The Membrane Binding Domains of Prostaglandin Endoperoxide H Synthases 1 and 2

PEPTIDE MAPPING AND MUTATIONAL ANALYSIS*

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Prostaglandin endoperoxide H synthases 1 and 2 (PGHS-1 and -2) are the major targets of nonsteroidal anti-inflammatory drugs. Both isozymes are integral membrane proteins but lack transmembrane domains. X-ray crystallographic studies have led to the hypothesis that PGHS-1 and -2 associate with only one face of the membrane bilayer through a novel, monotopic membrane binding domain (MBD) that is comprised of four short, consecutive, amphipathic α-helices (helices A–D) that include residues 74–122 in ovine PGHS-1 (oPGHS-1) and residues 59–108 in human PGHS-2 (hPGHS-2). Previous biochemical studies from our laboratory showed that the MBD of oPGHS-1 lies somewhere between amino acids 25 and 166. In studies reported here, membrane-associated forms of oPGHS-1 and hPGHS-2 were labeled using the hydrophobic, photoactivatable reagent 3-trifluoro-3-(m-125I)iodophenyl)diazirine, isolated, and cleaved with AspN and/or GluC, and the photolabeled peptides were sequenced. The results establish that the MBDs of oPGHS-1 and hPGHS-2 reside within residues 74–140 and 59–111, respectively, and thus provide direct biochemical support for the hypothesis that PGHS-1 and -2 do associate with membranes through a monotopic MBD. We also prepared HeIA, HeIB, and HeIC mutants of oPGHS-1, in which, for each helix, three or four hydrophobic residues expected to protrude into the membrane were replaced with small, neutral residues. When expressed in COS-1 cells, HeIA and HeIC mutants exhibited little or no catalytic activity and were present, at least in part, as misfolded aggregates. The HeIB mutant retained about 20% of the cyclooxygenase activity of native oPGHS-1 and partitioned in subcellular fractions like native oPGHS-1; however, the HeIB mutant exhibited an extra site of N-glycosylation at Asn104. When this glycosylation site was eliminated (HeIB/N104Q mutation), the mutant lacked cyclooxygenase activity. Thus, our mutational analyses indicate that the amphipathic character of each helix is important for the assembly and folding of oPGHS-1 to a cyclooxygenase active form.

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The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; PG, prostaglandin; hPGHS-2, human PGHS-2; oPGHS-1, ovine PGHS-1; MBD, membrane binding domain; NTA, nitritolriacetic acid; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; Tricine, N-2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine.
with an underlying lipid layer. These helices also surround an opening through which fatty acid substrates are believed to enter the cyclooxygenase active site.

It is important to our understanding of PGHS biology and the structural biology of membrane proteins in general to understand how PGHS-1 and -2 interact with cellular membranes. In this report, we describe experiments designed to identify and characterize biochemically the regions of PGHS-1 and PGHS-2 that interact with cellular membranes by using a combination of photoaffinity labeling with 3-trifluoro-3-[(125)I]iodoacetic acid ([125]IITID) (28) and mutagenic analyses of the α-helices proposed to be involved in membrane binding.

EXPERIMENTAL PROCEDURES

Materials—All materials were purchased from Sigma unless otherwise noted. [125]IITID, DEA-EE-Sepharose, and Protein A-Sepharose were purchased from Amersham Pharmacia Biotech. The nonionic detergent C10E6 was purchased from Anatrace, Inc. Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. Fetal bovine serum and bovine calf serum were from Hyclone. CsCl was from Roche Molecular Biochemicals. Arachidonic acid was purchased from Cayman Chemical Co. Oligonucleotides were from the Macromolecular Structure Facility at Michigan State University. Nickel-NTA was purchased from Qiagen. N-Isopropylidioacetic acid was from Molecular Probes, Inc. PVDF membranes were from Millipore Corp.

Preparation of Microsomal oPGHS-1 and hPGHS-2—Microsomes containing oPGHS-1 were prepared from sheep seminal vesicles essentially as described previously (29, 30) and assayed for protein using the Bio-Rad Bradford protein assay reagent. Microsomal hPGHS-2 was prepared from SF21 insect cells that had been infected with baculovirus containing the coding region for hPGHS-2 engineered to contain a hexahistidine tag near the N terminus.2 SF21 insect cells were grown in 500-ml spinner flasks or in a 12-liter bioreactor in Sigma TC-100 medium containing 10% fetal calf serum. When the cell density had reached 1.8 × 10^6 cells/ml, cells were infected with baculovirus encoding His-tagged hPGHS-2. Infection was allowed to proceed for 3 days. Approximately 9.0 × 10^6 SF21 cells were harvested by centrifugation and resuspended in 10 ml of 0.1 M Tris-HCl, pH 7.4. Cells were then disrupted by vigorous sonication using a microtip attachment to a Hornsonic separator. After low speed (10,000 × g for 10 min) centrifugation to pellet cell debris, microsomal membranes were collected by centrifugation at 200,000 × g for 40 min in a Beckman SW50.1 swinging bucket rotor. The microsomal pellets were resuspended in 2 ml of 0.3 M Tris-HCl, pH 7.4, Dounce-homogenized, and assayed for protein using the Bio-Rad Bradford protein assay reagent.

[125]IITID Labeling of PGHS-1 and -2—Steps in this procedure were carried out at 4 °C unless otherwise noted. [125]IITID was ordered as soon as possible after its synthesis and used within 1 week. Microsomal preparations of oPGHS-1 or hPGHS-2 were prepared as described above and resuspended to a final protein concentration of 15–20 mg/ml in the appropriate buffer. Aliquots (400 μl) were preincubated in the presence of 80 μM furibuprofen to block the ability of [125]IITID to label the PGHS active site (30). In some experiments, 200 μM reduced glutathione was included to scavenge [125]IITID exposed to the aqueous phase. In no case did glutathione scavenging affect the [125]IITID labeling pattern (data not shown), indicating that [125]IITID was not reacting with labile portions of PGHS within the hydrophobic core of the membrane (28, 30). To each 400-μl aliquot of microsomal protein, approximately 50 μCi of [125]IITID was added to a final concentration of 10–12 μM, depending on the lot. After incubation for 10 min, resuspended microsomes were transferred to a quartz cuvette (path length = 1 cm) and irradiated for 5 min with a UV illuminator (366 nm) held at a distance of 5 cm. Labeled membrane proteins were solubilized using the detergent C5E6 at a concentration of 1% (v/v) for 1 h with gentle shaking. oPGHS-1 or hPGHS-2 was purified from the solubilized membrane protein fraction using immunoprecipitation or nickel-agarose, respectively.

Immunoprecipitation of oPGHS-1 from Solubilized Microsomes—Following [125]IITID labeling, solubilized microsomal membrane proteins were transferred to a fresh tube. A monoclonal antibody directed against oPGHS-1 (50 ml, 0.1 mg/ml) (51) was added to the solubilized proteins, and the sample was allowed to incubate with shaking for 30 min at 4 °C. PGHS-1 was precipitated by the addition of 100 μl of Protein A-Sepharose as described by the manufacturer. The immunocomplex was collected by centrifugation at 500 × g for 10 min on a table top microcentrifuge and washed three times with 0.1 M Tris-HCl, pH 8.0, containing 0.1% Tween 20. To elute immunoprecipitated oPGHS-1, the immunocomplex was resuspended in 5.0 ml recrystallized SDS and exposed to 80 °C for 5 min. Protein A-Sepharose was added to this sample with periodic vortexing. After 10 min, the sample was centrifuged to pellet cell debris, microsomal membranes were collected by centrifugation at 1000 × g for 2 min in a Beckman table top centrifuge, and the resin was washed three times for 5 min in wash buffer (10 mM sodium phosphate, 500 mM NaCl, 30 mM imidazole, 0.1% C5E6, pH 8.0). Photoaffinity-labeled oPGHS-2 was eluted from the nickel-NTA by incubation in elution buffer (10 mM sodium phosphate, 200 mM NaCl, 200 mM imidazole, pH 7.2). The Protein A-Sepharose was collected by centrifugation at 1200 rpm for 2 min in a Beckman table top centrifuge, and the resin was washed three times for 5 min in wash buffer (10 mM sodium phosphate, 500 mM NaCl, 30 mM imidazole, 0.1% C5E6, pH 8.0). Photoaffinity-labeled hPGHS-2 was eluted from the nickel-NTA by incubation in elution buffer (10 mM sodium phosphate, 200 mM NaCl, 200 mM imidazole, pH 7.2) for 1 h at 4 °C. hPGHS-2 eluted from nickel-NTA was dialyzed against 10 mM Tris-HCl, pH 8.5, and further purified by anion exchange chromatography. hPGHS-2 bound to DEA-EE-Sepharose at pH 8.5 and was eluted with 50 mM Tris-HCl, pH 6.5. Eluted protein was concentrated using a Millipore Ultra-Free concentrator to a volume of 1 ml, and approximately 400 μl and 10 μl of protein were applied to the nickel-NTA, pH 8.2, prior to denaturation, reduction, and alkylation.

Denaturation, Alkylation, Reduction, and Proteolysis of oPGHS-1 and hPGHS-2—Prior to denaturation, [125]IITID-labeled protein samples were concentrated to a volume of 25 μl. Samples were denatured by incubation in 6 M guanidinium hydrochloride (final concentration) at 55 °C for 3 h. freshly prepared 1 M dithiothreitol was then added to a final concentration of 10 mM. After covering with argon, the sample was incubated at 55 °C for 2 h. Reduced disulfide bridges were prevented from reforming by the addition of 7 μl of freshly prepared N-isopropylidioacetic acid (2.3 mg/100 μl in 20% methanol) and incubation at room temperature in the dark for 1 h. After the alkylation step, samples were dialyzed against 4 liters of either 0.05 M NH4HCO3, pH 7.9 (for Glc digestion) or 10 mM Tris-HCl, pH 8.5 (for AspN digestion) for 12–15 h in a Fisher 0.5-m1 Slide-A-Lyzer (molecular mass = 10,000 Da). Prior to proteolysis, samples were concentrated in a SpeedVac to a volume of approximately 50 μl. Endoprotease Gluc or AspN was added such that the final ratio of protease to PGHS-1/2 was approximately 1:50. Digestions were performed at 37 °C for 20–24 h. Proteolytic fragments were electrophoresed as described below.

SDS-PAGE and Analysis of Proteolytic Products—The following precautions were employed to prevent the N-terminal blockade of peptides separated by SDS-PAGE: (a) highly purified, fresh acrylamide reagents and buffers were used to prepare slab gels; (b) the gels were allowed to polymerize overnight at room temperature before use; and (c) recrystallized SDS was used in all buffers and gels. Proteolytic peptides or unmodified controls were separated on 16% (w/v) SDS-polyacrylamide slab gels and transferred to PVDF membranes for Western analysis or autoradiography. Small molecular weight peptides generated by AspN treatment were separated using 10% T, 3% C Tris-Tricine gels exactly as described by Schagger and von Jagow (32). Autoradiography was performed by (a) sandwiching the PVDF membrane between two intensifying screens and exposing to Amersham Hyperfilm-MP for 4 days at ~80 °C or (b) exposing the PVDF membrane to a phosphor screen (Molecular Dynamics, Inc.) for 2 days. In the former case, Stratagen Glogos II fluorometric markers were used to orient the film to the PVDF membrane prior to excision of [125]IITID-labeled peptides. Immunoblot visualization of PGHS-1 or -2 and their proteolytic products was done as described previously (29). Antibodies against hPGHS-2 were directed against amino acids 20–40 at the N terminus (Santa Cruz Biotechnology, Inc.). An antibody recognizing the N terminus of oPGHS-1 was directed against amino acids 25–35 (29).

Microsequencing of Radiolabeled Proteolytic Peptides—Using the autoradiograph as a template, [125]IITID-labeled peptides were excised from PVDF membranes and cut into 1-mm^2 squares with clean razor blades. Amino-terminal amino acid sequence analysis was performed in collaboration with Dr. Joseph Leykam of the Michigan State University Macromolecular Structure Facility using automated Edman degradation in an Applied Biosystems 477A gas phase amino acid sequence analyzer. Phenylthiohydantoin-derivatives were identified by high pressure liquid chromatography.

Preparation of oPGHS-1 Mutants—Mutants were prepared starting with M13mp19-oPGHS-1, which contains a 2.5-kilobase pair Sau3A fragment.

2 A. C. Spencer, T. Smith, and D. L. DeWitt, unpublished results.
ment encoding native oPGHS-1, according to the method of Kunkel et al. (35) using a Bio-Rad kit essentially as described previously (34). The following mutagenic oligonucleotides were used in the preparation of mutations in the membrane binding domain of oPGHS-1: HelA (I74T/W75S/L76A); HelB (F88S/L91A/F92S) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3
HelC (W98S/L99A/F102S) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3
HelD (C25W/F78S/V79A); HelE (F88S/L91A/L92A) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3
HelF (F88S/L91A/L92A) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3
HelG (F88S/L91A/L92A) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3
HelH (F88S/L91A/L92A) 5'-3' 3'-CTTTGGGATTCTGTCAATGCCACC418-3

Predicted proteolytic cleavage products for GluC (8 kDa) and AspN (6.2 kDa) are shown (darrow) along with their predicted molecular masses.

Expression and Harvesting of Native and Mutant oPGHS-1 in COS-1 Cells—COS-1 cell transfections were performed essentially as described previously (29) except that cells were harvested 18 h post-transfection. Differential centrifugation was used to prepare membrane and soluble fractions of the cells. Cells were harvested and then homogenized by sonication using a Misonix microtip sonicator. Following sonication, the sample was centrifuged at 10,000 × g for 10 min to collect nuclei and dense cell debris. This latter pellet was designated 10P. The supernatant and pellet was determined by Western blotting analysis.

Cyclooxygenase and Peroxidase Assays—Cyclooxygenase assays were performed using an oxygen electrode as described with 100 μm arachidonate as substrate (36). Peroxidase assays were performed spectrophotometrically on a Perkin-Elmer model 552A Double Beam UV-visible spectrophotometer by measuring the oxidation of 3,3,3',3'-tetramethylethyleneendiamine at 611 nm (13, 37).

RESULTS

[125I]TID is a hydrophobic molecule that partitions into membranes and upon photoactivation yields a carbene that, in turn, reacts nonspecifically with both lipid and protein constituents of the membranes (28). Previous studies using [125I]TID labeling of oPGHS-1 in ovine seminal vesicle microsomes had established that the radiolabel was incorporated into a 20.5-kDa peptide that included residues 25–166 (30). The putative membrane binding domains of oPGHS-1 and hPGHS-2 are predicted to include residues 74–140 (Fig. 1). Accordingly, our experiments were designed to determine if a peptide containing only these residues could be photolabeled with [125I]TID.

Photolabeling of the oPGHS-1 Membrane Binding Domain—Ovine seminal vesicle microsomes containing oPGHS-1 were preincubated with flurbiprofen and photolabeled with [125I]TID, and, as expected (30), oPGHS-1 was the major [125I]TID-labeled membrane protein (data not shown). SDS-PAGE of [125I]TID-labeled oPGHS-1 immunoprecipitated from solubilized microsomes revealed a single radioactive band (Fig. 2A) that was recognized, upon Western transfer blotting, by an oPGHS-1-specific antibody (Fig. 2B). Analysis of the primary structure of oPGHS-1 predicts that cleavage of the enzyme with endoproteinase GluC will yield an 8-kDa peptide containing amino acids 74–140 (Fig. 1B). Accordingly, [125I]TID-labeled,
immunoprecipitated oPGHS-1 was denatured in guanidinium hydrochloride and subjected to exhaustive proteolysis with endoproteinase GluC. Separation of the GluC proteolysis products by SDS-PAGE, transfer to PVDF membranes, and subsequent autoradiography revealed an 8-kDa, \([^{125}\text{I}]\)TID-labeled peptide (Fig. 2A). A small amount of a radiolabeled 14-kDa peptide was found that was recognized by an antibody directed against amino acids 25–35 (Fig. 2B); the 14-kDa peptide probably represents incompletely digested oPGHS-1 containing residues 25–140. We attempted to confirm the identity of the 8-kDa photoaffinity-labeled peptide from Fig. 2A by N-terminal sequence analysis. Unfortunately, this was unsuccessful because the sequencing background was unacceptably high, apparently because of contaminating peptides derived from GluC-digested IgG molecules used to immunoprecipitate the oPGHS-1.

Photolabeling of the hPGHS-2 Membrane Binding Domain—hPGHS-2 was engineered to contain a hexa-His tag and expressed in Sf21 insect cells using a baculovirus expression system. The \(K_m\), \(V_{max}\), and subcellular localization of His-tagged hPGHS-2 were indistinguishable from those of the native enzyme, suggesting that the His-tagged hPGHS-2 is folded and associated with membranes in a native fashion.

To determine which regions of hPGHS-2 were associated with the endoplasmic reticulum, microsomal membranes from Sf21 cells expressing His-tagged hPGHS-2 were prepared and photoaffinity-labeled with \([^{125}\text{I}]\)TID. Analysis of the purified His-tagged hPGHS-2 was done by autoradiography revealed a single radioactive peptide of 8 kDa (Fig. 2C). The 8-kDa fragment was not recognized by antibodies directed against amino acids 20–40 of hPGHS-2 (Fig. 2D). These observations suggested that the membrane binding domain of hPGHS-2 lies between residues 59 and 126. To confirm this, the 8-kDa \([^{125}\text{I}]\)TID-labeled peptide from Fig. 2C was excised from the PVDF membrane and subjected to 15 rounds of N-terminal sequence analysis. The N-terminal sequence of this peptide was FLTRIKLFLKTPNT, which corresponds to amino acids 59–73. Thus, the 8-kDa radioactive peptide containing the \([^{125}\text{I}]\)TID-labeled portion of hPGHS-2 consists of amino acids 59–126. This region contains the corresponding four amphipathic \(\alpha\)-helices predicted to serve as the membrane binding domain of hPGHS-2.

Further mapping of the membrane-associated portion of hPGHS-2 was accomplished by exhaustive digestion of \([^{125}\text{I}]\)TID-labeled hPGHS-2 with endoproteinase AspN (Fig. 1B). AspN treatment of hPGHS-2 resulted in a single radioactive band of approximately 6.2 kDa (Fig. 2E). This radiolabeled peptide was smaller than the 8-kDa, radiolabeled GluC peptide and was of a size similar to that of the four-helix hPGHS-2 membrane binding domain containing amino acids 59–109. N-terminal sequence analysis of the radioactive AspN product identified three peptides, one including residues 59–111 of hPGHS-2 (6.2 kDa), another corresponding to residues 384–428 of hPGHS-2 (6.1 kDa), and an unknown trace contaminant. The hPGHS-2 fragment consisting of amino acids 59–111 apparently resulted from promiscuous cleavage of the protein by AspN at Glu\(^6\) and Asp\(^111\). AspN has been reported to cleave proteins at Glu residues under normal proteolytic conditions (38, 39). Thus, AspN peptide mapping provided further evidence consistent with an hPGHS-2 membrane binding domain involving amino acids 59–109.

Mutations in the Membrane Binding Domain of oPGHS-1—To begin examining the role of hydrophobic amino acids within the membrane binding domain, three different sets of mutations were introduced into helices A–C, respectively, of oPGHS-1 (Figs. 1 and 3). Hydrophobic residues oriented away from the body of the enzyme and expected to interdigitate with the membrane lipids were replaced by smaller neutral or hydrophilic side chains. The mutants were designated HelA.
and HelC mutants appear to result from an additional molecular mass of 72 kDa (29). The 74-kDa bands seen for HelB glycosylation at a consensus N-glycosylation site at Asn 104 of oPGHS-1 is glycosylated at three of four consensus glycosylation sites (Asn 68, Asn 144, and Asn 410) and has an observed molecular mass of 72 kDa. Native oPGHS-1 is glycosylated at three of four consensus glycosylation sites and has an observed molecular mass of 72 kDa. Native oPGHS-1 is normally associated with the membrane pellets derived from centrifugation at 10,000 × g and 200,000 × g (10P and 200P). To determine if mutations in the membrane binding domain of oPGHS-1 were sufficient to disrupt its association with membranes, COS-1 cells expressing native oPGHS-1 or HelA, HelB, or HelC mutants were homogenized and separated into 10P, 200P, and 200S fractions by differential centrifugation as detailed under “Experimental Procedures.” Proteins in each of the fractions were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis using an antibody against the amino terminus of oPGHS-1.

(174/175/176) HelB (F88S/F91S/L92A), and HelC (W98S/L99/S102A). Helix D was not altered, because this latter helix is part of the hydrophobic channel involved in binding arachidonic acid, and mutations in this region were expected to yield inactive enzyme (36, 40). Each of the mutant proteins was expressed in COS-1 cells. As summarized in Table I, enzymatic assays of microsomal membranes prepared from the cells revealed that the HelB mutant protein retained approximately 20% of the native cyclooxygenase and peroxidase activities, and HelC retained very low, but detectable levels of activity (~1% of native enzyme) measured by radio thin layer chromatography (not shown), while the HelA mutant was catalytically inactive. The electrophoretic mobilities of the HelA, HelB, and HelC mutants were examined by SDS-PAGE (Fig. 3A). Interestingly, the HelB and HelC mutants exhibited a 74-kDa band in addition to the native 72-kDa band. Native oPGHS-1 is glycosylated at three of four consensus N-glycosylation sites (Asn 68, Asn 144, and Asn 410) and has an observed molecular mass of 72 kDa (29). The 74-kDa bands seen for HelB and HelC mutants appear to result from an additional N-glycosylation at a consensus N-glycosylation site at Asn 104 located in helix C of the membrane binding domain that in native oPGHS-1 is not glycosylated. Consistent with this is the finding that treatment of each of the three mutant proteins with endoglycosidase H reduced their molecular masses by 66 kDa (Fig. 3A). Additionally, another HelB mutant was prepared and designated HelB/N104Q; this mutant exhibited a 72-kDa but not a 74-kDa band on SDS-PAGE (Fig. 3B). The HelB/N104Q mutant lacked cyclooxygenase activity but exhibited a low level of peroxidase activity. In contrast, N104Q oPGHS-1 retains 45–50% of both peroxidase and cyclooxygenase activities of native oPGHS-1 (29). Our results suggest that the additional N-glycosylation occurring in the HelB mutant partially compensates for a complete loss of cyclooxygenase activity induced by mutations in residues in helix B, which are expected to protrude into the membrane.

Microsomal membranes from COS-1 cells that were sham-transfected or transfected with plasmids encoding native oPGHS-1 or oPGHS-1 mutants were assayed for cyclooxygenase and peroxidase activities as described under “Experimental Procedures” and are displayed as a percentage ± S.D. of the activities of native oPGHS-1 expressed in COS-1 cells. These experiments were performed using a minimum of three transfections and with measurements made in triplicate and yielded similar results with each transfection.

Table I: Cyclooxygenase and peroxidase activities of oPGHS-1 membrane binding domain mutants

| Enzyme | Cyclooxygenase | Peroxidase |
|--------|----------------|------------|
| % of native activity |
| Native oPGHS-1 | 100 | 100 |
| Sham-transfected | 0 | 0 |
| HelA oPGHS-1 | 0 | 0 |
| HelB oPGHS-1 | 25 ± 2.3 | 20 ± 4.1 |
| HelC oPGHS-1 | 1± 0 | 0 |
| N104A/HelB | 0 | 5.6 ± 1.8 |
| oPGHS-1 | 49 | 42 |
| N104Q oPGHS-1 | 1% of native enzyme | measured by radio thin layer chromatography of cyclooxygenase products formed upon incubation of microsomes from cells expressing HelC oPGHS-1 with [1-14C]arachidonate. |

* From Ref. 29.
α-helices present in this segment of the protein are embedded in the lipid bilayer and thus compose the membrane binding domain of this isozyme. Parallel experiments with oPGHS-1 yielded results that also are consistent with the observations made with hPGHS-2. We were unable to confirm the identity of the [125I]TID-labeled peptide derived from oPGHS-1, but taken together with previous work demonstrating selective [125I]TID labeling of a peptide containing amino acids 25–166 (30), our observation of a single 8-kDa radioactive GluC-derived peptide indicates that a region of oPGHS-1 consisting of amino acids 74–140 is membrane-associated.

Our conclusions based on biochemical identification of the membrane-associated domain of PGHSs are consistent with recent functional studies of the PGHS membrane binding domains (49). When overexpressed in NIH 3T3 cells, green fluorescent protein fusion proteins containing the PGHS-1 and -2 membrane binding domains and epidermal growth factor domains associate with cellular membranes, but green fluorescent protein-PGHS proteins lacking the membrane binding domain do not. Again, these studies indicate that the domain containing the four amphipathic α-helices are necessary to permit the interactions of PGHSs with membranes. Moreover, PGHS-1 and -2 appear not to require other proteins in order to interact with membranes, since purified oPGHS-1 (50) and hPGHS-2b both bind to phospholipid vesicles.

A series of mutations were made in three of the four α-helices of oPGHS-1 in order to determine the relative importance of the individual α-helices in membrane binding. In each case, the mutants remained membrane-associated but were catalytically inactive or nearly catalytically inactive with respect to both peroxidase and cyclooxygenase activities. With the HelA and HelC mutants, at least a portion of the protein molecules appeared to form misfolded aggregates, which could not be solubilized efficiently. The HelB mutant retained activity, but this apparently resulted from an unexpected but compensatory glycosylation of Asp104. When this residue was replaced with a glutamine, the HelB mutant lost activity, whereas the corresponding mutant in native oPGHS-1 retains approximately 50% of its activity (29). Overall, we interpret the results of studies with the MBD mutants to indicate that appropriate folding of enzyme requires that each of the membrane binding domain α-helices needs to be structurally intact. The roles of individual amino acids need to be investigated further.

The structures of several types of protein domains involved in reversible Ca2+- and/or lipid-dependent monopatic associa
tion with membranes have been determined (51–53). However, until recently, PGHS-1 and -2 were the only integral membranes that appeared to interact with membranes monopatically. Crystallographic analysis of a bacterial squalene cyclase revealed a structural motif similar to that observed in PGHSs-1 and -2 and suggested that this type of domain may be used by integral membrane biosynthetic enzymes whose substrate is derived directly from the lipid bilayer (54, 55). Although the three-dimensional fold of PGHS and the squalene cyclase are similar, there is no sequence similarity between the proteins. However, both proteins are homodimeric and possess hydrophobic patches that protrude away from the hydrophilic catalytic domains. Monopatic membrane binding domains like those of the PGHSs and squalene cyclase appear to be positioned in such a way to allow direct substrate access to the active site. This would be thermodynamically favorable, since hydrophobic substrates like arachidonate and squalene are not likely to be released directly into an aqueous environment. The membrane binding domains of PGHS and squalene cyclase appear to function as (a) membrane anchors and (b) continuous hydrophobic tunnels from the site of substrate release to the

FIG. 4. Detergent solubilization of oPGHS-1 helix mutants. A, microsomes were prepared from COS-1 cells expressing native, HelA, HelB, or HelC oPGHS-1, and samples were incubated in the presence of Tween 20 for 1 h and subjected to ultracentrifugation as detailed under “Experimental Procedures.” Solubilized and membrane-associated oPGHS-1 was visualized with an antibody against amino acids 25–35. B, nuclear membranes were isolated from COS-1 cells expressing native oPGHS-1, HelA, HelB, or a double mutant, HelAB. Equivalent samples were incubated in the presence or absence of various detergents as described under “Experimental Procedures.” Following solubilization, proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized with an antibody against the amino terminus of oPGHS-1 in order to determine the relative importance of the individual α-helices in membrane binding. In each case, the mutants remained membrane-associated but were catalytically inactive or nearly catalytically inactive with respect to both peroxidase and cyclooxygenase activities. With the HelA and HelC mutants, at least a portion of the protein molecules appeared to form misfolded aggregates, which could not be solubilized efficiently. The HelB mutant retained activity, but this apparently resulted from an unexpected but compensatory glycosylation of Asp104. When this residue was replaced with a glutamine, the HelB mutant lost activity, whereas the corresponding mutant in native oPGHS-1 retains approximately 50% of its activity (29). Overall, we interpret the results of studies with the MBD mutants to indicate that appropriate折叠 of enzyme requires that each of the membrane binding domain α-helices needs to be structurally intact. The roles of individual amino acids need to be investigated further.

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Discussion

Integral membrane proteins utilize one or more of a variety of mechanisms to associate with lipid bilayers. G protein-linked receptors (41) and ion channels (42) as well as single pass membrane proteins such as cytochrome P450s (43, 44) use hydrophobic α-helices that span both leaflets of the membrane lipid bilayer, whereas porins traverse the bilayer using antiparallel β-barrels (45). Other proteins including monomeric and heteromeric G proteins associate with membranes via fatty acyl and/or prenyl groups (46). PGHS-1 and PGHS-2 are integral membrane proteins in that they can only be solubilized with detergents, but these enzymes lack established structural motifs known to confer membrane association. In 1994, Picot et al. (14) observed an amphipathic domain consisting of four short α-helices in the crystal structure of oPGHS-1 and proposed that this domain anchors the protein monopatically (47) within one face of the membrane (Fig. 1A). The major aim of this study was to test further this prediction using direct biochemical analyses.

Biochemical identification of PGHS membrane binding domains was based on the characterization of radiolabeled pep
tides derived from oPGHS-1 and hPGHS-2 after treatment of the proteins in their membrane-associated forms with [125I]TITD. This latter reagent partitions primarily into membranes and, under appropriate conditions, labels only those portions of proteins that reside within the lipid bilayer (28, 30, 48). Radioactive peptides resulting from GluC and AspN cleav
age of [125I]TID-labeled hPGHS-2 were unequivocally identified by N-terminal sequence analysis, and the results establish that a peptide consisting of amino acids 59–111 is associated with the lipid bilayer. Because the hypothesized membrane binding domain of hPGHS-2 is composed of residues 59–109, our studies support the concept that the four amphipathic α-helices present in this segment of the protein are embedded in the lipid bilayer and thus compose the membrane binding domain of this isozyme. Parallel experiments with oPGHS-1 yielded results that also are consistent with the observations made with hPGHS-2. We were unable to confirm the identity of the [125I]TID-labeled peptide derived from oPGHS-1, but taken together with previous work demonstrating selective [125I]TID labeling of a peptide containing amino acids 25–166 (30), our observation of a single 8-kDa radioactive GluC-derived peptide indicates that a region of oPGHS-1 consisting of amino acids 74–140 is membrane-associated.

Our conclusions based on biochemical identification of the membrane-associated domain of PGHSs are consistent with recent functional studies of the PGHS membrane binding domains (49). When overexpressed in NIH 3T3 cells, green fluorescent protein fusion proteins containing the PGHS-1 and -2 membrane binding domains and epidermal growth factor do
main domains associate with cellular membranes, but green fluorescent protein-PGHS proteins lacking the membrane binding domain do not. Again, these studies indicate that the domain containing the four amphipathic α-helices are necessary to permit the interactions of PGHSs with membranes. Moreover, PGHS-1 and -2 appear not to require other proteins in order to interact with membranes, since purified oPGHS-1 (50) and hPGHS-2b both bind to phospholipid vesicles.

A series of mutations were made in three of the four α-helices of oPGHS-1 in order to determine the relative importance of the individual α-helices in membrane binding. In each case, the mutants remained membrane-associated but were catalytically inactive or nearly catalytically inactive with respect to both peroxidase and cyclooxygenase activities. With the HelA and HelC mutants, at least a portion of the protein molecules appeared to form misfolded aggregates, which could not be solubilized efficiently. The HelB mutant retained activity, but this apparently resulted from an unexpected but compensatory glycosylation of Asp104. When this residue was replaced with a glutamine, the HelB mutant lost activity, whereas the corresponding mutant in native oPGHS-1 retains approximately 50% of its activity (29). Overall, we interpret the results of studies with the MBD mutants to indicate that appropriate folding of enzyme requires that each of the membrane binding domain α-helices needs to be structurally intact. The roles of individual amino acids need to be investigated further.

The structures of several types of protein domains involved in reversible Ca2+- and/or lipid-dependent monopotic associa
tion with membranes have been determined (51–53). However, until recently, PGHS-1 and -2 were the only integral membranes that appeared to interact with membranes monopatically. Crystallographic analysis of a bacterial squalene cyclase revealed a structural motif similar to that observed in PGHSs-1 and -2 and suggested that this type of domain may be used by integral membrane biosynthetic enzymes whose substrate is derived directly from the lipid bilayer (54, 55). Although the three-dimensional fold of PGHS and the squalene cyclase are similar, there is no sequence similarity between the proteins. However, both proteins are homodimeric and possess hydrophobic patches that protrude away from the hydrophilic catalytic domains. Monopotic membrane binding domains like those of the PGHSs and squalene cyclase appear to be positioned in such a way to allow direct substrate access to the active site. This would be thermodynamically favorable, since hydrophobic substrates like arachidonate and squalene are not likely to be released directly into an aqueous environment. The membrane binding domains of PGHS and squalene cyclase appear to function as (a) membrane anchors and (b) continuous hydrophobic tunnels from the site of substrate release to the
active site. There has not yet been biochemical confirmation that the predicted membrane binding domain of the squamaline cyclase is membrane-associated.

The catalytic domains of PGHS-1 and -2 share 70% of their primary structure, while the membrane binding domains of the enzymes are only 38% identical. An interesting question is whether sequence differences in the membrane binding domains of PGHS-1 and -2 may promote their association with distinct phospholipid microdomains within cellular membranes. Interactions between membrane proteins and polar head groups of phospholipids have been shown to be of biological importance and appear to form in vivo (54, 56–58). It is conceivable that the phospholipid microenvironment immediately surrounding an integral membrane protein may affect its biological activity by promoting or restricting access of the protein to various components of the cellular membrane. In the case of PGHS-1 and -2, the overall charge of the MBD is 2.75 and 6.0, respectively. It is not known whether these differences in the membrane binding domain confer differential activities of PGHS-1 or -2.

The depth at which the individual amino acids lie within the PGHS membrane binding domain is unknown. Although [125I]TID labels only those parts of a protein associated with the hydrophobic core of a lipid bilayer, it labels nonspecifically in a manner that makes depth determinations for individual amino acids difficult. In making the mutant versions of oPGHS-1 (HelA, HelB, and HelC), we changed residues that, based on charge and polarity, might be expected to interact with phospholipid head groups. Only HelB seemed to fold in a native state, retaining 20% of the native cyclooxygenase and peroxidase activities. Changing the amphipathic character of helices A and C resulted in their apparent misfolding. Interestingly, the three HelB mutations replaced native PGHS-1 residues with side chains similar in charge and polarity to corresponding residues in PGHS-2.

In summary, we have provided biochemical evidence that the membrane binding domains of ovine PGHS-1 and human PGHS-2 are contained within residues 74–140 and 59–111, respectively. These results are consistent with crystallographic predictions of a new kind of monotopic membrane binding domain. These results support this hypothesis and supply new information characterizing a novel membrane association strategy that may be used by other lipid biosynthetic enzymes.

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