Amino-terminal Topology of Thromboxane Synthase in the Endoplasmic Reticulum*

(Received for publication, April 2, 1993)

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The membrane topology of the NH2-terminal portion of human thromboxane synthase (TXS), a member of the cytochrome P450 superfamily, has been investigated. By sequence alignment, the first 6 residues of the mature TXS polypeptide are likely to form a distinctive "tail" structure not found in many other mammalian cytochromes P450 in the endoplasmic reticulum membrane. Peptides with either the ultimate 10 or 15 residues of the NH2 terminus of TXS were synthesized and used to produce site-directed antibodies. The resulting peptide antibodies were highly specific and recognized human TXS, as shown by binding assays and Western blot analysis. Binding of the peptide antibodies to recombinant TXS in transfected COS-1 and to endogenous TXS in THP-1 cells was analyzed by immunocytochemistry. Selective permeabilization of the plasma membrane to immunoglobulin was achieved with streptolysin O; general permeabilization, including the endoplasmic reticulum membrane, was accomplished with Triton X-100. Permeabilization of the plasma membrane was sufficient to produce binding of both peptide antibodies to their epitopes, indicating that the epitopes for both of the peptide antibodies were exposed on the cytoplasmic side of the endoplasmic reticulum membrane. The results with the peptide antibodies provide direct experimental evidence supporting the topological model for membrane-bound cytochrome P450 proposed by Nelson and Strobel (Nelson, D. R., and Strobel, H. W. (1988) J. Biol. Chem. 263, 6036-6050), in which the NH2 terminus is oriented toward the cytoplasmic side of the endoplasmic reticulum membrane.

Thromboxane synthase (TXS) catalyzes the isomerization of prostaglandin-H2 endoperoxide to the unstable proaggregatory and vasoconstrictive agent thromboxane A2 (Hamberg et al., 1975). The active enzyme has been purified to homogeneity from human platelets (Haurand and Ullrich, 1985) and porcine lung (Shen and Tai, 1986b) and characterized as a cytochrome P450, with one heme/69-kDa (Ullrich and Graf, 1984) or 53-kDa (Shen and Tai, 1986b) polypeptide.

The cDNA for human lung TXS has been cloned and sequenced, and the amino acid sequence was deduced (Wang et al., 1991; Ohashi et al., 1992). Two cDNA clones of TXS were found in the lung library. The longer predicted protein, designated TXS-1, contains 534 amino acids and has an M, of 60,684, whereas the shorter protein, designated TXS-2, contains 460 amino acids and has an M, of 52,408. TXS-2 lacks the conserved cysteine that is believed to serve as the proximal heme ligand in other cytochromes P450 (Ohashi et al., 1992). The primary structure of the enzyme from human platelets has also been deduced recently (Yokoyama et al., 1991). The predicted human lung TXS-1 and human platelet TXS amino acid sequences are essentially identical. The amino acid sequence of TXS has considerable similarity to those of other cytochromes P450, particularly those in family 3 (Ohashi et al., 1992).

The transmembrane topology of mammalian membrane-bound cytochromes P450, in particular the orientation of the NH2 terminus, remains rather controversial. Current models for the membrane topology of microsomal cytochromes P450 propose a large cytoplasmic domain anchored to the membrane by either one or two NH2-terminal transmembrane segments (Black, 1992). Support for these models is derived from studies using site-specific antibodies and chemical modification to probe the exposure of particular segments of the enzyme in the endoplasmic reticulum (ER) membrane. The results indicate that the bulk of the protein is exposed on the cytoplasmic surface of the ER (Thomas et al., 1977; De Lemos-Chiarandini et al., 1987; Edwards et al., 1991). Nelson and Strobel (1988) conducted an extensive analysis of hydrophathy profiles and of previously reported results and proposed an NH2-terminal transmembrane hairpin loop as the sole membrane anchor, with the NH2 terminus of the polypeptide exposed to the cytoplasmic face of the ER. This proposed loop, involving parts of the first 66 amino acids of microsomal P450, has two transmembrane segments. The more widely accepted structural model has only one transmembrane segment in the NH2-terminal region and the NH2 terminus oriented toward the lumen (Edwards et al., 1991).

As a member of the cytochrome P450 superfamily, thromboxane synthase shares considerable primary and secondary structure with other cytochromes P450 (Yokoyama et al., 1991; Ohashi et al., 1992). However, the initial 6 residues of the NH2 terminus of mature TXS appear to make up an additional "tail" segment that extends beyond the NH2 terminus of other microsomal cytochromes P450 (Ohashi et al., 1992). Assuming that all microsomal cytochromes P450 adopt a similar membrane topology, the additional tail segment of TXS in the ER membrane provides an important opportunity to determine the orientation of the NH2 terminus in microsomal cytochromes P450.

We have used two peptide antibodies directed against the NH2-terminal segment of human TXS to investigate the transmembrane topology of the NH2-terminal domain of this cytochrome P450 in the ER membrane. The results suggest that some of the first 10–15 residues of the NH2-terminal segment of TXS are exposed on the surface of the cytoplasmic side in the ER membranes, supporting the model proposed by Nelson and Strobel (1988).

*This work was supported in part by National Institute of Health Grant NS-23327. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: TXS, thromboxane synthase; ER, endoplasmic reticulum; PM, phospholipids; 12-myristate 13-acetate; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; STO, streptolysin O.
Thromboxane Synthase Amino-terminal Topology

EXPERIMENTAL PROCEDURES

Structure Prediction—Hydropathy calculations were performed by the method of Kyte and Doolittle (1982). Analysis of sequence similarity and alignment and prediction of secondary structures were performed with the EUGene software package developed by C. B. Lawrence, T. Shalom, and S. Honda at the Molecular Biology Information Resource at Baylor College of Medicine.

Preparation of Human Platelet Microsomes—Human platelet microsomes were prepared essentially by the methods described by Shew and Thistlethwaite (1966a). Platelet-rich plasma (Gulf Coast Regional Blood Center, Houston, TX) was centrifuged at 200 x g for 30 min to remove leukocytes and red blood cells. Platelets were collected by centrifugation at 2000 x g for 15 min and washed once with one-third of the original volume of 25 mM Tris-HCl, pH 7.5, in 0.9% NaCl. The platelets were suspended in 3 volumes of saline buffer at 4°C, sonicated (5 x 10 s) with a Soniflex Cell Disruptor (Model W185, Heat Systems-Ultrasonics, Inc.), and then centrifuged at 8000 x g for 30 min. The supernatant liquid was filtered through a layer of glass wool and centrifuged at 100,000 x g for 60 min at 4°C. The microsomal pellet was suspended in 25 mM Tris-HCl, pH 7.5, in 0.9% NaCl.

Construction of TXS Expression Vectors—To construct a prokaryotic expression vector, 2 oligonucleotides corresponding to the sequences at the translation start and termination sites were used as primers to amplify the TXS cDNA, which is identical to that of TXS-1 except for 1 nucleotide in the COOH terminus. The primer sequences were 5'-CGAGATCTG- and 5'-CGAGATCTGAGGGCGGCTT-3'. Both primers contained an EcoRI site. The amplified TXS-2 cDNA was digested with BglII and subcloned in-frame in the BamHI site adjacent to the coding region for glutathione S-transferase in the pGEX-2T expression vector (Pharmacia LKB Biotechnology Inc.). The desired orientation was confirmed by restriction enzyme mapping. In all cases the adjacent coding region was from the synthetic peptides were affinity-purified by the appropriate peptide immobilized on a Sepharose 4B column as described by the manufacturer (Pharmacia). Briefly, serum (20 ml) was loaded onto the peptide-Sepharose 4B column (0.9 x 3.5 cm) to remove contaminating antibodies against the carrier protein.

Immunoblotting.—Human platelet microsomal TXS or crude recombinant glutathione S-transferase-TXS-2 expressed in E. coli cells was solubilized with 1% SDS, mixed with sample buffer (60 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, and 0.005% bromophenol blue) and heated at 100°C for 5 min. The samples were separated by electrophoresis on a 10 or 13% polyacrylamide gel (Laemmli, 1970) and then transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979). Treatment with 1% nonfat powdered milk in PBS at room temperature for 1 h, the membranes were incubated with either antiserum or the affinity-purified anti-peptide antibody at room temperature overnight. After washing three times with PBS, the membranes were incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) at room temperature for 3 h. The membranes were washed three times with PBS, and the bands were visualized with a mixture of 0.5% 4-chloro-1-naphthol in methanol and 50 ml of 0.03% H2O2 in PBS.

Assay of Antibody Binding.—Binding of antibody to the corresponding synthetic peptides was assessed using microtiter plate assays (Cornsing, Cornying, NY). The wells were coated with 50 ml of the appropriate peptide solution (30 pg/ml) in PBS at 4°C overnight. After washing twice with PBS, nonpecific binding sites were blocked by incubation with 1% lysine in PBS at 37°C for 1 h. Incubations with 50 ml of antibody in PBS containing 1% lysine proceeded at room temperature for 2 h, and the plate was washed three times with PBS, followed by a 2-h incubation with 50 ml of goat anti-rabbit IgG-horseradish peroxidase conjugate in PBS. After washing twice with PBS, 50 ml of a fresh mixture of equal volumes (1:1) of 3,3',5,5'-tetramethyldibenzoic acid and H2O2 solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added. The peroxidase reaction was stopped by addition of 200 ml of 2% H2SO4, and the reaction product was measured by its absorbance at 450 nm using an enzyme immunosorbent assay microplate reader (Dynatech Model MR 5000).

Immunocytochemical Studies—THP-1 cells or transfected COS-1 cells grown on coverslips were incubated with TXS antibody at room temperature for 1 h, washed with the same buffer, and then mounted on glass slides with Mowiol (Calbiochem) containing 2.5% thiyethylendiamine (Sigma). For permeabilization of cells with Triton X-100, the cells were washed three times with PBS, fixed with 5% formaldehyde in PBS, blocked with 1% myoglobin, and then incubated with the primary and secondary antibodies as described above. Cells stained with the FITC conjugate were examined by fluorescence microscopy (Campbell et

Peptide Synthesis.—Peptide synthesis was assayed by incubating 0.1 ml of a 0.1 M Tris (pH 8.0) buffer containing H2SO4 buffer (Ulmacz and Lands, 1977) in 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) for 1 min and then adding 50 ml of microsomes from human platelets or from transfected COS-1 cells in PBS containing 0.1% glycine, 0.15 M NaCl (PBS) for 1 h, washed with the same buffer, and then mounted on glass slides with Mowiol (Calbiochem) containing 2.5% thiyethylendiamine (Sigma). For permeabilization of cells with Triton X-100, the cells were washed three times with PBS, fixed with 5% formaldehyde in PBS, blocked with 1% myoglobin, and then incubated with the primary and secondary antibodies as described above. Cells stained with the FITC conjugate were examined by fluorescence microscopy (Campbell et
RESULTS

Design of \( \text{NH}_2 \)-terminal Peptides—The \( \text{NH}_2 \)-terminal region of the other mammalian cytochromes P450 is predicted to be a membrane-spanning domain based on the hydropathy profiles and predicted secondary structures, and indeed all models of P450 topology have the \( \text{NH}_2 \) terminus as some sort of transmembrane segment (Heinemann and Ozols, 1982; Heinemann and Johnsson, 1985; Black and Coon, 1987; De Lemos-Chiarandini et al., 1987; Sakaguchi et al., 1987). Comparison of the deduced TXS sequences with data bank protein sequences indicate that the \( \text{NH}_2 \)-terminal region of mature TXS, beginning at about residue 7, has considerable similarity to other cytochromes P450, particularly those in family 3 (Fig. 1) (Ohashi et al., 1992). A dot matrix homology domain comparison of TXS-1 with a family 3 member, P450 IIIA4, indicated that ~60% similarity (i.e. identical residues or conservative mutations) extended over 90% of the length of the TXS polypeptide. This strong overall similarity supports the validity of using TXS as a model to investigate the \( \text{NH}_2 \)-terminal orientation of cytochromes P450. From an alignment analysis, the first 6 residues or so at the \( \text{NH}_2 \) terminus of mature TXS make up a segment that extends beyond those of the other membrane-bound mammalian cytochromes P450 (Fig. 1A) (Nelson and Strobel, 1988).

To characterize the topology of this additional segment in the \( \text{NH}_2 \) terminus of mature TXS, peptides containing either the first 10 or 15 residues were synthesized, purified, characterized, and used to raise site-specific antibodies as described under "Experimental Procedures."

Characterization of Site-specific Antibodies—Binding of the affinity-purified antibodies to the two synthetic peptides was evaluated by enzyme immunoassay as described under "Experimental Procedures." Each peptide antibody, even at low levels, bound to the corresponding \( \text{NH}_2 \)-terminal peptide and did not react with the carrier protein (BSA or ovalbumin) (Fig. 2A). In contrast, preimmune IgG did not bind significantly to either peptide (Fig. 2B). The affinity-purified site-specific antibodies thus exhibited a high titer and specificity for recognition of their target \( \text{NH}_2 \)-terminal peptide sequence.

The interactions of the NT-10 antibody with the NT-15 peptide-ovalbumin conjugate and of the NT-15 antibody with the NT-10 peptide-BSA conjugate were also examined. As expected, each affinity-purified antibody cross-reacted with the other \( \text{NH}_2 \)-terminal peptide-albumin conjugate (Fig. 3). This further confirmed the ability of the peptide antibodies to recognize the \( \text{NH}_2 \)-terminal epitope of TXS.

Binding of the site-specific peptide antibodies to TXS itself was examined with immunoblotting techniques. The affinity-purified antibody against the NT-10 peptide recognized a human platelet microsomal protein with a molecular mass of ~61 kDa (Fig. 4A, lane 1), which is very close to the reported molecular mass of human TXS-1 (Yokoyama et al., 1991; Ohashi et al., 1992). However, the purified NT-15 peptide antibody did not exhibit reactivity with the 61-kDa protein band (data not shown) even though it did bind the TXS \( \text{NH}_2 \)-terminal peptide coated on a microtiter plate (Fig. 2). This may indicate that recognition of TXS by the NT-15 antibody is dependent on a structural conformation that is lost upon denaturation of the protein during the immunoblot procedure. As would be expected, unpurified NT-10 antiserum recognized both TXS and albumin (Fig. 4A, lane 2), and preimmune IgG did not show

![Fig. 1. \( \text{NH}_2 \)-terminal alignment of TXS with family 3 cytochromes P450.](image)

![Fig. 2. Binding of peptide antibodies to synthetic \( \text{NH}_2 \)-terminal peptides.](image)
significant binding to any platelet microsomal proteins (Fig. 4A, lane 3).

To further establish the specificity of the peptide antibodies for TXS, a recombinant fusion protein made up of TXS-2 and glutathione S-transferase-TXS-2 fusion protein (100 μg of protein) (B) were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide for A and 13% acrylamide for B) and blotted on nitrocellulose membranes (except for B, lane 1). The membranes were incubated with 10 μg/ml affinity-purified antibody against NT-10 (A, lane 1; B, lane 2), or a 1:500 dilution of unpurified NT-10 antisera (A, lane 2), or a 1:500 dilution of preimmune rabbit serum (A, lane 3) and then reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate. B, lane 1, Coomassie Blue-stained gel after SDS-polyacrylamide gel electrophoresis analysis of the E. coli extract. The positions of molecular mass standards are indicated by the horizontal bars to the left of each panel (from top to bottom: 106, 80, 49.5, and 32.5 kDa for A and 106, 80, 49.5, 32.5, and 27.5 kDa for B). The position of the glutathione S-transferase-TXS-2 fusion protein is shown by the arrow.

**Fig. 3.** Binding of NT-15 antibody to NT-10 conjugate and of NT-10 antibody to NT-15 conjugate. A, a microtiter plate coated with the NT-10 peptide-BSA conjugate was incubated with the indicated levels of the affinity-purified NT-15 antibody (●) or preimmune IgG (○). B, a microtiter plate coated with the NT-15 peptide-ovalbumin conjugate was incubated with the indicated levels of the affinity-purified NT-10 antibody (●) or preimmune IgG (○). Bound antibody was quantitated as described under "Experimental Procedures."

**Fig. 4.** Recognition of endogenous and recombinant TXS by peptide antibodies. Detergent extracts of human platelet microsomes (100 μg of protein) (A) or of E. coli cells expressing a recombinant glutathione S-transferase-TXS-2 fusion protein (100 μg of protein) (B) were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide for A and 13% acrylamide for B) and blotted on nitrocellulose membranes (except for B, lane 1). The membranes were incubated with 10 μg/ml affinity-purified antibody against NT-10 (A, lane 1; B, lane 2), or a 1:500 dilution of unpurified NT-10 antisera (A, lane 2), or a 1:500 dilution of preimmune rabbit serum (A, lane 3) and then reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate. B, lane 1, Coomassie Blue-stained gel after SDS-polyacrylamide gel electrophoresis analysis of the E. coli extract. The positions of molecular mass standards are indicated by the horizontal bars to the left of each panel (from top to bottom: 106, 80, 49.5, and 32.5 kDa for A and 106, 80, 49.5, 32.5, and 27.5 kDa for B). The position of the glutathione S-transferase-TXS-2 fusion protein is shown by the arrow.

Recombinant TXS—The TXS activity of microsomes from COS-1 cells transfected with a vector containing the cDNA of TXS-1 or with the vector alone was assayed as described under "Experimental Procedures." Microsomes from COS-1 cells transfected with TXS-1 cDNA had about seven times the TXS-specific activity compared with human platelet microsomes, and the activity was blocked by a TXS inhibitor (Table I). In contrast, microsomes from COS-1 cells transfected with a control plasmid had no detectable TXS activity (Table I). These results show that the recombinant TXS in the COS-1 cells was highly active and therefore that at least the catalytic region of the protein was likely to be in the native conformation.

**Table I.** TXS activity in microsomes from platelets and COS-1 cells

| Cells                          | TXS activity \( +0.7 \text{ mm U63557A} \) |
|-------------------------------|------------------------------------------|
| COS-1 cells transfected with plasmid DNA either with (pSVL-TXS-1) or without (pSVL) the TXS-1 cDNA sequencing as described under "Experimental Procedures." Each assay contained 100–120 μg of microsomal protein. | \( \text{pmol TXB}_2^+ \text{ mg protein} \) |
| Human platelets               | 68 ± 2                                   |
| COS-1, pSVL-TXS-1             | 517 ± 13                                 |
| COS-1, pSVL                   | ND                                       |

\( \text{a} \) The values shown are the averages from two experiments.

\( \text{b} \) TXB<sub>2</sub>, thromboxane B<sub>2</sub>; ND, not detectable.
antibodies gave the same staining in the PMA-induced THP-1 cells permeabilized with either STO (Fig. 8, A and B) or Triton X-100 (C and D). The significant amount of antibody binding in STO-permeabilized cells indicates that NH$_2$-terminal TXS epitopes recognized by the two antibodies were exposed to the cytoplasmic compartment. Much less intense fluorescence was observed with the STO-permeabilized quiescent THP-1 cells (Fig. 8, E and F), as expected from the lower levels of TXS activity (Sanduja et al., 1991). Thus, the endogenous TXS in the THP-1 cells had the same NH$_2$-terminal topology as the recombinant TXS transiently expressed in COS-1 cells. The diffuse staining of TXS in the THP-1 cells in Fig. 8 was thought to reflect the limited resolution of conventional light microscopy. Therefore, confocal microscopy was used to examine the sub-

Fig. 5. Schematic representation of permeabilization of cells with STO or Triton X-100 illustrating access of antibodies for protein disulfide-isomerase (PDI) (lightface forks) and for TXS (boldface forks) to their target molecules in ER.

Fig. 6. Immunofluorescence micrographs of cultured cells stained with anti-protein disulfide-isomerase antibody. COS-1 cells (A and C) and THP-1 cells (B and D) permeabilized with Triton X-100 (A and B) or with STO (C and D) were reacted with 200-fold diluted rabbit anti-protein disulfide-isomerase serum and then stained with the FITC-labeled second antibody as described under "Experimental Procedures." All photomicrographs were taken with the same exposure times (20 s) and are printed at the same magnification (x ~500).

Fig. 7. Immunofluorescence micrographs of COS-1 cells stained with anti-TXS NH$_2$ terminus antibodies. COS-1 cells transfected with a vector containing TXS cDNA (A-D) or with the vector alone (E and F) were permeabilized with STO (A, B, E, and F) or with Triton X-100 (C and D). Permeabilized cells were incubated with 75 μg/ml affinity-purified NT-10 (A, C, and E) or NT-15 (B, D, and F) antibody and stained with the FITC-labeled second antibody (see "Experimental Procedures"). Photomicrographs were obtained as described for Fig. 6.

Fig. 8. Immunofluorescence micrographs of THP-1 cells stained with anti-TXS NH$_2$ terminus antibodies. PMA-induced THP-1 cells (A-D) or quiescent THP-1 cells (E and F) were permeabilized with STO (A, B, E, and F) or with Triton X-100 (C and D). After reaction with the affinity-purified NT-10 (A, C, and E) or NT-15 (B, D, and F) antibody, the cells were stained with the FITC-labeled second antibody (see "Experimental Procedures" for details). Photomicrographs were obtained as described for Fig. 6.
cellular localization of TXS in the THP-1 cells and revealed a distinctly reticular pattern of TXS staining (Fig. 9), consistent with an ER location, as found with other cytochromes P450.

DISCUSSION

The similarities in amino acid sequence between TXS and cytochromes P450 establish that TXS belongs to the cytochrome P450 superfamily (Yokoyama et al., 1991; Ohashi et al., 1992). The notable conservation of sequence motifs among the various cytochromes P450 (Nelson and Strobel, 1988) argues for a conservation of general elements of secondary and tertiary structure in the cytochromes P450. A number of alternative models for the membrane topology of mammalian cytochromes P450 have been proposed. The currently favored concepts have the protein anchored by its NH₂ terminus to the ER membrane (Nelson and Strobel, 1988; Edwards et al., 1991). This arrangement is consistent with the observation by Sakaguchi et al. (1987) that the NH₂ terminus acts as an uncleaved signal sequence, interacting with the signal recognition particle during insertion of cytochrome P450 in the membrane. Other evidence supporting the NH₂-terminal region as the membrane anchor of cytochromes P450 has come from studies using site-specific antibodies (De Lemos-Chiarandini et al., 1987; Edwards et al., 1991), alignment of hydropathy profiles (Nelson and Strobel, 1988), and site-directed mutagenesis (Monier et al., 1988; Kemper and Szczesna-Skorupa, 1989).

De Lemos-Chiarandini et al. (1987) found that an antibody directed to the first 31 residues of native P450 IIB1 bound strongly to the purified solubilized enzyme, but not to the microsomal enzyme, and that another antibody directed to residues 24–38 of the same P450 bound equally well to both soluble and microsomal forms of the protein. Edwards et al. (1991) interpreted these results to imply the presence of a single membrane-spanning segment in the first 20 residues. The ability of FITC to react with the NH₂-terminal residue of P450 in a mixture of the protein and liposomes (Bernhardt et al., 1983) was cited by Nelson and Strobel (1988) as evidence that the NH₂-terminal residue was exposed to the aqueous environment, a key prediction of the hairpin model they proposed. However, incorporation of the P450 into the liposomal membranes was not demonstrated by Bernhardt et al. (1983), and thorough examinations by Vergeres et al. (1989, 1991) convincingly showed that the NH₂-terminal residue could be labeled by FITC in soluble P450, but not in the microsomal enzyme or in the enzyme incorporated into liposomes. Still, the lack of labeling in the membrane-bound P450 does not rule out the presence of the NH₂-terminal residue on the cytoplasmic side of the membrane.

Recently, Larson et al. (1991a, 1991b) expressed rabbit liver P450 11E1 in E. coli cells and found that the active enzyme was localized to the inner membrane even when residues 3–29 were deleted. Yabusaki et al. (1988) obtained similar results when residues 1–30 were removed from a P450 expressed in yeast. Although the nature of the interactions of the mutant cytochromes P450 with the membranes were not established, these results suggest that a single membrane-spanning α-helix at the NH₂ terminus might not be the only component of the membrane anchor in the protein. The first hydrophobic segment at the P450 NH₂ terminus has, however, been found to be sufficient to direct correct membrane insertion of test proteins in vitro (Sakaguchi et al., 1987; Vergeres et al., 1989). During revision of this manuscript, Szczesna-Skorupa and Kemper (1993) reported that a chimeric P450 with a 29-amino acid insert containing a potential N-glycosylation site appended to the NH₂ terminus of P450 2C1 was in fact glycosylated. This result indicates that the NH₂-terminal region of the chimeric P450 was oriented toward the ER lumen. However, the rather long peptide addition in the chimera may have disrupted the normal membrane insertion pattern or prevented reorientation of a part of the protein that is normally only transiently exposed to the ER lumen. The topological arrangement of the P450 NH₂ terminus in the ER membrane thus has remained controversial, although the single transmembrane segment anchor model is more generally accepted. Resolution of the question centers on obtaining unambiguous information about the disposition of the extreme NH₂-terminal segment in native cytochromes P450. One of the difficulties with most mammalian cytochromes P450 is that most of the extreme NH₂-terminal segment is hydrophobic, presumably leaving very few amino acid residues to protrude away from the membrane and thus limiting the usefulness of the immunological techniques that have proven so effective in analyzing the topology of the rest of the polypeptide. The NH₂-terminal tail present in mature TXS gives this particular P450 additional antigenic epitopes at the NH₂ terminus, allowing direct immunological study. The tail is not especially long, extending only 6 residues beyond the end of family 3 cytochromes P450 (Fig. 1A), and it is not particularly hydrophobic (Fig. 1C). The hydropathy profile of the very hydrophobic segment following the extension at the NH₂ terminus of TXS coincides very well with the putative membrane anchor segment at the NH₂ terminus of P450 IIIA4 (Fig. 1C). In addition, a dot matrix homology domain comparison of TXS with P450 IIIA4 showed ~60% similarity over 90% of the length of the protein sequences. All of this suggests that the presence of the NH₂-terminal extension in TXS is not likely to give it an overall topology different from that in the other cytochromes P450, although such a perturbation cannot be ruled out.

Fig. 9. Subcellular localization of TXS in PMA-induced THP-1 cells. PMA-induced THP-1 cells permeabilized with STO were reacted with the affinity-purified NT-15 antibody, stained with the FITC-labeled second antibody, and then analyzed by confocal microscopy using Imagespace software and a Screendstar 35-mm camera system.
Two antibodies directed to the NH₂-terminal segment of TXS were used in this study. One (NT-10) was directed to the ultimate 10 residues of mature TXS, and the other (NT-15) to the ultimate 15 residues. Both antibodies specifically recognized their target peptide sequences (Figs. 2–4), and one (NT-10) recognized authentic denatured TXS on nitrocellulose membranes (Fig. 4). The specificity of the interaction of the two peptide antibodies with native TXS was demonstrated by the observation of their binding only to COS-1 cells expressing enzymatically active TXS, and not to COS-1 cells transfected with a vector lacking TXS cDNA (Fig. 7). These results confirm that the antibodies were indeed specific for the intended targets in the NH₂-terminal segment of TXS.

The ability of the NT-15 antibody to bind the cellular TXS in spite of its inability to bind the denatured TXS on nitrocellulose membranes suggests that the epitope recognized by NT-15 requires the native secondary structure in the NH₂-terminal segment. Residues 11–15 may contribute part of the NT-15 epitope or be required to preserve some element of secondary structure in residues 1–10. In any case, the binding of both NT-10 and NT-15 to TXS in STO-permeabilized cells indicates that at least some of the first 10 residues at the NH₂ terminus of TXS protrude from the membrane into the aqueous surroundings. The somewhat more intense staining with both antibodies seen after treatment of THP-1 cells with Triton X-100 (Fig. 8) is consistent with increased access to the NH₂ terminus near the membrane surface upon lipid extraction by the detergents. Cytosolic exposure of the NH₂-terminal epitopes was observed both with recombiant TXS expressed in COS-1 cells (Fig. 7) and with endogenous TXS in THP-1 cells (Fig. 8). This offers some reassurance that the observed TXS membrane orientation was not peculiar to the recombiant protein. It remains possible that the mitogen-induced TXS in THP-1 cells has an NH₂-terminal topology different from that in quiescent THP-1 cells or in other cells.

Larson et al. (1991a, 1991b) found that the NH₂-terminal segment of P450 IIE1 was not necessary for the oxygenase catalytic function of the cytochrome or for its interaction with the reductase or with cytochrome b₅. Similarly, the extreme NH₂ terminus of TXS does not appear necessary for catalytic activity because neither the NT-10 nor the NT-15 antibody inhibited formation of thromboxane A₂ from the enzyme in platelet microsomes (data not shown).

Considerable experimental support has accumulated for the concept that mammalian ER cytochromes P450 are essentially globular proteins anchored to the ER membrane by a segment near their NH₂ termini (Black, 1992). The results of the present study with site-directed antibodies indicate that in one of these cytochromes P450, TXS, the NH₂ terminus itself is on the cytoplasmic side of the ER membrane. Given these topological constraints, the membrane anchor arrangements of the two models lead to opposite predictions about the orientation of the globular domain of TXS with respect to the ER membrane. The hairpin loop anchor model predicts that the globular domain of TXS is on the same side as the NH₂ terminus, in the cytoplasm, whereas the single transmembrane anchor model predicts that this domain is on the side opposite the NH₂ terminus, in the ER lumen. Because a cytoplasmic orientation of the globular domain has been found for several other cytochromes P450 (Thomas et al., 1977; De Lemos-Chiarandini et al., 1987; Edwards et al., 1991), the simplest interpretation of the present data favors the hairpin anchor model.

Some details of the hairpin anchor model are difficult to reconcile with current concepts of the structure of α-helical transmembrane polypeptide segments. Current sequence analysis algorithms predict only one full-length transmembrane α-helical segment at the TXS NH₂ terminus (roughly residues 15–32) (Fig. 1). The portion of TXS that would correspond to the second leg of the hairpin proposed by Nelson and Strobel (1988) is strongly conserved with other cytochromes P450, particularly residues 42–57 (Fig. 1), consistent with some functional importance. However, there are 3 conserved proline residues in this segment of TXS, and such an accumulation of proline residues is unprecedented in the limited number of verified transmembrane polypeptides examined so far. This suggests that the hairpin does not completely traverse the membrane or that the second leg of the hairpin has an unconventional structure.

Despite the extensive similarities between TXS and other cytochromes P450 (Fig. 1), it remains possible that TXS has a membrane anchor arrangement different from those in other cytochromes P450. The NH₂ terminus of TXS is different from that of other cytochromes P450 and could contain unsuspected signals for a distinct membrane insertion process. In this regard, alteration of just 2 amino acid residues in P450 IIC2 was reported to alter the subcellular location of that protein (Kemper and Szczesna-Skorupa, 1989). Further studies will be needed to determine just how homogeneous the membrane topologies of the ER cytochromes P450 are. In any case, this study defines the membrane topology of the NH₂ terminus of TXS and sets the stage for detailed studies of the arrangement of the rest of the protein and of the influence of TXS membrane topology on the physiological function of this important protein.

Acknowledgments—We thank Dr. William T. Moore (Analytical Chemistry Center, University of Texas Medical School, Houston, TX) for peptide syntheses and mass spectrometric analyses, Dr. Hiram Gilbert (Baylor College of Medicine) for the anti-protein disulfide-isomerase antibody, Yong Ren for help with the immunofluorescence experiments, and Aiqing Leng for excellent technical assistance. Dr. K. Angeles’s laboratory (Baylor College of Medicine) provided valuable assistance with the initial confocal microscopy experiments. Dr. Barry Van Winkle and Dr. ConfoLab Microscopy Laboratory (Department of Pathology, University of Texas Health Science Center, Houston, TX) provided additional confocal microscopy services.

REFERENCES

Bernhardt, R., Ngec Dao, N. T., Stiel, H., Schwarze, W., Friedreich, J., Janig, G.-R., and Rückpaal, K. (1985) Biochem. Biophys. Acts 745, 140–148
Black, S. D. (1992) FASEB J. 6, 680–685
Black, S. D., and Coon, M. J. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 60, 35–87
Coon, M. J., Kessler, P., and Pamboukh, D. M. (1990) J. Biol. Chem. 267, 9321–9325
De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D., and Kreibich, G. (1987) J. Biol. Chem. 262, 209–219
DeWitt, D. L., El-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., Heinemann, F., Larson, J. R., Coon, M. J., and Porter, R. B. (1963) Biochemistry 2, 147–148
Doolittle, R. F. (1982) Nature 298, 131–133
Edwards, R. L., Murray, B. F., Singleton, A. M., and Boobis, A. R. (1991) Biochem. Biophys. Curr. Top. Ser. 40, 1–76
Erlanger, B. F., Borek, F., Reiser, S. M., and Lieberman, S. (1989) J. Biol. Chem. 264, 1099–1094
Guieu, K., and Dixon, J. (1991) Anal. Biochem. 192, 262–267
Hamberg, M., Svensson, J., and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2984–2988
Houard, M., and Ulrich, V. (1985) J. Biol. Chem. 260, 15059–15067
Heinemann, F. U., and Oszlo, J. (1985) J. Biol. Chem. 260, 14988–14999
Kemper, B., and Szczesna-Skorupa, E. (1989) Drug Metab. Rev. 20, 811–820
Kanai, Y., and Langs, W. F. (1987) in Prostaglandins and Related Substances: A Practical Approach (Benedetto, C., McDonald-Gibson, R. C., Nigam, S., and Slater, T. F., eda) pp. 209–227, IRL Press, Oxford
Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–152
Lemberm, U. K. (1970) Nature 227, 685–686
Larson, J. R., Coon, M. J., and Porter, T. D. (1991a) J. Biol. Chem. 266, 7321–7324
Larson, J. R., Coon, M. J., and Porter, T. D. (1991b) Proc. Natl. Acad. Sci. U. S. A. 88, 9141–9145
Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149–2154
Monier, S., Van Luc, P., Krebich, G., Sabatini, D. D., and Adesnik, M. (1988) J. Cell Biol. 107, 457–470
Moore, W. T., and Caprilli, R. M. (1991) in Techniques in Protein Chemistry (Vilainfrance, J. J., ed) Vol. 11, pp. 511–529, Academic Press, New York
Nelson, D. R., and Strobel, H. W. (1988) J. Biol. Chem. 263, 6058–6060
Ohashi, K., Ruan, K.-H., Kulmace, R. J., Wu, K. K., and Wang, L.-H. (1992) J. Biol. Chem. 267, 789–793
Oshio, S., Heinemann, P. S., and Johnson, E. F. (1985) J. Biol. Chem. 260, 5427–5434
Ruan, K.-H., Hashida, S., Yoshitake, S., Ishikawa, W., Wakesaka, O., Yamamoto, Y., Ichikawa, T., and Nakajima, K. (1985) Clin. Chim. Acta 147, 167–172
Sakaguchi, M., Mihara, K., and Sato, R. (1987) EMBO J. 6, 2425–2431
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Sanduja, S. K., Mehta, K., Xu, X. M., Sanduja, R., and Wu, K. K. (1991) Blood 78, 3178–3185
Shen, R.-F., and Tai, H.-H. (1986a) J. Biol. Chem. 261, 11585–11591
Shen, R.-F., and Tai, H.-H. (1986b) J. Biol. Chem. 261, 11592–11599
Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 77, 31–40
Szczerba-Skorupa, E., and Kemper, B. (1993) J. Biol. Chem. 268, 1757–1762
Thomas, P. G., Lu, A. Y. H., Wurt, S. B., Ryan, D., Mowa, G. T., and Levin, W. (1977) Mol. Pharmacol. 13, 819–831
Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
Ullrich, V., and Graf, H. (1984) Trends Pharmacol. Sci. 5, 352–355
Vergeres, G., Winterhalter, K. H., and Richter, C. (1989) Biochemistry 28, 3650–3655
Vergeres, G., Winterhalter, K. H., and Richter, C. (1989) Biochem. Biophys. Acts 1063, 235–241
Wang, L.-H., Ohashi, K., and Wu, K. K. (1991) Biochem. Biophys. Res. Commun. 178, 286–299
Yabusaki, Y., Murakami, H., Sakaki, T., Shibata, M., and Ohkawa, H. (1988) DNA (N. Y.) 7, 701–711
Yamamoto, S., Yokota, K., Tanai, T., Sheno, F., and Hayashi, Y. (1987) in Prostaglandins and Related Substances (Benedictio, C., McDonald-Gibson, R. G., Nigam, S., and Slater, T. F., eds) pp. 197–208, IRL Press, Oxford
Yokoyama, C., Miyata, A., Ibara, H., Ullrich, V., and Tanabe, T. (1991) Biochem. Biophys. Res. Commun. 178, 1479–1484