Membrane Topology and Cell Surface Targeting of Microsomal Epoxide Hydrolase

EVIDENCE FOR MULTIPLE TOPOLOGICAL ORIENTATIONS

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Microsomal epoxide hydrolase (mEH) is a bifunctional membrane protein that plays a central role in the metabolism of xenobiotics and in the hepatocyte uptake of bile acids. Numerous studies have established that this protein is expressed both in the endoplasmic reticulum and at the sinusoidal plasma membrane. Preliminary evidence has suggested that mEH is expressed in the endoplasmic reticulum (ER) membrane with two distinct topological orientations. To further characterize the membrane topology and targeting of this protein, an N-glycosylation site was engineered into mEH to serve as a topological probe for the elucidation of the cellular location of mEH domains. The cDNAs for mEH and this mEH derivative (mEHg) were then expressed in vitro and in COS-7 cells. Analysis of total expressed protein in these systems indicated that mEHg was largely unglycosylated, suggesting that expression in the ER was primarily of a type I orientation (Ccyt/Nexo). However, analysis, by biotin/avidin labeling procedures, of mEHg expressed at the surface of transfected COS-7 cells, showed it to be fully glycosylated, indicating that the topological form targeted to this site originally had a type II orientation (Cexo/Ncyt) in the ER. The surface expression of mEH was also confirmed by confocal fluorescence scanning microscopy. The sensitivity of mEH topology to the charge at the N-terminal domain was demonstrated by altering the net charge over a range of 0 to +3. The introduction of one positive charge led to a significant inversion in mEH topology based on glycosylation site analysis. A truncated form of mEH lacking the N-terminal hydrophobic transmembrane domain was also detected on the extracellular surface of transfected COS-7 cells, demonstrating the existence of at least one additional transmembrane segment. These results suggest that mEH may be integrated into the membrane with multiple transmembrane domains and is inserted into the ER membrane with two topological orientations, one of which is targeted to the plasma membrane where it mediates bile acid transport.

Hepatic microsomal epoxide hydrolase (mEH)† (EC 3.3.2.3)

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The abbreviations used are: mEH, microsomal epoxide hydrolase; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; mEHg, mEH with introduced glycosylation site; mEHN, N-terminal truncated mEH; Pgpp, P-glycoprotein fragment; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; nt, nucleotide(s); NYT, Asn-Tyr-Thr.

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EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Procedures—COS-7 cells were obtained from American Type Culture Collection and were transfected with rat cDNA for mEH or mEH derivatives in the pcDNA1.1/Amp vector (Invitrogen) using the DEAE-dextran procedure to obtain transient transformants (25). Cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

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mEH DNA/pBSK Construct—The cDNA for mEH was prepared by reverse transcription and the polymerase chain reaction procedure (26). The 5′ primer used, ATGG-GTCGACGGGACCTTGGTTTCCAGGAAA, was targeted to the cDNA sequence 45–106 and the second primer (underlined) at position 1559–1579 (Fig. 1A). The polymerase chain reaction product was digested with SalI and KpnI and cloned into the vector pBSV. The mEH cDNA was then isolated as an SalI/EcoRI (linker site) fragment and inserted into pBSK (Bluescript/Stratagene) digested with SalI and EcoRI. The fidelity of the cDNA, the amino acids was used to terminate the translation of the chimeric protein. The mEH termination codon following the last 15 amino acids was included to radiolabel newly synthesized protein. The effect of the mutation on the C-terminal amino acids was made by replacing a wild type fragment with a synthesized short extragenic DNA fragment. The fragment was then translated in vitro with T3 RNA polymerase (Ambion/MEGAscript) and visualized by autoradiography.

In Vitro Transcription/Translation of mEH and mEH Derivatives—The expression of mEH and mEH derivatives was investigated by in vitro transcription/translation with the various mEH cDNA constructs inserted in pBSK. These constructs were linearized by EcoRI digestion and in vitro transcribed with T3 RNA polymerase (Ambion/MEGAscript T3 kit). The resultant mEH mRNA (1 μg) was then translated in vitro with the rabbit reticulocyte lysate system (Promega) using conditions recommended by the manufacturer in the presence and absence of canine microsomal membranes (Promega). [35S]Methionine (40 μCi) was included to radiolabel newly synthesized protein. The effect of the glycosylation inhibitor tripeptide Asn-Tyr-Thr (NYT) on the synthesis of mEH and mEH derivatives was monitored using a tritiated [3H]leucine glycoprotein as described previously (29). The translated products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

Addition of an Internal Glycosylation Site—A glycosylation site (Asn-Tyr-Thr) was introduced near the C terminus of mEH (mEH$_{5}$) by replacing the wild type Phe-Ala-Ala (residue 432–434, Fig. 2). The double stranded DNA fragment, plus the Pgp Patl/HindIII fragment, and the mEH/pBSK construct in which the Xhol/HindIII fragment had been removed as shown in Fig. 1A. The resulting construct encoded the entire mEH polypeptide (455 amino acids), followed by a Pgp poly peptide tail (Pgp amino acid 439 to 678), which contains a glycosylation site at position 491, followed by the last 15 amino acids (440 to 455) of mEH (Fig. 1A). The cDNA was inserted in pBSK in the sense orientation (Fig. 1A), which encoded a mEH/Pgp fusion protein with the Ser codon at residue 20 of the wild type mEH polypeptide. Thus, this replacement links the ATG codon directly to residue 20, deleting the Ser codon at residue 20 of the wild type mEH polypeptide. Thus, this replacement links the ATG codon directly to residue 20, deleting the Ser codon at residue 20 of the wild type mEH polypeptide.

Deletion of the N-terminal Transmembrane Domain—This deletion was made by replacing the wild type fragment with a synthesized shortened 5′ end of the mEH/pBSK construct (Fig. 1B). The mEH fragment was then digested with restriction enzymes SplI and HindIII and inserted into a wild type SplI/HindIII fragment. The sequence was then isolated as a SalI/EcoRI fragment and inserted into the pBSK/Pgp vector.

Cell Surface Biointylation—The expression of mEH and mEH derivatives on the plasma membrane of COS-7 cells was established using a cell surface biointylation labeling procedure with sulfosuccinimidyl-6-(bixinnamidoo)hexanate (SuFl-NHS-Biotin) (Pierce) on transfected and untransfected cells as described previously (17). The labeled cell surface proteins were isolated using avidin-agarose beads, and mEH and the mEH derivatives were detected by SDS-PAGE and Western blot analysis. To establish that the reagent labeled only cell surface protein, the biointylation of the cytoplasmic protein, Hsp 70 was also determined using an anti-Hsp 72/23 monoclonal antibody (Roche Molecular Biochemicals) as described previously (21). In addition, COS-7 cells were
disrupted by suspending in 20 mM Tris, pH 7.4 (1 ml), frozen and thawed (2×) and homogenized with 100 strokes in a tight Dounce homogenizer. The soluble fraction isolated by centrifugation was biotinylated as described for intact cells. Following biotinylation, the excess reagent was removed by dialysis against phosphate-buffered saline for 24 h, and the resultant protein was treated as described for intact cells and the biotinylated Hsp 70 determined as described above.

Deglycosylation of mEH Derivatives—Glycosylated forms of mEH derived from the COS-7 cell plasma membrane or directly from the microsome fraction were deglycosylated with Endo H (New England Biolabs) using procedures supplied by the manufacturer. Proteins were then detected by SDS-PAGE and Western blot analysis. Inhibition of the Glycosylation Reaction by the Tripeptide NYT—The tripeptide Asn-Tyr-Thr, with both the N and C termini blocked as acetyl-Asn-Tyr-Thr-amide, has been shown to be an effective substrate for oligosaccharyl transferase (29) and to inhibit glycosylation reactions in vitro (30). This tripeptide was kindly provided by Dr. R. A. F. Reithmeier (University of Toronto, Canada) and was included in the in vitro translation mixture at a final concentration of 46 μM to inhibit the in vitro glycosylation reaction of the mEH(+/+) derivative.

Proteinase K Digestion—After in vitro translation, proteinase K was added to a final concentration of 0.1 mg/ml, followed by incubation at 0 °C for 60 min as recommended by Roche Molecular Biochemicals. Phenylmethylsulfonyl fluoride was then added to a final concentration of 4 mM to stop the digestion before the reaction product was analyzed with SDS-PAGE.

Confocal Immunofluorescence Analysis of Transfected COS-7 Cells—COS-7 cells transfected with the cDNA for mEH, mEH(0), mEH(+3), and mEH were incubated with anti-mEH monoclonal antibody, mAb 25D-1 (1 μg/ml) for 18 h at 4 °C, and fixed with 4% paraformaldehyde before incubation with a secondary antibody, cy3-conjugated antimouse IgG for 4 h at 24 °C. Antibody labeling was characterized by cy3 epifluorescence using a Zeiss LSM-510 scanning confocal microscope equipped with a barrier filter. Image analysis was performed using the standard system operating software.

RESULTS

Addition of an N-Glycosylation Site and N-terminal Charge Modification of mEH—The membrane topology of mEH was characterized by the introduction of N-glycosylation sites into two different loci of mEH, which itself lacks such sites, to establish whether a particular protein domain is expressed in the lumen of the endoplasmic reticulum. A fragment of P-glycoprotein (Pgpf) amino acids 394–678 containing a potential glycosylation site at amino acid 439 (28), which has been used to study the topology of the cystic fibrosis transmembrane conductance regulator (19), has been linked to mEH at position 454 as shown in Fig. 1A. A glycosylation site was also directly introduced into full-length mEH at position 432 by site-directed mutagenesis (Fig. 1B) resulting in the conversion of Phe-Ala-Ala to Asn-Gly-Thr (Fig. 2). In addition, the net charge of the N-terminal end preceding the 16-residue hydrophobic domain was modified over a range of 0 to +5 as shown in Fig. 2 to investigate the role of charge in regulating mEH insertion into the ER membrane in vitro and COS cell expression systems, and also to define the topological form of mEH that is targeted to the plasma membrane.

In Vitro Translation of mEH and mEH Derivatives—The various mEH constructs in pBSK were in vitro transcribed with T7 RNA polymerase and then translated with the rabbit reticulocyte lysate system (Promega) in the presence and absence of canine pancreas microsomes. The mEH(0)/Pgpf chimera containing a glycosylation site was translated yielding a peptide with the expected molecular mass (80.7 kDa). However, in the presence of microsomes, no glycosylated higher molecular weight species was observed indicating an undetectable amount of the Pgpf tail on mEH was located in the ER lumen, thus suggesting primarily a type I topology in the ER (Fig. 3A, lanes 1 and 2). A mEH derivative in which Trp (position 2) was replaced with Asn was translated yielding a peptide representing the N-terminal end preceding the 16-residue hydrophobic domain (Fig. 2). In addition, the net charge of the N-terminal end preceding the 16-residue hydrophobic domain was modified over a range of 0 to +5 as shown in Fig. 2 to investigate the role of charge in regulating mEH insertion into the ER membrane in vitro and COS cell expression systems, and also to define the topological form of mEH that is targeted to the plasma membrane.

in the cell-free system. In the absence of microsomes, a single band was observed that had the same mobility as the chimera composed of mEH(0)/Pgpf (Fig. 3A, lane 3). In the presence of microsomes, however, two bands were observed, indicating that approximately 40% of the expressed protein was glycosylated, and suggesting that the glycosylation site must be partially located in the lumen (Fig. 3A, lane 4).
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The partial glycosylation of this derivative observed in vitro indicated that the cell-free system was not as efficient as the COS-7 cell system in expressing the type II orientation. Glycosylation of the mEHg(+3) product was confirmed by deglycosylation with Endo H, resulting in a single band with the mobility of mEH (Fig. 4, lane 4).

The expression of mEH and mEHg(0) at the cell surface of transfected COS-7 cells was next investigated using biotinylation labeling procedures as previously described for aromatase P450 (17) and the ileal sodium-dependent bile acid transporter (21), in an effort to obtain definitive evidence that mEHg(0) as well as mEH with a type II topology in the ER membrane, was targeted to the plasma membrane. As shown in Fig. 5 (lane 1), the biotinylated cell surface product obtained from the COS-7 cells expressing mEH without an inserted glycosylation site, afforded a single band with a mobility identical to mEH obtained from the total cell lysate (Fig. 4, lane 1), suggesting that some percentage of the expressed protein was accessible to the labeling reagent on the cell surface. In contrast, the surface biotinylation of COS-7 cells expressing mEHg(0) afforded only a protein with a higher molecular weight (Fig. 5, lane 2) than the main protein found in the total cell lysate (Fig. 4, lane 2). This material was also treated with Endo H as described above for mEHg(+3) (Fig. 4, lane 4), yielding a product with the same molecular weight as mEH (Fig. 5, lane 3), thereby establishing that the increased size of this product resulted from glycosylation of the introduced glycosylation site in the ER lumen. This result confirmed the thesis that mEH can exist in two topological forms in the ER with the type II form targeted to the plasma membrane. mEHg(+3), which afforded only a fully glycosylated product (Fig. 4, lane 3) in the ER, was also targeted to the plasma membrane, where biotinylation identified the glycosylated derivative (Fig. 5, lane 4). It was established that the biotinylating reagent reacted only with surface proteins by demonstrating that unglycosylated mEHg(0) was not labeled in intact cells (Fig. 5, lane 2), although it is the major intracellular form of the protein (Fig. 4, lane 2). This conclusion was confirmed by demonstrating that the endogenous cytoplasmic protein, Hsp 70 was labeled only in cell lysates and not in intact cells (data not shown).

The Effect of N-terminal Charge on mEH Topology—The alteration of the net N-terminal charge from 0 to +3 has been shown to result in a change in mEH topology from primarily a type I to a type II orientation. To further evaluate the role of charge on the topological orientation of mEH, three additional derivatives were prepared with altered N-terminal charges at different positions. When the negative charge at position 4 was removed (Glu to Gly), the resultant derivative (mEHg(+1a)) (Fig. 2) was, like mEHg(0), primarily expressed in the ER with a type I orientation (Fig. 4, lane 5) as estimated by the level of glycosylation (Table I). When a positive charge was added at
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FIG. 5. Analysis of cell surface expression of mEH and mEH derivatives in transfected COS-7 cells by biotinylation procedures. mEH and mEH derivatives were transiently expressed in COS-7 cells. Cell surface proteins were labeled with succinimidyl-6-(biotinamido)hexanoate and isolated using avidin-agarose. Bound proteins were then analyzed by SDS-PAGE and immunoblotting as described in Fig. 3. Biotinylated surface protein from 2 to 150-100 nm plates was applied to each lane. mEHg(0)d indicates deglycosylated mEHg(0).

TABLE I

| Derivative (net charge) | N-terminal charge display a | Type II orientation | % |
|------------------------|---------------------------|---------------------|---|
| mEHg(0)                | (+000)                    |                     | 2 |
| mEHg(+1)a              | (+000)                    |                     | 3 |
| mEHg(+1)b              | (+000)                    |                     | 3 |
| mEHg(+2)               | (+000)                    |                     | 3 |
| mEHg(+3)               | (+000)                    |                     | 3 |

a Refers to the N-terminal 4 amino acids as described in Fig. 2.

FIG. 6. Confocal immunofluorescence analysis of transfected COS-7 cells. Transfected COS-7 cells expressing mEH and mEH derivatives were incubated with an anti-mEH monoclonal antibody (25D-1) followed by cy3-conjugated goat anti-mouse IgG. Cell diameter: 18–22 μm. A, mEHg(0); B, mEH; C, mEHg(+3); D, mEHt; E, nonimmune control for A; F, untransfected COS cells; G, mEHg(0) transfected cells permeabilized with digitonin.

Previous studies have demonstrated that mEH is targeted to both the ER and plasma membrane, where it mediates the transport of bile acids (8, 11, 12, 18). Epitope accessibility and resistance to proteolysis suggested that mEH was expressed in the ER membrane with two distinct topological orientations (18), because a common epitope for mEH was found on both the hepatocyte cell surface and on the cytoplasmic face of the ER. To further establish the topology and targeting properties of mEH, use has been made of glycosylation site insertion, avidin/ avidin labeling technology and confocal fluorescence scanning microscopy. Initial studies using an in vitro expression system with a glycosylation site located either in a P-glycoprotein fragment linked to mEH (mEH/Pgp) or directly engineered into mEH at position 432 (mEHg(0)) indicated that a single unglycosylated product was formed (Fig. 3A). Alteration of the N-terminal charge from 0 to +3 (mEHg(+3)), however, afforded a glycosylated product, establishing that this alteration in charge led to a topological inversion that placed the glycosylation site in the ER lumen. The addition of the internal glycosylation site at position 432 was affected with conservative substitutions (Fig. 2) and located far from the N-terminal transmembrane domain in a region of mEH that exhibits numerous amino acid variations between species (31, 34, 35) where total protein expression levels were approximately the same as observed for unmodified mEH.

The expression of mEHg(0) was also carried out in an intact COS-7 cell system where, in a total cell lysate, mEHg(0) afforded primarily an unglycosylated form, with a small percent of the higher molecular weight glycosylated protein (Fig. 4, lane 2), whereas the modified mEHg(+3) was expressed only in the glycosylated form (Fig. 4, lane 3). These glycosylation results are qualitatively similar to those previously reported for mEH when the potential glycosylation site was inserted at position 39 or 303 (20). These studies, however, analyzed only total expressed protein with an indicated lower limit of detec-
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...tion of 5–10% (36) and did not investigate mEH expressed at the plasma membrane. Analysis of intact transfected COS-7 cells with the biotin/avidin surface labeling procedure established that mEH(0) that was targeted to the plasma membrane was in the glycosylated form (Fig. 5, lane 2), thereby establishing a luminal type II orientation for a fraction of the total expressed protein. Native mEH was also biotinylated on the cell surface, but the protein targeted to the plasma membrane had an unaltered molecular weight, as expected in the absence of a glycosylation site. The presence of mEH(0) (Fig. 6) as well as mEH, mEH(+3), and mEHt on the surface of transfected COS-7 cells was corroborated using confocal fluorescence spectroscopy, confirming that a portion of the 25D-1 epitope was originally in the ER lumen, because it reacts with the antibody on the cell surface. Similar results were observed for Madin-Darby canine kidney cells stably transfected with mEH and for intact hepatocytes. These results are consistent with our earlier studies, which showed that an anti-mEH monoclonal antibody (25A-3) could protect both hepatocytes (8) and transfected Madin-Darby canine kidney cells expressing mEH (12) from DIDS inhibition of taurocholate transport, and which demonstrates that these cells possess a percentage of the type II form of mEH, which would result in expression of mEH on the extracellular surface where it could mediate the binding and transport of bile acids. The relationship between total mEH expression and cell surface expression is under investigation.

Various factors have been shown to influence the topological orientation of proteins in the ER membrane, such as the distribution of charged residues flanking the signal anchor sequence, where positive charges are enriched on the cytosolic side and depleted from the luminal (exoplasmic) side of the first transmembrane domain (37–39). The role of charge on membrane protein topology has been confirmed by site-directed mutagenesis (40). Protein topology is also influenced by the folding properties of the N-terminal sequence (41) as well as the length and hydrophobicity of the transmembrane segment (39, 42, 43). The resultant topology (or topologies) of a membrane protein is thus determined by a complex interaction of these factors. The charge distribution (\(\Delta(C-N)\)) around the N-terminal anchor of mEH (-2 for rat, see Ref. 31; -3 for human, see Ref. 34; and -4 for rabbit, see Ref. 35) predicts a type II orientation. However, a majority of mEH in the ER is found in the opposite type I orientation, stressing that multiple factors acting in concert determine protein topology, and can lead, in some cases, to the expression of more than one topological form. The effect of charge alterations reported in this study (Table 1) stresses the exquisite sensitivity of mEH topology to this variable so that subtle variations have a dramatic effect on the ratio of the two topological forms. Studies with aromatase cytochrome P450 offer a clear illustration of the above paradigm. Based on the degree of glycosylation of a naturally occurring glycosylation site, this protein is expressed in two topological orientations (17), with one of the two forms expressed at the plasma membrane. Several other membrane proteins have been described, which are expressed in more than one topological form, such as the prion protein (44), P-glycoprotein (45), and ductin (46).

Sequence motifs known to act as retrieval signals for resident ER proteins with a Nexo (47) or Ncyt (48) orientation are not found in mEH. Structural features within the N-terminal hydrophobic domain may also function as an ER retention signal (49) as described for cytochrome P450 2C1, perhaps by mediating the formation of homo- or hetero-oligomers, which prevent transport from the ER compartment. Other proteins destined to reside in the plasma membrane leave the ER by default and travel along the exocytic pathway (50). The two topological forms of mEH may undergo differential oligomerization, resulting in the expression of mEH in the plasma membrane by default or through a specific targeting pathway. This idea is again illustrated by studies with aromatase cytochrome P450 that have established that only one of the two topological forms present in the ER membrane is targeted to the plasma membrane (17). Several other members of the cytochrome P450 superfamily together with NADPH:cytochrome P450 reductase (17) have also been shown to be expressed on the hepatocyte cell surface (51) and in the ER. The two topological forms of ductin described above are targeted to different cellular domains where this protein functions as either a component of a connexon channel of gap junctions or as subunit c of the vacuolar H+-ATPase (46).

The number of transmembrane domains that anchor mEH in the ER has been the subject of numerous studies. Hydropathy analysis indicates the presence of a hydrophobic N-terminal region as well as several additional domains that could integrate into the membrane (11, 31). Studies using truncated mEH suggested that mEH has only a single transmembrane domain, based on the results of alkaline extraction of membranes containing mEH or the truncated derivative (32). The expression of this truncated derivative, however, was only 5% of the level observed for mEH in BHK cells, and the authors failed to point out that approximately 40% of the truncated mEH appeared to be resistant to this extraction procedure, thereby leaving the conclusion of this study open to question. The identification of a truncated mEH derivative (mEHt) on the surface of transfected COS-7 cells (Fig. 5, lane 5) clearly establishes that mEH must integrate into the membrane with more than one transmembrane domain to have mEHt targeted and expressed on the extracellular surface. Similar conclusions have been obtained with aromatase P450, where a truncated version of this protein was also identified on the surface of transfected COS-7 cells (17), suggesting the presence of at least one additional transmembrane domain apart from the N-terminal hydrophobic domain. These results are supported by previous studies on cytochrome P450 IIE1 (52) and P450 1A1 (53). Because mEH residues 39, 303 (20), and 432 (this study) are expressed on the same side of the membrane in either the type I or type II orientation, based on glycosylation site analysis, a model with three transmembrane domains would be consistent with the current available data. The oligomerization of mEH could then lead to a system spanning the membrane multiple times, which would be consistent with its role as a bile acid transporter. Evidence for mEH oligomerization with itself as well as with cytochrome P450 and NADPH:cytochrome P450 reductase has been reported (54).

In conclusion, these studies have demonstrated that mEH is expressed with two distinct topological orientations in the ER membrane and that orientation is extremely sensitive to alterations in charge in the N-terminal 5 amino acids. The use of glycosylation site insertion and surface biotinylation has also established that one of these topological forms is targeted to the plasma membrane where it can function as a bile acid transport protein. Expression of a truncated derivative of mEH on the cell surface also establishes that mEH is integrated into the ER with more than one transmembrane domain. In addition to the bile acid transport function of mEH, the presence of various cytochrome P450s and the cytochrome P450 reductase on the hepatocyte cell surface raises provocative questions concerning the role of these proteins in extracellular carcinogen metabolism.

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