, and 9-week-old (*3w, 6w,* and *9w*, respectively) female rats and in liver microsomes from 9-week-old female rats. Each lane contains 10 μg of microsomal protein.

Fig. 3 shows results from the Ames test, which was used to assess the inhibitory capacity of the antibody on the metabolic activation of IQ in lung microsomes from 9-week-old rats. With the CYP2A3 antiserum, 65% of the metabolic activation of IQ could be inhibited.

The control with preimmune serum also showed an inhibition of 30%. Preincubation of the antiserum with the BSA-coupled CYP2A3 peptide could bring the metabolic activation only up to the level with the preimmune serum. Breast microsomes from 9-week-old rats also were tested in the Ames test. After background subtraction, the activation was <100 revertants/mg of microsomal protein, with 4 μg of IQ. This activity, although clearly over background, was too low to permit inhibition studies.

Western blot analysis. Fig. 4 shows Western blots of lung microsomes from 3-, 6-, and 9-week-old rats with the addition of a monoclonal rat P4502B1 antibody and a polyclonal rat CYP2E1 antibody. There were intense signals of CYP2A3 and CYP2B1 in 6- and 9-week-old rat lung, whereas lower intensities were observed in 3-week-old rat lung and similar levels of CYP2E1 were detected in the lung from 3-, 6-, and 9-week-old rats.

Fig. 5 shows Western blot analysis of CYP2A3 in fractions

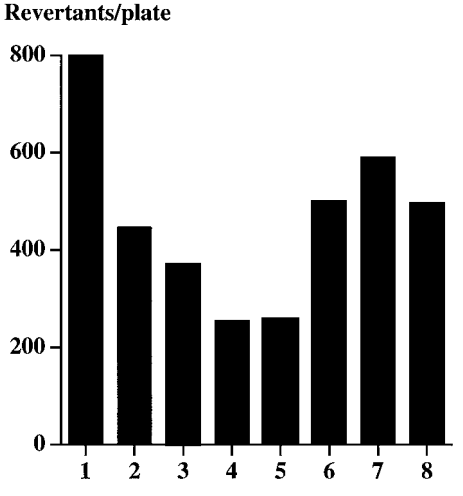

Fig. 3. Ames mutagenicity test. Inhibition of the metabolic activation of IQ (4 μg/plate) by rat lung microsomes (0.5 mg/plate) from 9-week-old rats with the CYP2A3 peptide antiserum. *1*, Initial value. *2–5*, Antiserum (5, 10, 20, and 40 μl). *6 and 7*, Negative controls, CYP2A3 antiserum preincubated with 20 and 40 μl of BSA-coupled CYP2A3 peptide (1 μg/μl) before addition to the microsomes. *8*, Negative control, microsomes preincubated with 20 μl of preimmune serum.

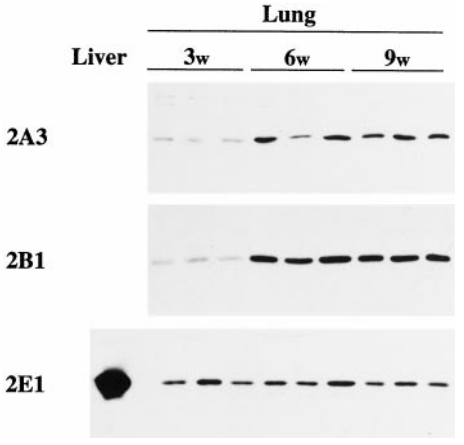

Fig. 4. Western blot analysis of lung microsomes from 3-, 6-, and 9-week-old (*3w, 6w,* and *9w*, respectively) female rats using antibodies against CYP2A3, CYP2B1, and CYP2E1. In the case of CYP2E1, rat liver microsomes from 9-week-old female rats were used as a positive control. *Lanes*, 10 μg of microsomal protein.

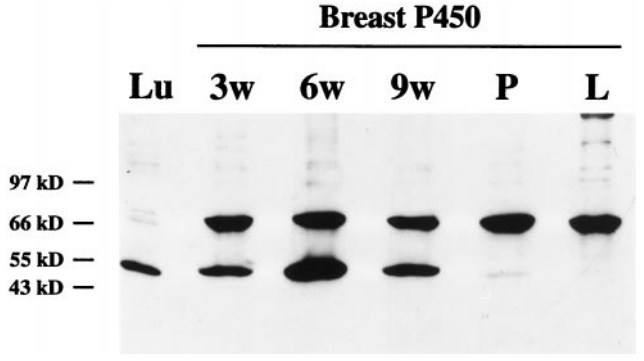

Fig. 5. Western blot analysis of CYP2A3 in breast cytochrome P450 fractions from 3-, 6-, and 9- week-old (*3w, 6w,* and *9w*, respectively) pregnant (*P*) and lactating (*L*) rats. Breast cytochrome P450 (5 pmol) was loaded in each lane. Lung microsomes (*Lu*) from 9-week-old rats (10 μg of microsomal protein) were used as positive control. Representative of experiments performed in triplicate with different cytochrome P450 preparations.

of partially purified breast P450 from 3-, 6-, and 9-week-old pregnant and lactating rats, with 5 pmol of cytochrome P450 loaded in each lane. A band comigrating with the band in lung microsomes was visible in 3-, 6-, and 9-week-old and pregnant rat breast, with the strongest signal in 6-week-old breast. The upper 67-kDa band is caused by albumin. The absence of the albumin band in the lung lane is due to the small amount of loaded lung microsomes.

The yield of cytochrome P450 per gram of tissue is approximately two to three times higher from lactating rat breast than that from 6-week-old rat breast (Hellmold *et al*., 1995). In Fig. 6, 15 pmol of cytochrome P450 from lactating breast was loaded to detect CYP2A3 in the lactating breast.

The specificity of the antibody was checked further by adsorption with CYP2A6-containing microsomes coupled to activated Sepharose. A Western blot using the antibody eluted from this CYP2A6 column is shown in Fig. 7. The eluted antibody recognized bands in lung microsomes, CYP2A6 microsomes, and the cytochrome P450 fraction from 6-week-old rat breast to the same extent as before adsorption on the CYP2A6 column. These results verify that the protein band in the breast contains the CYP2A3 peptide.

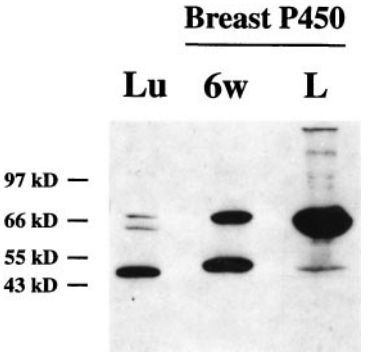

Fig. 6. Western blot analysis of CYP2A3 in breast cytochrome P450 fractions from 6-week-old (*6w*) (5 pmol) and lactating (*L*) (15 pmol) rats. Lung microsomes (*Lu*) from 9-week-old rats (10 μg of microsomal protein) were used as positive control.

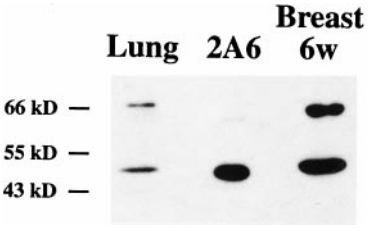

Fig. 7. Western blot analysis of CYP2A3 in lung microsomes from 6-week-old rats (10 μg of microsomal protein), CYP2A6-containing microsomes (0.5 pmol), and a cytochrome P450 fraction from 6-week-old (*6w*) rat breast (5 pmol) after adsorption of the antibody on a CYP2A6 column as described in the text.

RT-PCR analysis. The RT-PCR analysis was performed to confirm the presence and identity of CYP2A3 in the 6-week-old rat breast. Conserved primers for CYP2A1, CYP2A2, and CYP2A3 were used, and intense signals were observed in the liver and lung on ethidium bromide-stained agarose gels, whereas no band was visible in the breast (Fig. 8A). On Southern blot analysis using a biotinylated probe directed to the corresponding peptide sequence used for antibody production, a band of the correct size was detected in the lung and breast from 6-week-old rats (Fig. 8B), but no band was detectable in the liver. On sequencing of the PCR products, CYP2A3 was identified in the lung and the breast, whereas the PCR product in the liver was found to be CYP2A1 (data not shown).

Coumarin-7-hydroxylation. CYP2A3-specific 7-hydroxylation of coumarin was measured to determine whether signals detected on Western blots are indicative of functionally active protein (Yamano *et al*., 1990; Salonpää *et al*., 1993). The formation of 7-hydroxycoumarin was linear with time up to 20 min and with protein concentration up to 1 mg when mouse and human liver microsomes were used (data not shown). Fig. 9 shows coumarin-7-hydroxylation measured in lung and breast microsomes. The data correlate well with the Western blots results in Figs. 2 and 4. In the lung from 3-week-old rats, the coumarin-7-hydroxylation was 28 $\pm$ 6 pmol/mg/min. The levels increased in 6- and 9-week-old rats to 46 $\pm$ 12 and 77 $\pm$ 7 pmol/mg/min, respectively. In the breast, the highest levels, 7 $\pm$ 2 pmol/mg/min, were found in 6-week-old rats, and low levels, $<$1 pmol/mg/min, were found in 3- and 12-week-old pregnant and lactating rats.

Immunohistochemistry. In 6-week-old breast, CYP2A3-immunoreactive cells could be seen in single cells in the

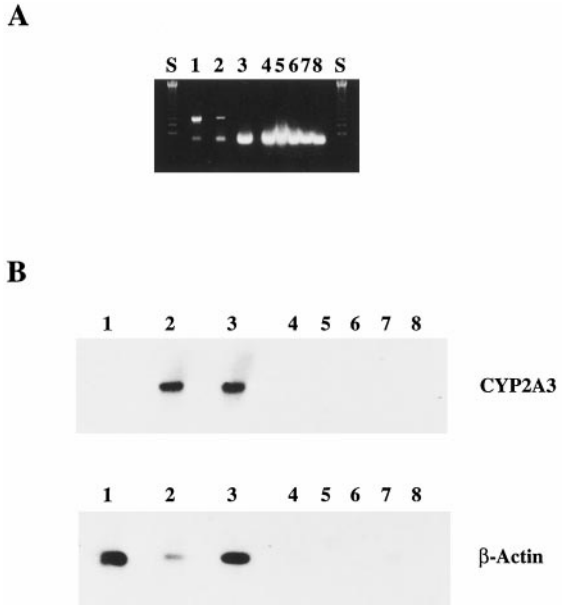

Fig. 8. RT-PCR analysis of CYP2A3 in liver, lung, and breast from 6-week-old rats. A, Ethidium bromide-stained agarose gel. *Lane 1*, liver, 10% of the PCR. *Lane 2*, lung, 10% of the PCR. *Lane 3*, breast, 100% of the PCR. *Lanes 4–8*, negative controls, 100% of the PCR. *Lanes 4–6*, without reverse transcriptase liver, lung, and breast. *Lane 7*, without RNA. *Lane 8*, without cDNA. B, Southern blot analysis of CYP2A3 as described in the text. *Lane 1*, liver. *Lane 2*, lung. *Lane 3*, breast. *Lanes 4–8*, negative controls.

epithelium of the ducts, as shown in Fig. 10a. The number of labeled cells varied among cross sections. Some of the CYP2A3-labeled cells were cuboidal, resembling epithelial cells, but most of them were stellate, sending short processes among neighboring cells. The labeled cells often were situated in the middle or basal portion of the epithelium.

Double staining of sections with antibodies for CYP2A3 and actin, a marker for myoepithelial cells, showed that CYP2A3-immunoreactive cells did not stain with the actin antibody, whereas staining of myoepithelial cells was clearly seen (Fig. 10, b and c).

The affinity purified CYP2A3-antibody gave weak nonspecific staining in most epithelial cells, some fibroblasts, and fat cells, but this staining could not be abolished by preadsorption of the antibody with the antigen. Mast cells and erythrocytes exhibited strong nonspecific labeling due to high levels of peroxidases in these cells.

Discussion

A specific enzyme, CYP2A3, which catalyzes the metabolic activation of food mutagens, was shown to be expressed in the breast of rats between 6 and 9 weeks of age. To study the regulation of expression of this enzyme and identify the cells in which it is localized, a specific CYP2A3 peptide antibody was raised in rabbits.

CYP2A3 was first identified in rat lung (Kimura *et al.*, 1989) and shown recently to be expressed in rat olfactory mucosa (Béréziat *et al.*, 1995), but this enzyme has not been detected in the liver. Microsomes from both lung and olfactory mucosa catalyze the 7-hydroxlyation of coumarin (Honkakoski *et al.*, 1993; Béréziat *et al.*, 1995). Surprisingly, the Baculovirus-expressed CYP2A3 exhibited low activity toward coumarin (Liu *et al.*, 1996). CYP2A3 and CYP2A6

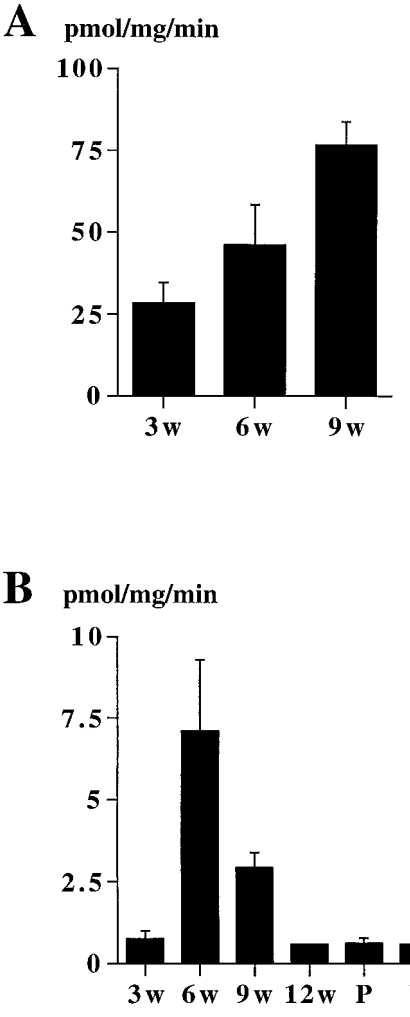

Fig. 9. Coumarin-7-hydroxylation measured in lung (A) and breast (B) microsomes as described in the text [3-, 6-, and 9-week-old (*3w, 6w, and 9w*, respectively) pregnant (*P*) and lactating (*L*) rats]. Standard deviations are presented on measurements of three independent preparations. Two preparations were made of 12-week-old (*12w*) rat breast microsomes.

have been shown to metabolize a number of toxic compounds and procarcinogens, such as nitrosamines, aflatoxin B1, and heterocyclic amines (Salonpää *et al.*, 1993; Fernandez-Salguero and Gonzalez, 1995; Liu *et al.*, 1996). Furthermore, a member of the CYP2A subfamily has been shown previously to be involved in the activation of the food mutagen IQ in the rat lung (Hellmold *et al.*, 1993).

The CYP2A3 peptide antibody recognized a single band of 50 kDa in the rat lung, the human homolog CYP2A6, and several bands of unknown identity in the rat liver. The antibody did not recognize CYP2A1 in microsomes from a cell line expressing this enzyme, and therefore it is not likely that the 50-kDa band in the liver microsomes is CYP2A1. Based on previous studies that show no expression of CYP2A3 in the rat liver (Kimura *et al.*, 1989) and the results obtained from RT-PCR analysis of 6-week-old female rat liver confirming these data, we conclude that the 50-kDa band recognized by the CYP2A3 antibody is not CYP2A3. It is possible that there exists a as-yet-unknown P450 enzyme in the rat liver that contains the peptide sequence used for immunization, and further studies are necessary to elucidate the identity of this protein.

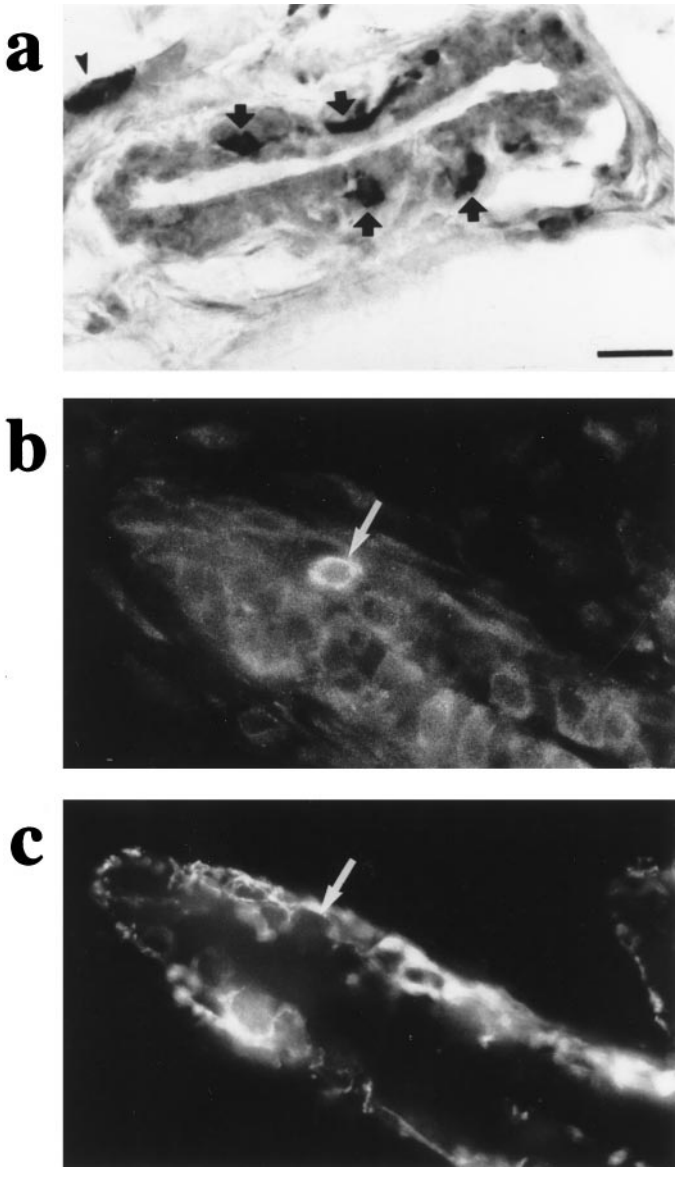

Fig. 10. Immunocytochemical demonstration of CYP2A3 in virgin rat breast (a). *Arrows*, CYP2A3 immunoreactive cells. Mast cells and erythrocytes exhibited strong nonspecific reaction (*arrowheads*) due to high levels of peroxidases in these cells. In sections double-labeled for CYP2A3 (b) and smooth muscle actin (c), CYP2A3 immunoreactive cells (*arrow*) were devoid of actin immunoreactivity. *Bar in a*, 40 μm for a–c.

Through assessment of the inhibitory capacity of the CYP2A3 peptide antibody with the Ames test, it was confirmed that part of the metabolic activation of IQ by rat lung microsomes can be attributed to CYP2A3. When breast microsomes were used in the Ames test, it was impossible to obtain reliable evidence for involvement of CYP2A3 in the formation of mutagens. This is not surprising because in the breast, the cytochrome P450 levels are <10% of those in the lung and the Ames test is not designed to detect low levels of activating enzymes such as those found in the breast. This low expression of cytochrome P450 does not necessarily mean that breast cytochrome P450 is unimportant, particularly if the enzymes are localized in a limited number of cells. In the case of CYP2A3, immunocytochemical studies revealed specific localization in some epithelial cells in virgin rat breast. The staining was prevented by preincubation of the antibody with the CYP2A3 peptide.

There are three major cell types in the breast: epithelial cells, which compose the inner lining of ducts and ductules; smooth muscle-like myoepithelial cells, which form a sometimes discontinuous outer lining associated with the basement membrane; and casein-secretory alveolar cells, which are found in distended ductules and alveoli in pregnant and lactating breast. In addition to these cell types, the existence of a stem cell, which is intermediate in immunocytochemical staining characteristics between epithelial and myoepithelial cells, has been suggested (Böcker *et al.*, 1992; Rudland, 1993). This cell type is believed to be involved in malignant breast disease. Double staining with an antibody to smooth muscle actin, a marker for myoepithelial cells, showed that the CYP2A3 immunoreactive cells were not myoepithelial. The negative staining for actin together with the basal location of the CYP2A3 immunoreactive cells and their stellate nature indicates that these cells may be stem cells. Further immunocytochemical staining with a combination of antibodies is, however, needed to elucidate the possibility that CYP2A3 may be located in stem cells.

On Western blots, CYP2A3 was present at low levels in cytochrome P450 isolated from 3-week-old rat breast. It was highly expressed in the breast of 6-week-old rats and began to decline after 9 weeks of age. In pregnant and lactating rat breast, in which the overall cytochrome P450 and specific cytochrome P450 contents are elevated (Hellmold *et al.*, 1995), weak CYP2A3 signals were observed. The weak signals on Western blots during pregnancy and lactation indicate that CYP2A3 is not one of the cytochrome P450 forms induced during this period. The developmental profile of CYP2A3 in the lung was similar to that in the breast, with peak levels between 6 and 9 weeks of age. In addition, there seems to be a coregulation of CYP2B1 and CYP2A3 in the breast, whereas CYP2E1 remained unchanged.

Coumarin-7-hydroxylation, a catalytic activity characteristic of CYP2A, was 10-fold lower in the breast than in the lung. This value is compatible with the relative cytochrome P450 contents of the respective tissues. Interestingly, CYP2A3 increased in the lung during the peripubertal period, but the highest levels were found at 9 rather than 6 weeks of age. The developmental pattern as assessed by catalytic activity was in accordance with that observed by Western blot analysis. For comparisons between Western blot data and catalytic activity in the breast, it should be noted that the catalytic activity was measured on microsomes and was expressed as activity/mg of microsomal protein. The Western blots were performed with partially purified cytochrome P450 fractions, and the same amount of cytochrome P450 was loaded in each lane in Fig. 5. CYP2A3 was detectable in lactating rat breast cytochrome P450 when a 3-fold higher amount of cytochrome P450 was loaded on the gels, as shown in Fig. 6. The catalytic activity data revealed that there is some CYP2A3 in pregnant and lactating rat breast, and the Western blots show CYP2A3 is not the major enzyme present.

In conclusion, we have shown that in the peripubertal rat breast, CYP2A3 is specifically concentrated in some epithelial cells. Because this enzyme is known to activate procarcinogens, such as the mammary carcinogen IQ, it is likely that these cells are at higher risk. This risk would be highest

in 6–9-week-old rats, in which the level of this enzyme is highest. Further studies are needed to elucidate the identity and features of these cells and delineate the role of CYP2A3 in mammary carcinogenesis.

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