Membrane Topology of Liver Microsomal Cytochrome P450 2B4 Determined via Monoclonal Antibodies Directed to the Halt-Transfer Signal†

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ABSTRACT: The membrane topology of cytochrome P450 2B4 from the endoplasmic reticulum has been studied with highly-purified liver microsomes in a site-directed immunochemical approach. Microsomes were prepared from phenobarbital-induced rabbits, and the resulting microsomal fraction was washed 6 additional times with 0.1 M pyrophosphate buffer to effect removal of significant quantities of adventitiously-bound protein. Monoclonal antibodies were prepared against residues 18–29 of P450 2B4 (Leu[18]-Leu-Phe-Arg-Gly-His-Pro-Lys-Ala-His-Gly-Arg[29]), essentially corresponding to the halt-transfer signal. This region was chosen due to its mutually-exclusive location in the two alternative membrane topology models currently tenable [Black, S.D. (1992) *FASEB J. 6*, 680–685]. Model "A" contains a single transmembrane anchor peptide with the amino terminus projecting into the lumen of the endoplasmic reticulum, while model "B" exhibits a hairpin loop of the first approximately 46 residues inserted into the membrane with the amino terminus located on the cytosolic side of the lipid bilayer; the halt-transfer signal peptide would be located at the cytosolic surface of the membrane in model "A" or as a loop on the lumenal side of the membrane in model "B". Nine antibodies, denoted as MmAbA, MmAbC, MmAbD, MmAbF, MmAbH, MmAbI, MmAbK, MmAbL, and MmAbP, were produced, and all were identified as IgM/κ subtypes. Western blotting demonstrated that the antibodies could readily recognize P450 2B4 in microsomes. ELISA assays showed that all of the antibodies exhibited strong binding to intact microsomes. Similar assays in the presence of detergent (0.5% Tergitol NP-10) or high ionic strength (0.3 M potassium phosphate buffer) revealed no additional binding by any antibody. Thus, the halt-transfer signal is located at the cytosolic side of the microsomal membrane, and no fraction of this peptide segment is found in the lumen. Our results indicate that only membrane topology model "A" is correct. This structure is discussed in light of the topologies of other microsomal proteins.

Cytochrome P450[1] refers to a superfamily of heme proteins having versatile substrate specificity and catalytic capability (Ortiz de Montellano, 1986; Schenkman & Greim, 1993; Waterman & Johnson, 1991). Various isozymes have been described in a wide variety of species, including humans, and metabolic activities are known to be essential in lipid biosynthesis and xenobiotic detoxification. The mammalian P450s are found associated with virtually all cellular membranes, and are most plentiful in the endoplasmic reticulum.

The membrane topology of microsomal cytochrome P450 has been a topic of intense interest as well as controversy. Much has been learned in recent years about the properties of biological membranes and the forces and mechanisms that promote cellular targeting, assembly, and stabilization of membranous proteins (Cramer et al., 1992; Milik & Skolnick, 1992; Popot & Engelman, 1990). The mechanisms of membrane insertion for microsomal proteins in particular are complex, involving a number of ribonucleoprotein and membrane–protein complexes (Rapoport, 1992a,b; Sanders & Schekman, 1992). The membrane topology of mammalian P450s has been studied by a number of investigators employing a variety of experimental approaches, and such results have been reviewed (Black, 1992). Two general topological models, as shown in Figure 1, have emerged as being tenable: model "A" in which residues ~1–20 are embedded in the membrane with the NH_2-terminus located in the lumen of the endoplasmic reticulum and the catalytic heme domain located in the cytosol, or model "B" in which residues ~1–46 are membrane-enmeshed with both amino and carboxyl termini located on the cytosolic side of the membrane. Either model was feasible based on microsomal proteolysis studies (Brown & Black, 1989). Theoretical studies independently supported model "A" (Hartmann et al., 1989) and model "B" (Nelson & Strobel, 1988). Chemical modification of P450s with fluorescein isothiocyanate yielded opposite conclusions (Bernhardt et al., 1988; Vergères et al., 1991). Deletion of the NH_2-terminal signal anchor by molecular biological means has yielded a variety of results showing that the given P450 cytochrome either was still membrane-bound (Cullin, 1992) or was partially membrane-bound (Clark & Waterman, 1991; Hsu et al., 1993; Li & Chiang, 1991; Pernecky et al., 1993; Sanglard

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[1] Abbreviations: BSA, bovine serum albumin; cytochrome P450 2B4, major isozyme in barbiturate-induced rabbit liver microsomes in accordance with proposed systematic nomenclature (Nelson et al., 1993); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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et al., 1993). Insertion of a glycosylation signal at the NH_2-terminus netted glycosylation upon insertion into the endoplasmic reticulum membrane (Szczesna-Skorupa & Kemper, 1993), thereby providing support for model "A". The balance between hydrophobicity and amino-terminal charge was shown to control the insertion characteristics of the signal-anchor sequence (Sakaguchi et al., 1992). Insertion into the microsomal membrane was suggested to proceed through intermediates which resembled both topological models (Monier et al., 1988). Finally, an immunochemical approach (De Lemos-Chiarandini et al., 1987) suggested that only model "A" was tenable; however, the lack of binding by an antibody raised to residues 1–31 and the relatively poor binding by an antibody raised against residues 24–38 call this assignment into question. Overall, more evidence has accumulated in support of model "A".

Because data were available to support both topological models, the possibility existed that some fraction of each might be present in the endoplasmic reticulum. The present studies were designed to decide critically between both alternatives or to quantify the fraction of each model present in microsomal cytochrome P450 2B4. We have made use of the observation that the cationic halt-transfer sequence in P450 2B4 (residues 21–29) had a topologically-opposite orientation in models "A" and "B". That is, in the former model, residues 21–29 are located on the cytoplasmic surface of the membrane, while in the latter, the halt-transfer signal is located as a loop on the lumenal surface of the endoplasmic reticulum. Furthermore, we recognized the importance of studying the membrane topology with the natively-incorporated, native sequence of the cytochrome. Thus, we have raised a panel of site-specific monoclonal antibodies against the halt-transfer sequence and have studied binding to phenobarbital-induced rabbit liver microsomes in the presence and absence of detergent. By this approach, the fraction each of models "A" and "B" could be determined. Results are presented to show that only model "A" is correct. A preliminary report of these findings has been presented (Black & Smith, 1993).

EXPERIMENTAL PROCEDURES

Preparation of Extensively-Washed Microsomes. Microsomes were prepared from male New Zealand White rabbits fed 0.1% sodium phenobarbital, pH 7.0, in the drinking water for 6 days prior to sacrifice, as described (Brown & Black, 1989). The resulting microsomes (50 mL) were sedimented from the storage buffer by ultracentrifugation at 105000*g* for 90 min at 4 °C. The microsomal sediment was resuspended in 50 mL of "wash buffer" (0.1 M sodium pyrophosphate/1 mM EDTA, pH 7.4) with a Teflon–glass homogenizer and resedimented at 105000*g* for 90 min at 4 °C. A small sample ("wash 1") of the supernatant fluid was carefully removed for spectral, protein, and electrophoretic analyses, and then the bulk of the supernate was removed. Suspension in wash buffer, ultracentrifugation, and supernatant fraction removal were repeated, as above, 5 additional times, resulting in wash fractions 2 through 6. Protein concentration was determined by the bicinchoninic acid (BCA) assay (Smith et al., 1985) in the 30-min standard procedure with crystalline bovine serum albumin as standard. SDS–PAGE was performed in 7.5% acrylamide gels (Bio-Rad MiniProteanII apparatus) run with 200-V constant voltage at 25 °C according to the procedure of Laemmli, as modified (Haugen & Coon, 1976). Spectral properties of microsomes and wash fractions were determined with a Kontron 941+ double-beam scanning spectrophotometer at 25 °C; samples were diluted in the appropriate buffer to obtain absorbance readings in the 0–4 range. P450 content was measured in the ferrous carbonyl state after dithionite reduction in the presence of saturating carbon monoxide and 0.1 μM methyl viologen, with use of a molar extinction coefficient of 91 000 M^{-1} cm^{-1} (Omura & Sato, 1964). Final extensively-washed microsomes contained 48.4 μM P450, 15.5 mg/mL protein, and 3.12 nmol of P450/mg of protein.

Synthesis of P450 2B4 Halt-Transfer Signal Peptide L^{18}LFRGHPKAHGR^{29}. The sequence H_3N^+-Leu-Leu-Phe-Arg-Gly-His-Pro-Lys-Ala-His-Gly-Arg-COO^-, corresponding to residues 18–29 of P450 2B4 and the membrane-insertion halt-transfer signal, was synthesized by solid-phase FMOC chemistry with a Milligen-Biosearch Model 9600 synthesizer using standard programs and recommended blocking groups. The product proved to be exceptionally complex, with over 20 peaks seen in the reversed-phase HPLC chromatogram; chromatography was performed as described by Brown and Black (1989). Fractions were collected, and peptides were submitted to PTC-amino acid analysis (Tarr, 1986). Appropriate candidate fractions were submitted to sequencing and fast-atom bombardment (FAB) mass spectral analysis. The peptide which eluted at 28 min on preparative HPLC analysis proved to have the correct sequence and exhibited the expected molecular mass (1389 g/mol). The molar extinction coefficient at 205 nm was determined to be 48 000 M^{-1} cm^{-1}. The final yield of purified L^{18}LFRGHPKAHGR^{29} was about 8% of the crude mixture.

Conjugation of L^{18}LFRGHPKAHGR^{29} to Carrier Proteins. Halt-transfer peptide (2.0 mg) at a concentration of 4 mg/mL in 0.1 M MES, 0.9 M NaCl, and 0.02% NaN_3, pH 4.7, was combined with either bovine serum albumin (2.0 mg) or keyhole limpet hemocyanin (2.0 mg) at concentrations of 10 mg/mL in pure water. For reaction mixtures with BSA, 10 mg of neat EDC was added with mixing; for reaction mixtures with KLH, 0.5 mg of EDC was added (10 mg/mL stock solution in water). Reaction was at ambient temperature for 2 h. Any precipitate formed during reaction was removed by centrifugation. Peptide–carrier conjugates were desalted on cross-linked dextran columns ("Presto" 1 × 5 cm, Pierce Chemical Co.) with a mobile phase of 83 mM sodium phosphate/0.9 M NaCl, pH 7.2. Pooled fractions were stored at −20 °C until use. Protein concentration was determined by BCA assay; the BSA–peptide conjugate and the KLH–peptide conjugate proved to be 0.34 and 0.88 mg/mL protein, respectively.

Preparation of Anti-L^{18}LFRGHPKAHGR^{29} Monoclonal Antibodies. Briefly, a stable water-in-oil emulsion of 1:1 (v/v) KLH-L^{18}LFRGHPKAHGR^{29} conjugate/Freund's complete adjuvant was prepared, and Balb/C mice were immunized intradermally with 20 μg of conjugate each in multiple sites. Mice received a booster immunization of KLH–peptide conjugate (20 μg/animal intraperitoneally, 1:1 v/v conjugate/Freund's incomplete adjuvant water-in-oil emulsion) after 28 days. Serum was screened by ELISA with the BSA–L^{18}LFRGHPKAHGR^{29} conjugate. At 79 days after primary immunization when sufficient titers were obtained, mice were sacrificed, and spleen cells were isolated. Somatic cell hybridization was performed essentially as described (Oi & Herzenberg, 1980). After selection in "HAT" medium, hybridomas were cloned twice by limiting dilution, and 16 positive clones were identified. Further testing showed that nine of these, MmAbA, MmAbC, MmAbD, MmAbF, MmAbH, MmAbI, MmAbK, MmAbL, and MmAbP, were useful for the purposes of the present study. Cell-culture

supermatants were obtained from the respective hybridomas cultured in RPMI-1640 medium with 15% fetal calf serum and 2 mM Gln (Gibco). Ascites were obtained after intraperitoneal injection of (1–3) × 10^6 hybridoma cells per pristane-primed mouse; these hybridomas produced predominantly solid tumors, but sufficient quantities of ascites fluid were obtained.

Determination of Mouse Monoclonal Antibody Subclass. Antibody subclass was determined by ELISA assay in 96-well microtiter plates (Corning no. 25805-96) with P450 2B4 as "antigen" and the nine monoclonals (see above) as "primary antibody". Rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, IgA, κ light-chain, and λ light-chain antibodies (Bio-Rad Laboratories) were used as "secondary antibodies", and a goat anti-rabbit horseradish peroxidase conjugate served as the "detecting antibody" (Bio-Rad Laboratories). P450 2B4 (2 μM) was added to microtiter plates at 100 μL per well and was permitted to bind for 2 h at 25 °C or overnight at 4 °C. All further incubations were carried out at 25 °C, and plates were covered with plastic film to minimize evaporation between all manipulations. Unbound antigen was removed by washing 3 times with 200 μL per well of PBS-T (0.144% Na_2HPO_4, 0.024% KH_2PO_4, 0.8% NaCl, 0.02% KCl, and 0.05% Tween-20, pH 7.2). Residual binding sites were blocked for 2 h with 200 μL per well of 1% BSA/0.02% NaN_3 in PBS. The washing procedure, as above, was repeated. Primary antibodies MmAbA, MmAbC, MmAbD, MmAbF, MmAbH, MmAbI, and MmAbK were used as ascites fluid at 1:100 dilutions in PBS, and MmAbL and MmAbP were from cell-culture supernate, undiluted; these antibodies were utilized at 100 μL per well, with incubation for 2 h. The wells were washed, as above. Rabbit anti-mouse isotype antisera described above were used undiluted in 100-μL volumes per well and were permitted to bind for 2 h. Wells were washed, as above. Detecting antibody, diluted 1:500 in PBS, was added at 100 μL per well, and binding occurred for 1 h, followed by the wash procedure stated above. Horseradish peroxidase substrate solution [hydrogen peroxide and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); Bio-Rad Laboratories] was added at 100 μL per well, and the absorbance at 405 nm was measured *versus* time at 25 °C with a Titertek Multiskan Plus microtiter plate reader. Initial rates were calculated, and these values were corrected by subtraction of the appropriate blanks. Rates associated with the blank/control experiments were typically in the range of 2–10% of the total velocity observed.

Western Blotting. After SDS–PAGE, as described above, purified cytochrome P450 2B4 and extensively-washed microsomes were electrophoretically transferred to a Westran PVDF membrane (Schleicher & Schuell) according to Towbin et al. (1979), modified as follows. The transfer buffer contained 25 mM Tris-HCl, 190 mM glycine, 0.01% SDS, and 20% methanol, pH 8.3, and transfer was effected for 1 h at 4 °C with a field of 100 V (22 V/cm, 230 mA). After transfer, the acrylamide gel was stained with Coomassie Blue R250 to assess the completeness of transfer. A portion of the PVDF membrane was stained for total protein with 0.1% amido black in 10% methanol/2% glacial acetic acid. The remaining portion of the membrane was immunostained with the procedure of Sheng and Schuster (1992), aimed to reduce nonspecific background. The primary antibodies used in the immunostaining procedure were MmAbA (ascites, diluted 1:300 in PBS) and MmAbL (cell-culture supernatant, undiluted) in volumes of 25 mL; the detecting antibody, goat anti-mouse horseradish peroxidase conjugate (Bio-Rad Laboratories), was diluted 1:300 in PBS. Antibody binding was visualized with a substrate solution containing hydrogen peroxide and 2-chloro-1-naphthol (Bio-Rad Laboratories).

Enzyme-Linked Immunosorbent Assays (ELISA) To Assess Membrane Topology. Extensively-washed microsomes were utilized in assays at 1 mg/mL protein, and three treatments of the microsomes were tested, which included "no treatment" (in PBS), "treatment with detergent" (inclusion of 0.5% v/v Tergitol NP-10 in PBS), or "treatment at high ionic strength" (inclusion of 0.3 M potassium phosphate buffer, pH 7.4, in PBS). Microsomes of various treatments were added to 96-well microtiter plates (Corning no. 25805-96) at 100 μL per well and were permitted to bind for 2 h at 25 °C or overnight at 4 °C. The 1 mg/mL protein concentration was used to ensure complete binding of microsomes to titer plate wells, and, in the case of experiments with detergent-solubilized microsomes, to accommodate the need for a Tergitol:protein ratio significantly below 1000:1 (w/w). Binding of microsomal proteins to polystyrene is inhibited at or above this ratio (Shires et al., 1987). Tergitol NP-10 was employed at 0.5% as this concentration was required to solubilize the microsomal membrane fully. All further incubations with proteins were carried out for 2 h at 25 °C, and plates were covered with plastic film to minimize evaporation between manipulations. Unbound microsomes were removed by washing 5 times with 200 μL of PBS per well. Residual binding sites were blocked with 200 μL of 1% BSA/0.02% NaN_3 in PBS per well. Wells were washed 5 times, as above. Monoclonal antibodies (MmAbA,C,D,-F,H,I,K,L,P as ascites, and MmAbL,P as hybridoma cell-culture supernatants) were added at 100 μL per well. MmAbA,I,L, and -P ascites were utilized at 1:5000 dilution in 1% BSA/0.02% NaN_3 in PBS, MmAb C,D,F,H, and -K ascites were used at 1:1000 dilution in 1% BSA/0.02% NaN_3 in PBS, and MmAbL and -P cell-culture supernatants were employed without dilution. Detection was via goat anti-mouse γ heavy-chain/κ light-chain antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories) at 100 μL per well, diluted 1:1000 with 1% BSA in PBS. The ABTS/H_2O_2 chromogenic substrate solution (Bio-Rad Laboratories) was added at 100 μL per well, and the absorbance at 405 nm was measured *versus* time, as above. Rates for each treatment were corrected relative to the appropriate controls.

Computer Searching of Sequence Databases. The uniqueness of the L^{18}LFRGHPKAHGR^{29} sequence was assessed by searching the Protein Information Resource (PIR) version 36.0, SwissProt database version 24.0, and GenPept (the protein translation of GenBank) version 76.0 via the National Institutes of Health, National Center for Biotechnology Information BLAST server version 1.3.5MP (Altshul et al., 1990). The search internet address was blast@ncbi.nlm.nih.gov, and search parameters were "PROGRAM blastp" and "DATALIB nr". The halt-transfer signal peptide was searched through 95 352 sequences (26 378 928 residues).

RESULTS

Properties of the Synthetic L^{18}LFRGHPKAHGR^{29} Halt-Transfer Signal Peptide. Choice of the proper sequence for preparation of antipeptide antibodies for the present membrane topology studies was vital to our experimental design. As shown in Figure 1, the halt-transfer region of cytochrome P450 2B4 was theoretically ideal in view of its mutually-exclusive location at the cytosolic surface of the endoplasmic reticulum membrane in topology model "A" but at the lumenal surface in model "B". Antibodies, having access solely to the

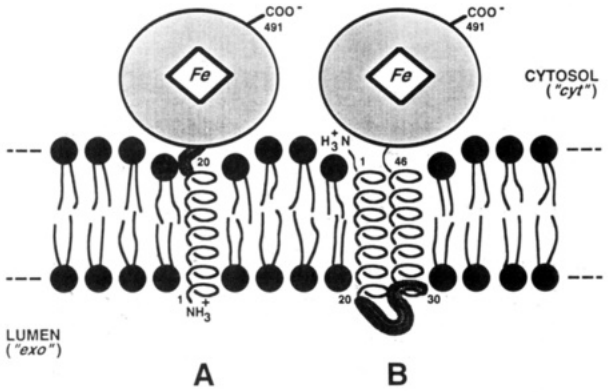

FIGURE 1: Theoretically feasible membrane topological models for cytochrome P450 2B4. Alternative membrane topological models "A" and "B" are depicted, with the halt-transfer signal shown in each by bold highlighting.

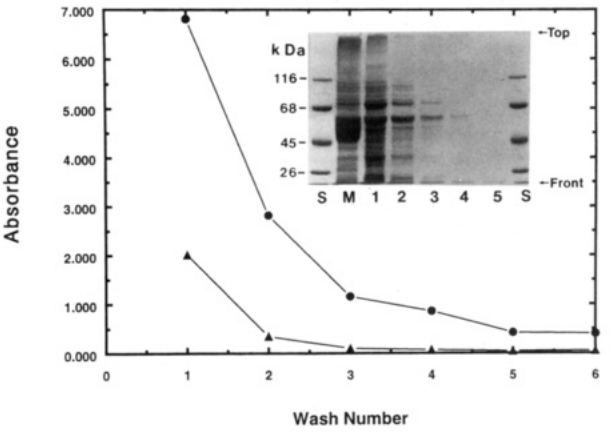

FIGURE 2: Preparation of highly-purified phenobarbital-induced rabbit liver microsomes by extensive washing. Microsomes purified by standard procedures were submitted to six additional washes with 0.1 M sodium pyrophosphate buffer, pH 7.4. The absorbance at 280 nm (●) and 418 nm (▲) was followed for the supernatant fraction of each wash. In addition, the final washed microsomes and samples of each wash were submitted to SDS–PAGE (*inset*). Lanes "S" contain molecular weight standards (β-galactosidase, M_r 116 000; bovine serum albumin, M_r 68 000; ovalbumin, M_r 45 000; and concanavalin A, M_r 26 000), lane "M" contains 9 μg of the final washed microsomal fraction, and lanes "1" through "5" contain equal (1:3) dilutions of wash supernatants 1 through 5, respectively; protein loads in the lanes were 26.0, 8.7, 2.8, 1.3, and 0.6 μg, respectively.

cytosolic side of the intact membrane, could bind to the halt-transfer signal region only in model "A".

The L[18]LFRGHPKAHGR[29] peptide proved rather difficult to synthesize. The yield with the FMOC solid-phase approach was slightly less than 10%; however, equivalent results were obtained when manual synthesis was attempted with *t*-Boc/Merrifield chemistry on methylbenzhydrylamine resin (data not shown). This suggested that the sequence, rather than the chemistry employed during synthesis, was the underlying cause of low synthetic yield. Certainly, the presence of histidyl and prolyl residues in the sequence and the highly basic character of the peptide are contributory factors.

Extensive Washing of Rabbit Liver Microsomes To Obtain a Purified Microsomal Fraction. Previous studies (Brown & Black, 1989) showed by microsequence analysis that at least albumin and hemoglobin were present artifactually in typical microsomal preparations. Thus, we sought to remove such adventitious components from our phenobarbital-induced preparation so as to achieve the most pure microsomal sample for use in membrane topology experiments. Accordingly, we submitted microsomes to six high ionic strength washes (0.1 M sodium pyrophosphate/1 mM EDTA, pH 7.4), with results as shown in Figure 2. Protein absorbance (A_{280nm}) and heme absorbance (A_{418nm}) were quite high in the initial wash, but diminished in an approximately exponential fashion with subsequent washes. By the sixth wash, the absorbance of the supernatant fraction had reached a plateau. Spectral analysis of these fractions in the oxidized and dithionite-reduced states (data not shown) showed spectra identical with hemoglobin. The *inset* in Figure 2 shows the SDS–PAGE profile of proteins present in wash fractions 1 through 5. The proteins removed by washing were clearly of a different composition than those in the microsomal fraction. The two major proteins present had molecular weights of 60 000 and 71 000; the latter component has been identified as rabbit albumin (data not shown). The extensively-washed microsomal preparation appeared to be essentially free of albumin. Ferrous carbonyl difference spectra (data not shown) of the extensively-washed microsomes demonstrated a native P450 spectrum with no evidence of P420 present. Thus, washing apparently removed adventitiously-bound protein without any deleterious effect on the cytochrome P450 present in the membrane.

Characterization of Anti-P450 2B4 Halt-Transfer Signal Monoclonal Antibodies. Nine antipeptide monoclonal antibodies directed against the L[18]LFRGHPKAHGR[29] halt-transfer sequence were prepared; these were designated MmAbA,C,D,F,H,I,K,L, and -P. Each antibody showed acceptable titers when assayed against the BSA–peptide conjugate. As expected, the ascites preparations had significantly higher titers than did hybridoma cell-culture supernatant fractions, but MmAbL and MmAbP showed good binding even as cell-culture supernates.

It was anticipated from the immunization schedule used that these specific antibodies should have been of the IgG subclass. However, somewhat surprisingly, ELISA isotyping assays showed that MmAbA,C,D,F,H,I,K,L, and -P were all IgM antibodies with κ light chains (data not shown). These results were confirmed by the observation that γ heavy-chain-specific anti-antibodies could not recognize any of the monoclonal antibodies. In addition, MmAbA ascites fluid was submitted to fractionation on protein G–Sepharose (data not shown). The majority of the binding activity was present in the nonbound fraction; the IgG eluant was essentially inactive. Immunoblot analysis of MmAbA and MmAbI ascites fluid and MmAbL and MmAbP hybridoma cell-culture supernate showed the presence of κ light chains but no γ heavy chain. Apparently, the L[18]LFRGHPKAHGR[29] peptide may elicit only an IgM response, or may suppress the secondary immune response for IgG.

ELISA of the nine monoclonal antibody ascites fluid with P450 2B4 as antigen showed strong binding, and titers were determined to be in the range of $1:10^3$ to $1:10^4$. The relative ranking of titers was MmAbL > A > P > I > C > D > H > F > K; MmAbL, -A, and -P ascites were still active at $1:10^6$ dilution. Corrected ELISA rates for 1:500 dilutions of the monoclonal antibody ascites preparations fell in the range of 0.052–0.218 A_{405nm}/min. For the hybridoma cell-culture supernatants (undiluted), MmAbL and MmAbP were the most active with velocities of 0.074 and 0.170 A_{405nm}/min, respectively.

Analysis of Antibody Specificity by Western Blotting. An extensive search of the L[18]LFRGHPKAHGR[29] peptide with the BLAST e-mail server revealed that this sequence matched only rabbit P450 isozymes 2B4 and 2B5 with identity (score = 68); all other P450 cytochromes and other known polypeptides were poorly related or unrelated. Such a finding was in

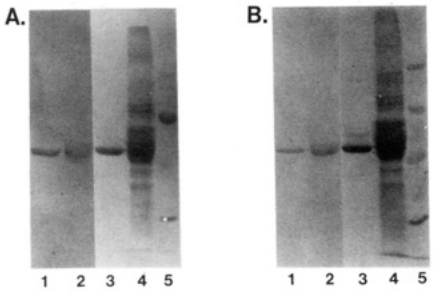

FIGURE 3: Specificity of anti-P450 2B4 halt-transfer signal monoclonal antibodies. Panels "A" and "B" show Western blotting results obtained with MmAbA ascites and MmAbL cell-culture supernatant fluid, respectively. Lanes 1 and 2 of each panel show the immunostained PVDF membranes, while lanes 3–5 show parallel membranes stained for total protein with amido black. Samples applied to the original SDS–PAGE experiment were as follows: lanes 1 and 3, purified cytochrome P450 2B4 (1 μg); lanes 2 and 4, extensively-washed liver microsomes (9 μg); and lane 5, molecular weight standards as described in Figure 2, with the exception that panel "A" lane 5 also contains phosphorylase *b* (M_r 97 500).

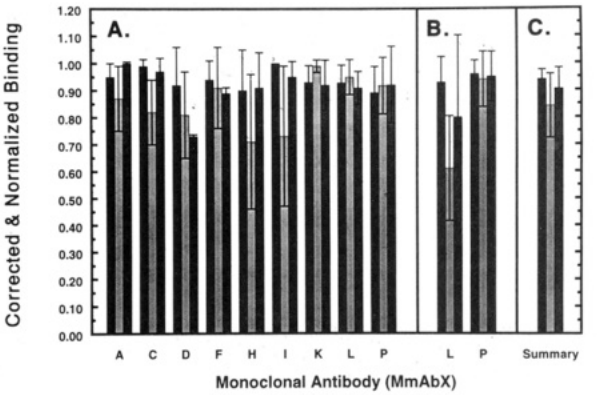

FIGURE 4: ELISA assays of the binding of various anti-P450 2B4 halt-transfer signal monoclonal antibodies to extensively-washed liver microsomes under various treatments. Binding of each monoclonal antibody was calculated relative to an appropriate blank and is the result of 3–5 separate determinations. Results were normalized to 1.0 for the highest signal obtained in each group. Bars represent mean binding with error bars indicating the standard deviation. *Black bars* (left) show antibody binding to extensively-washed liver microsomes, *light-shaded bars* (middle) show binding to extensively-washed microsomes in the presence of 0.5% Tergitol NP-10, and *medium-shaded bars* (right) show binding to extensively-washed microsomes in the presence of 0.3 M potassium phosphate buffer, pH 7.4. Results in panel "A" pertain to antibody acites preparations, and panel "B" pertains to antibody cell-culture supernatant fractions; panel "C" shows a summary of all results for all antibodies and treatments.

accord with the observation that while halt-transfer signal sequences serve similar biological functions and are found in many membrane proteins, little sequence homology is observed (Sabatini et al., 1982; Kuriowa et al., 1991). This result also indicated that the monoclonal antibodies produced in the present study were likely of adequate specificity to serve as membrane topology probes for cytochrome P450 2B4. To confirm this hypothesis, we performed Western blots with the various antibodies against extensively-washed rabbit phenobarbital-induced microsomes. The specificity appeared to be good, and representative examples of results are shown in Figure 3; MmAbA acites is studied in panel A of the figure, while MmAbL from hybridoma cell-culture supernatant fluid is examined in panel B. MmAbA ascites clearly identified P450 2B4, and also detected two minor bands of similar molecular weight. MmAbL cell supernate showed high specificity for only the cytochrome P450 2B4 band. Thus, in addition to having acceptable ELISA properties, the nine monoclonal antibodies were suitably specific.

Determination of P450 2B4 Membrane Topology. Six-fold-washed rabbit liver phenobarbital-induced microsomes were studied with anti-L^{18}LFRGHPKAHGR^{29} monoclonal antibodies (MmAbA,C,D,F,H,I,L,P) in ELISA assays either alone, in the presence of 0.5% Tergitol NP-10 detergent, or in 0.3 M potassium phosphate buffer (high ionic strength). Assays were performed generally in quadruplicate, and corrected rates were averaged and normalized to 1.0 for the highest activity per antibody in each set of experiments. Results are shown in Figure 4. The data in panel A are for the nine monoclonal antibodies from ascites fluid, while those in panel B indicate studies performed with MmAbL and MmAbP from cell-culture supernatant. Rates associated with negative controls in the ascites experiments represented 8.6 ± 5.5% of the total velocity, while such control values were 6.5 ± 4.2% for the cell-culture supernatant fractions; unrelated ascitic fluid and unrelated cell-culture supernatant were used for negative controls. Generally, the normalized averaged rates were similar for the microsomes alone, plus detergent, and plus high ionic strength treatments with each antibody. Also, the means were essentially unity in each case. This can also be seen in panel C in which grand averages for each treatment with all monoclonal antibodies have been calculated. The overall average for microsomes alone was 0.940 ± 0.034, for detergent-solubilized microsomes was 0.841 ± 0.118, and for microsomes at high ionic strength was 0.904 ± 0.077. It is also of importance to note that in no case was binding for the microsomes plus detergent statistically greater than microsomes alone. Thus, added detergent or salt does not result in enhanced binding of the anti-halt-transfer signal monoclonal antibodies to microsomal P450 2B4.

DISCUSSION

In view of the possibility that two modes of membrane binding might exist for P450 cytochromes from the endoplasmic reticulum, we chose an experimental design which had the potential to quantify the presence of either topological model ("A" or "B", as shown in Figure 1) or some fraction of each. Our approach utilized site-specific monoclonal antibodies targeted to the halt-transfer signal region of P450 2B4. Analysis by ELISA offered the advantage that the fraction of each model present could be quantified sensitively. One possible caveat existed in our experimental design if the 18–29 peptide were somehow inaccessible to antibody binding in model "A". To test for this possibility, microsomes were treated at high ionic strength to dissociate potential ionic interactions between the membrane surface, the halt-transfer signal sequence, and the globular P450 heme domain. Thus, if model "A" were quantitatively correct, antibody binding would be detected with native and/or high ionic strength treated microsomes; if model "B" were quantitatively correct, no binding would be detected under the above conditions, but full antibody binding would result with detergent-solubilized microsomes. Intermediate results would indicate the presence of some fraction of each model.

Our membrane topology experiments were carried out with extensively-washed phenobarbital-induced microsomes. The importance of the washing was demonstrated by experiments as shown in Figure 2 where significant quantities of protein were removed, and the electrophoretic pattern of the material separated was quite different from that of microsomes. Thus, experimental results were not complicated by the presence of such adventitiously-bound protein. Further, the fact that achievement of such a purified microsomal preparation

Table 1: Membrane Topology of Microsomal P450 Monooxygenase System Components and Related Microsomal Proteins

microsomal protein[a]	molecular weight	anchor location in sequence[b]	NH_2-terminal modification	topology[b,c]	reference
cytochrome P450 2B4	56K	N	none	N_{exo}-C_{cyt}	present work
NADPH–cytochrome P450 reductase	77K	N	acetyl-	N_{exo}-C_{cyt} or N_{cyt}-C_{cyt}	Black & Coon (1982)
NADH–cytochrome b_5 reductase	31K	N	myristoyl-	N_{exo}-C_{cyt} or N_{cyt}-C_{cyt}	Kensil & Strittmatter (1986)
epoxide hydrolase	53K	N	none	N_{exo}-C_{cyt}	Craft et al. (1990)
prostaglandin H synthase	69K	central	signal peptide-	N_{exo}-C_{cyt}	Merlie et al. (1988)
cytochrome b_5	15K	C	acetyl-	N_{cyt}-C_{cyt}	Ozols (1989a)
flavin monooxygenase	60K	C?	acetyl- or none	N_{cyt}-C_{exo} or N_{cyt}-C_{cyt}	Dolphin et al. (1992), Lawton & Philpot (1993)
heme oxygenase	30–36K	C	blocked or none	N_{cyt}-C_{exo} or N_{cyt}-C_{cyt}	McCoubrey & Maines (1993), Schacter et al. (1990)
UDP glucuronosyltransferase	58K	C	signal peptide-	N_{exo}-C_{cyt}	Shepherd et al. (1989)
glycoprotein esterase	60K	C	PyroGlu- or none	N_{exo}-C_{cyt}?	Ozols (1989b)

[a] Proteins in the table contain a single-segment integral-membrane anchor, according to present knowledge. [b] Amino- and carboxyl-terminal regions are indicated as N and C, respectively. [c] Terminal regions of proteins are indicated exo if located in the lumen of the endoplasmic reticulum, or cyt if located in the cytosol.

required six high ionic strength washes perhaps indicates that new standards may be necessary for topological and catalytic experiments carried out with microsomal fractions.

The monoclonal antibodies prepared against the halt-transfer segment of P450 2B4 exhibited good titers, binding characteristics, and specificity. Binding was observed not only in the case of the BSA–peptide conjugate but also with intact microsomes as well (see Figure 4). Clearly, no significant steric hindrance or other antibody accessibility problem existed. Furthermore, the extent of binding detected in ELISA assays with microsomes was essentially the same as was seen with microsomes at high ionic strength or after detergent solubilization. In accord with the logic discussed above, such findings are consistent with only model "A" and not model "B" or any combination of models "A" and "B". Therefore, the correct membrane topology of cytochrome P450 2B4 is a single transmembrane anchor at the NH_2-terminus [N_{exo}-C_{cyt}; for nomenclature, see Hartmann et al. (1989)], as shown in Figure 1A.

The membrane topology of all microsomal P450 cytochromes is anticipated to be similar, both on the basis of significant sequence homology (Nelson & Strobel, 1988) as well as on the basis of having comparable hydrophobicity (Black & Coon, 1986) and detergent solubilization characteristics. Thus, the model shown in Figure 1 (model "A") should be archetypic of the topology of other microsomal P450s. However, comparison of the P450 membrane topology to that of other components of the monooxygenase system reveals both similarities and differences, as shown in Table 1. NADPH–cytochrome P450 reductase, NADH–cytochrome b_5 reductase, and epoxide hydrolase are bound to the endoplasmic reticulum by membrane anchors at the amino terminus, but both reductases contain NH_2-terminal acylation whereas the P450s and hydrolase do not. Curiously, the P450s and epoxide hydrolase exhibit an N_{exo}-C_{cyt} topology, but that of the reductases is not known precisely. Either N_{exo}-C_{cyt} or N_{cyt}-C_{cyt} topologies are possible based on current experimental evidence. Both acetyl- and myristoyltransferases are known to be cytosolic, ribosomally-associated proteins, and cotranslational transferase activity could potentially acylate any microsomal protein regardless of topology. However, it is of interest to note that P450 2B4 contains an amino-terminal sequence of Met-Glu-Phe-Ser---, which should serve as an excellent substrate for acetyltransferases (Yamada & Bradshaw, 1991), but no such modification has ever been observed. Thus, the N_{cyt}-C_{cyt} "loop" topology appears likely for the reductases. Like the P450s, prostaglandin synthase has an N_{exo}-C_{cyt} topology, but a significant portion of the polypeptide chain is translocated through the membrane and the anchor is localized in the central portion of the sequence. The remaining microsomal proteins in the table are believed to be bound to the membrane by anchor peptides located in the carboxyl-terminal region. Cytochrome b_5 has been shown to possess an N_{cyt}-C_{cyt} loop topology with an acetylated amino terminus; acylation of the cytosolic NH_2-terminus provides further support for the topology suggested above for the reductases. Flavin monooxygenases and heme oxygenases are either NH_2-terminally blocked or free, and their topology is not yet known with certainty. Either N_{cyt}-C_{exo} or N_{cyt}-C_{cyt} alternatives are possible, depending upon whether the COOH-terminus passes through the membrane or forms a loop. The fact that heme oxygenase and cytochrome b_5 are both inserted into the endoplasmic reticulum posttranslationally perhaps suggests that the N_{cyt}-C_{cyt} alternative for heme oxygenase is pertinent. UDP-glucuronosyltransferase and glycoprotein esterases both have N_{exo}-C_{cyt} topologies like the P450s, but unlike the other microsomal proteins in Table 1, the catalytic domain and a majority of the polypeptide are located in the lumen of the endoplasmic reticulum.

While the essential features of the cytochrome P450 2B4 membrane topology have now been solved, it is clear that secondary, peripheral interactions of the heme domain with the membrane must exist as well. P450 mutants in which the NH_2-terminal membrane anchor was removed were yet found partially associated with the membrane (Clark & Waterman, 1991; Li & Chiang, 1991; Cullin, 1992; Pernecky et al., 1993). Proteolysis studies reached essentially the same conclusion (Brown & Black, 1989). Clearly, a knowledge of these secondary membrane interactions is essential toward a complete knowledge of the cytochrome P450 membrane topology.

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