Based on the crystal structures of the prostaglandin endoperoxide H synthases-1 and -2 (PGHS-1 and PGHS-2), four short amphipathic helices near the amino termini of these proteins have been proposed to act as membrane binding domains. We constructed a series of plasmids coding for amino-terminal sequences of the PGHS-1 and PGHS-2, and now show that fusion proteins containing various portions of the amino termini of the PGHS-1 and PGHS-2 isozymes act as membrane anchors.

Prostaglandins are synthesized by a pair of isozymes referred to as prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1 and PGHS-2); or cyclooxygenase-1 and -2 (1). The first of these enzymes is expressed constitutively and produces prostaglandins acutely that modulate cellular responses to hormonal stimulation. Prostaglandins produced by PGHS-1 are also responsible for regulating platelet aggregation and vascular homeostasis. In contrast, PGHS-2 is constitutively present in only a few tissues, but its expression can be induced in almost any cell in response to mitogenic and inflammatory cytokines, suggesting that it produces prostaglandins that mediate inflammation. The anti-inflammatory properties of selective inhibitors of PGHS-2 support this role (2, 3). PGHS-2 is also expressed in colonic tumors (4–6) indicating that this enzyme may be involved in the transformation process in these tissues.

PGHS-1 and PGHS-2 are both integral membrane proteins that have been localized to the endoplasmic reticulum and nuclear membranes (7–9) and that require detergents for solubilization (10). How these enzymes might associate with membranes has until recently been unclear. Examination of the primary amino acid sequence predicted from the ovine PGHS-1 cDNA (11, 12) and the murine and chicken PGHS-2 (13, 14) revealed that these proteins do not contain amino acid sequences of sufficient length and hydrophobicity to act as classical transmembrane anchors (11). Recently, analysis of the crystal structure of the ovine PGHS-1 (15–16) has suggested an alternative mechanism for membrane association of the PGHS isozymes. Picot et al. (15) found that the PGHS-1 is a compact globular protein with no obvious hydrophobic hinge regions or loops that might act as a transmembrane anchor. Instead, they proposed that hydrophobic amino acids contained in four short α-helices located between residues 71 and 120 of PGHS-1 and residues 58 and 115 of PGHS-2 form hydrophobic surfaces that bind membranes, most likely by interacting with a single leaflet of the lipid bilayer (15). These amphipathic α-helices represent a new structural motif for stable membrane association distinct from the classical hydrophobic α-helices that transverse the entire lipid bilayer.

Subsequent work using labeling with 3-trifluoro-3-(m-125I)iodophenyl)diazirine, a hydrophobic photo-activated cross-linking agent, have confirmed that sequences located in the first half of the PGHS-1 are in contact with the membrane (17), but until now the membrane-binding properties of the α-helices have not been tested directly. We have constructed fusion proteins containing various portions of the amino termini of the PGHS-1 and PGHS-2 isozymes and now show that only those constructs that contain the α-helical amphipathic helices are correctly targeted to and associate with the ER and nuclear membranes.

MATERIALS AND METHODS

Construction of Plasmids

Six different expression plasmids were constructed containing amino-terminal sequences of PGHS-1 and PGHS-2 joined to the amino terminus of the green fluorescent protein gene from Aequorea victoria (GFP). These plasmids were constructed using the parent plasmid pEGFP-N1 (CLONTECH), which contains the coding sequence for GFP. Fig. 1 presents a diagram of the plasmid constructs, and their construction is described below.

pPGHS-1-EGF-STE1L (Fig. 1A)—A 0.26-kb Xhol/PstI fragment containing the coding sequence for the first 72 amino acids of the human
PGHS-1<sup>2</sup> was constructed by PCR amplification using oligonucleotide primers that contained added XhoI and PvuII recognition sites. Amino acids 1–72 contain the signal peptide, EGF homology domain, and first N-glycosylation site but not the putative membrane-binding helices of PGHS-1 (Fig. 1). The PGHS-1 fragment was subcloned into pGFP-N1 plasmid that had been prepared by first digesting with EcoRI, filling in the overhanging ends with polymerase I Klenow fragment, and digesting a second time with XhoI. The amino acids, RRRSTEL, which are present at the carboxyl termini of mouse PGHS-1 and PGHS-2, have been proposed to act as ER retention signal for these isozymes (18). Homologous sequences are also present in all other PGHSs that presumably function similarly, although only the final four amino acids, STEL, are strictly conserved between species. To test what effect this putative retention signal had on protein localization in mouse 3T3 cells, plasmids were constructed that expressed fusion proteins with and without the murine RRRSTEL sequence. These amino acids were added to the end of the PGHS-GFP by ligating a complementary pair of oligonucleotides (5′-TACAAGACGCGTCGTTCAACTGAGCTGTAA-G3′ and 5′-GGGCGGCGTCGAGTCGCTGAA-G3′) into the BsrGI/Ndel-digested pEGF-PGHS-1 plasmid.

### Membrane Binding Domains of PGHS-1 and PGHS-2

*Membrane Binding Domains of PGHS-1 and PGHS-2*

| DNA Transformants | Western Blotting |
|------------------|------------------|
| PGHS-1-EGF-MBD  | FRAP Analysis of Fusion Protein Mobility |
| PGHS-2-EGF-MBD  | |
| PGHS-1-EGF-STEL | |
| PGHS-2-EGF-STEL | |
| PGHS-1-EGF-MBD-STEL | |
| PGHS-2-EGF-MBD-STEL | |
| PGHS-1-EGF-MBD-STEL | |
| PGHS-2-EGF-MBD-STEL | |

### RESULTS

A series of expression plasmids in which the amino termini of the PGHS-1 and PGHS-2 isozymes were joined to the cDNA of the GFP of A. victoria (Fig. 1) were constructed to test whether these amphipathic α-helices are responsible for association of the PGHS isozymes with the endoplasmic and nuclear membranes. These plasmids expressed chimeric proteins of GFP joined with the amino-terminal sequences of PGHS-1 and PGHS-2, with and without the putative membrane binding domains. The plasmids PGHS-1-EGF-MBD and PGHS-2-EGF-MBD (Fig. 1) code for the first 139 and 136 amino acids of human PGHS-1 and PGHS-2, respectively, which includes, in order, the signal peptide sequences, EGF homology domains, and the putative membrane-binding (MBD) helices, followed by coding sequence for GFP. The plasmids PGHS-1-EGF-MBD and PGHS-2-EGF-MBD have in addition the amino acids RRRSTEL added to the carboxyl terminus of the GFP protein. This sequence, found on the carboxyl termini of all PGHS-1 and PGHS-2, has been reported to be necessary for retention of these enzymes within the endoplasmic reticulum (18). In the plasmids pPGHS-1-EGF-STEL and pPGHS-2-EGF-STEL, the putative membrane binding domain has been deleted. The plasmids GFP and GFP-MBD code for the GFP protein alone and GFP protein with the added RRRSTEL sequence.

### Characterization of the GFP Fusion Proteins

To determine whether the PGHS-GFP fusion proteins were synthesized correctly following transfection into 3T3 fibroblasts, their expres-
sion was examined using Western blotting (Fig. 2). Proteins from homogenates of 3T3 cells transfected with the various expression plasmids were separated by PAGE and transferred to nitrocellulose membranes. The GFP and PGHS-GFP fusion proteins were visualized using a monoclonal antibody against GFP. As can be seen in Fig. 2, this monoclonal anti-GFP antibody reacted specifically with proteins of the correct apparent Mr (29,000) in homogenates of cells transfected with the plasmids pGFP (GFP) or pGFP-STEL (GFP-STEL). This antibody also cross-reacted nonspecifically with a protein that migrates with an apparent Mr of 50,000 (Fig. 2) that is present constitutively in 3T3 cells.

The molecular masses of the PGHS-1- and PGHS-2-GFP fusion proteins predicted from their amino acid sequences, and assuming that their signal peptides are removed, are 41.7 and 41.5 kDa, respectively. The apparent Mr of the major immunoreactive bands in cells transfected with the PGHS-1 plasmids pPGHS-1-EGF-MBD and pPGHS-1-EGF-MBD-STEL was 43,000 (Fig. 2), and a minor band of about 42,000 was also observed with PGHS-1-EGF-MBD. The GFP fusion proteins expressed in cells transfected with the PGHS-2 plasmids PGHS-2-MBD-EGF and PGHS-2-MBD-EGF-STEL had a slightly higher apparent Mr of 44,500.

The 43,000–44,500 apparent Mr of the PGHS-1 and PGHS-2 fusion proteins is 2000–3000 larger than would have been predicted from the amino acid sequences of these proteins alone. This apparent size increase could have resulted from the expected N-glycosylation of these proteins, at asparagine 68 in PGHS-1 and asparagines 53 and 130 in PGHS-2 (22), or from failure to cleave the signal peptides. To differentiate between these two possibilities, tunicamycin, an inhibitor of N-glycosylation, was added to cells 5 h following transfection. Treatment with tunicamycin reduced the apparent Mr of the PGHS-1-EGF-MBD protein to 41,500 from 43,000 (Fig. 2) and of the PGHS-2-EGF-MBD protein to 41,500 from 44,500 (Fig. 2). A similar reduction in the presence of tunicamycin was observed for the proteins expressed by the PGHS-1-MBD-EGF-STEL and PGHS-2-MBD-EGF-STEL plasmids. These results demonstrate that the fusion proteins were glycosylated and indicate that the signal peptides were removed. The most likely explanation for the larger size of the glycosylated PGHS-2-GFP compared with glycosylated PGHS-1-GFP is that the PGHS-2-GFP fusion proteins were glycosylated at both Asn-53 and Asn-130, whereas the PGHS-1-GFP fusion proteins were glycosylated only once, at Asn-68.

Subcellular Targeting of the PGHS-GFP Fusion Proteins—Confocal fluorescent microscopy was used to determine if the putative MBD domains of the PGHS proteins could anchor the PGHS-GFP proteins to ER and nuclear membranes (Fig. 3). About 30% of 3T3 cells had visible fluorescence 18 h following transfection with the PGHS-GFP expression plasmids. Cells transfected with PGHS-1-MBD-GFP and PGHS-2-MBD-GFP, which express the PGHS-1-GFP and PGHS-2-GFP fusion proteins that contain amphipathic α-helices, showed an intense peri-nuclear ring of fluorescence and strong fluorescence in the cytoplasmic space which was irregular and appeared to be

![Fig. 1. Diagrams of the PGHS-GFP expression plasmids.](image)
associated with distinct reticular structures (Fig. 3, A and D), and was excluded from the nuclear space. An identical pattern of fluorescence was observed in cells transfected with PGHS-1-MBD-STEL and PGHS-2-MBD-STEL (Fig. 3, B and E). The localization of these proteins contrasted with that of GFP (Fig. 3G), which was uniform throughout the cytoplasmic and nuclear spaces, did not form a perinuclear ring, and did not appear to be associated with any subcellular structures. The staining pattern obtained from expression of all four PGHS-GFP fusion proteins containing membrane binding domains was identical to the fluorescent patterns observed previously using indirect immunofluorescent staining to localize the native PGHS-1 and PGHS-2 proteins in 3T3 cells (9).

A distinctly different staining pattern was observed following transfection of the plasmids PGHS-1-EGF-STEL and PGHS-2-EGF-STEL, which code for the PGHS-GFP fusion proteins that do not contain the putative membrane association helices. The fluorescence from these proteins was irregularly distributed throughout the cytoplasmic space and was excluded from the nucleus as were the MBD-containing chimeras (Fig. 3, C and F); however, unlike the MBD-containing chimeras, these proteins gave a very diffuse fluorescence which had the appearance of being fuzzy and unfocused in the micrographs. Another notable difference in the staining pattern of PGHS-GFP fusion proteins with no MBD sequences was the absence of a distinct perinuclear ring of fluorescence (compared Fig. 3, C and F; with Fig. 3, A and B and D and E) that is characteristic of MBD-containing constructs and was also observed during immunocytochemical localization of the native PGHS-1 and PGHS-2 enzymes (9). The diffuse fluorescence and lack of a defined nuclear ring suggested that proteins without the MBD might be targeted to the ER lumen but were not membrane-associated.

To determine directly whether the PGHS-GFP chimeric proteins were membrane-associated, we next examined the distribution of native PGHS-1 and PGHS-2 and the PGHS-GFP fusion proteins in particulate and soluble fractions of the transfected cells using Western blotting analysis. Cells transfected with the PGHS-GFP fusion plasmids or the control GFP plasmids were disrupted by sonication and separated into three subcellular fractions that corresponded roughly to nuclei, endoplasmic reticular membranes, and soluble cytoplasmic proteins (Fig. 4, S).

It has previously been demonstrated that the PGHS-1 and PGHS-2 are integral membrane proteins that are associated exclusively with the nuclear pellet and microsomal fractions (Fig. 4, N and M). Those fusion proteins that contained the amphipathic α-helices, PGHS-1-EGF-MBD-STEL and PGHS-2-EGF-MBD-STEL, also fractionated with the microsomal and nuclear membrane fractions in a ratio similar to that observed for the native PGHS-1 and PGHS-2 (Fig. 4, A and B). In some transfections, the PGHS-2-EGF-MBD-STEL fusion protein was present in cytoplasmic fractions at higher concentrations than is observed for the native proteins, suggesting that processing of the fusion proteins may not be as efficient as for the native proteins. As would be predicted from the results of the fluorescent localization experiments, the fusion proteins that do not contain the membrane binding domains, PGHS-1-EGF-STEL and PGHS-2-EGF-STEL, do not associate with particulate cells fractions and were present only in the soluble cytoplasmic fractions (Fig. 4, A and B). The control GFP and GFP-STEL proteins were found exclusively in the cytoplasmic fraction (Fig. 4C, S).

**Examination of the Mobility of PGHS-GFP Fusion Proteins in Live Cells**—The constraints of molecular movement within the lipid bilayer result in coefficients of diffusion (D) for integral membrane proteins that are approximately 2 orders of magnitude slower than soluble proteins. To provide further evidence for the subcellular localization of the PGHS-GFP fusion proteins, we examined the diffusional mobility of GFP and the various PGHS-GFP chimeras using fluorescent recovery after photo-bleaching (FRAP) (19). In this method, a laser attached to a microscope bleaches the fluorescence from a small defined area within a live cell. The time required for fluorescence to return to that site can be used to estimate the mobility of the fluorescent protein.

The coefficient of diffusion (D) for GFP has previously been measured as $8.7 \times 10^{-7}$ cm$^2$ s$^{-1}$ in saline buffer (22). By analyzing GFP expressed in Chinese hamster ovary cells, these same researchers demonstrated that translational diffusion of this protein is approximately 3.2-fold slower in live cells, a change that would lower its coefficient of diffusion to approximately $2 \times 10^{-7}$ cm$^2$ s$^{-1}$ in cell cytoplasm (22). The most rapid rate of diffusion that could be measured under the conditions we employed corresponds to a $D = 1.5 \times 10^{-7}$ cm$^2$ s$^{-1}$. For the majority of measurements (17/26), D values for GFP in live transfected 3T3 cells were greater than $1 \times 10^{-7}$ cm$^2$ s$^{-1}$; most were too fast to measure (Table I). These observations are consistent with the previous measurements (22). In a distinct subset of measurements (9/26), however, D values were obtained for GFP that averaged $2.5 \pm 1.8 \times 10^{-8}$ cm$^2$ s$^{-1}$. Although we have no explanation for the existence of two populations of GFP molecules with widely varying diffusion characteristics, the second slower population may represents dimers or other aggregates of GFP formed due to high level expression of this protein within cells (26).

The diffusion coefficients of PGHS-GFP chimeras without the MBD sequences averaged $1.3 \times 10^{-8}$ cm$^2$ s$^{-1}$ (Table I). Although these values are 10 times slower than the fastest values obtained for GFP, they are 10-fold faster than the rates we obtained for GFP constructs that contain the MBD and also an order of magnitude faster than rates previously measured for other integral membrane proteins (19).
sional coefficient of the GFP chimeras containing the MBD sequences was $2 \times 10^{-9}$ cm$^2$ s$^{-1}$ (Table I), a rate which is similar to the diffusion coefficients determined for ER-localized galactosyltransferase-GFP ($D = 2.1 \times 10^{-9}$ cm$^2$ s$^{-1}$) and the KDEL receptor GFP ($D = 2.6 \times 10^{-9}$ cm$^2$ s$^{-1}$) (19). The presence of the RRSTEL tail on the PGHS-GFP constructs did not have any significant effect on the mobility of the PGHS-GFP fusion proteins (Table I).

**DISCUSSION**

To test the hypothesis that four amphipathic $\alpha$-helices near the amino-terminal of PGHS-1 and PGHS-2 act as membrane binding domains for these enzymes, we constructed a series of chimeric proteins with PGHS sequences joined to the green fluorescent protein of *A. victoria*, and we examined the subcellular distribution and membrane association of these proteins by fluorescent microscopy, Western blotting, and FRAP analysis. We examined fusion proteins containing the entire amino termini of PGHS-1 and PGHS-2 with and without the putative

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**FIG. 3. Fluorescence localization of the PGHS-GFP chimeric proteins.**

NIH 3T3 cells grown in Nunc cover glass chambers were transfected with the indicated fusion protein expression plasmid (see Fig. 1). 18 h following transfection, fusion protein expression was examined using an InsightII confocal fluorescent microscope (Meridian Instruments) as described previously (9). Approximately 30% of cells displayed fluorescent staining, and the patterns of staining was similar for all cells transfected with a given plasmid. Plasmids used for transfection (A–H) are as indicated in Fig. 1. Digital images were collected with a 1-s integration time. Magnification, 300×.
measurements gave values of $D \geq 1 \times 10^{-7} \text{cm}^2 \text{s}^{-1}$. (Although the instrumenta-
tion used could not collect data fast enough during the recovery phase
to calculate values of $D$ greater than $1.5 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, in those
measurements bleaching and rapid recovery were still observed.) Nine
determinations gave values of $D$ that were 10-fold slower, averaging
$2.5 \times 1.8 \times 10^{-8} \text{cm}^2 \text{s}^{-1}$. One explanation for this bimodal distribution
may be that at high levels of expression aggregation of GFP may occur.
Although we have no evidence for this, it has been described previously
(26) and could account for the slower rates of diffusion observed.

The PGHS-2-EGF construct is identical to the PGHS-2-EGF-STEL, except the RRSTEL sequence is missing.

membrane binding sequences, instead of fusion proteins containing
only the putative membrane binding domains (MBD), because it seemed probable that the amino-terminal sequences
preceding these MBDs would be required for targeting of the
PGHS fusion proteins to the luminal endoplasmic reticulum
where they normally are integrated into the lipid bilayer.
It also seems likely that correct folding of the membrane associ-
ation helices would be required for membrane association and
folding, in turn, would depend on the proper co-translational
and post-translational modifications, e.g. signal peptide cleav-
gage, glycosylation, and disulfide bond formation, of these same
preceding amino-terminal sequences.

Our analysis indicates that signal peptides are removed from
the fusion proteins, and these proteins are glycosylated. In the
presence of tunicamycin, the observed molecular weights of the
largest unglycosylated PGHS-1 and PGHS-2 fusion proteins
were close to the size (41,500) that would be predicted for these
proteins following signal peptide cleavage. In the absence of
tunicamycin, the apparent molecular weights of the PGHS-1
and PGHS-2 fusion proteins were 43,000 and 44,500, sizes that
are consistent with modification of a single N-glycosylation site
present in PGHS-1 sequence and of the two N-glycosylation sites
present in the PGHS-2 sequence.

Our experimental results indicate that the putative mem-
brane binding domains in PGHS-1 and PGHS-2 are responsible
for membrane association, as only those fusion proteins con-
taining the amphipathic $\alpha$-helices associated with membranes.
Four separate lines of evidence support the conclusion that
proteins that contain the putative membrane binding domain
proteins are targeted to and stably associated with the ER and
nuclear membranes. First, the fluorescent patterns of these
proteins was identical to that observed for immunofluorescent
staining of the native PGHS-1 and PGHS-2 proteins in 3T3

**TABLE I**

Diffusion coefficients ($D$) of GFP and GFP-PGHS
chimeras in intact 3T3 cells

| Chimera               | $D$ value | Measurements |
|-----------------------|-----------|--------------|
| GFP                   | $<10^{-9}$ cm$^2$ s$^{-1}$ | n           |
| PGHS-1-EGF-STEL       | 10.6 ± 5.9 | 14           |
| PGHS-2-EGF-STEL       | 13.2 ± 5.0 | 8            |
| PGHS-2-EGF*           | 14.9 ± 5.9 | 8            |
| PGHS-1-EGF-MBD-STEL   | 3.1 ± 1.6  | 8            |
| PGHS-1-EGF-MBD        | 1.0 ± 0.5  | 6            |
| PGHS-2-EGF-MBD-STEL   | 2.2 ± 0.7  | 12           |
| PGHS-2-EGF-MBD        | 1.5 ± 1.1  | 6            |

* There was a distinct bimodal distribution of $D$ values for GFP. 17/26
measurements gave $D \geq 1 \times 10^{-7} \text{cm}^2 \text{s}^{-1}$. (Although the instrumenta-
tion used could not collect data fast enough during the recovery phase
to calculate values of $D$ greater than $1.5 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, in those
measurements bleaching and rapid recovery were still observed.) Nine
determinations gave values of $D$ that were 10-fold slower, averaging
$2.5 \times 1.8 \times 10^{-8} \text{cm}^2 \text{s}^{-1}$. One explanation for this bimodal distribution
may be that at high levels of expression aggregation of GFP may occur.
Although we have no evidence for this, it has been described previously
(26) and could account for the slower rates of diffusion observed.

The PGHS-2-EGF construct is identical to the PGHS-2-EGF-STEL, except the RRSTEL sequence is missing.

**FIG. 4.** Subcellular localization of the PGHS-GFP chimeric proteins using Western blot analysis of nuclear, microsomal,
and soluble protein fractions from transfected NIH 3T3 cells.

**FIG. 5.** Detergent solubility of the PGHS-GFP chimeric proteins. NIH 3T3 cells were transfected with the indicated PGHS-GFP or
PGHS fusion plasmids (see Fig. 1). After 18 h, microsomal membranes
were isolated from transfected cells and from untransfected NIH 3T3
cells that had been serum-stimulated to induce expression of PGHS-2
(20). Microsomal membranes (500 mgl) were resuspended in 0.5 ml of 0.1
M Tris-Cl, pH 8.0 (No Detergent), or in 0.1 M Tris-Cl, pH 8.0, containing
0.5% Nonidet P-40 detergent (0.5% NP-40), then centrifuged at
100,000 $\times$ g for 45 min. The supernatants from these high speed spins
(Sup) were removed, and the membrane pellets (Pel) were resuspended
in 0.5 ml of 0.1 M Tris-Cl, pH 8.0. Equal volumes of each of these
fractions were then analyzed by Western blotting as described under
“Materials and Methods” and in the legend for Fig. 4.
cells (Fig. 3) (9). Second, Western blot analysis of these proteins showed the same distributions of PGHS-GFP proteins in the particulate and soluble cell fractions as the native PGHSs (Fig. 4). Third, the mobility of the PGHS-GFP proteins was similar to other membrane proteins and 2 orders of magnitude slower than GFP alone. Finally, these proteins can be solubilized from microsomal membranes, demonstrating that they are not simply aggregated within the cell (Fig. 5).

Deletion of the membrane binding domains in PGHS-1-EGF-STEL and PGHS-2-EGF-STEL prevented membrane association but did not appear to affect targeting of these proteins to the lumen of the ER. Evidence for this assumption derives from observations of the pattern of fluorescence following expression of chimeras that do not contain the MBD sequences. This pattern was similar to that of the fusion proteins that contain membrane binding domains, in that it was restricted to the cytoplasmic space and appeared to be associated with specific structures within that space. However, the fluorescence pattern of proteins without MBDs was diffuse and lacked a defined perinuclear ring (Fig. 3). Our interpretation of this staining pattern is that the proteins lacking the MBD were targeted to the lumen of the ER but did not then associate with the membrane. We hypothesize that the diffuse distribution of these soluble proteins within the ER lumen leads to the hazy fluorescence and that the lack of membrane association prevents the concentration of these proteins on the membranes surrounding the nucleus, an organization which was observed as a perinuclear ring. Western blot analysis confirms that proteins without the MBD were soluble and were released upon homogenization of cells.

It is curious that the diffusion coefficients for the PGHS-GFP chimeric proteins that do not contain membrane binding domains are intermediate between soluble GFP and the membrane-associated PGHS-GFP proteins that do contain MBD domains. We know of no other studies that have examined the mobility of soluble luminal ER proteins. One possible explanation for the apparently slower rate of diffusion is that, as suggested above, the chimeric proteins are present in the reticular lumen of the ER, which may geometrically constrain diffusion of macromolecules, at least within the relatively large area (0.5 μm) where bleaching occurs. Alternatively, the luminal cytoplasm may be more viscous than the extra-luminal cytoplasm. Another possibility is that the chimeras proteins aggregate to form multimers and that the observed diffusional coefficients reflect the true rate of translation diffusion of these higher molecular weight oligomers. Finally, proteins that lack the MBD may nevertheless be constrained by protein-protein interactions within the cell. It is important to note, however, that in the absence of the MBD domain, the chimeric proteins all had coefficients of diffusion that are at least 10 times faster than any known membrane proteins, so it is unlikely that these proteins are stably associated with membranes.

The role of the peptide sequence STEL, which is found in the carboxyl termini of all PGHS, was also investigated. This sequence has been postulated to serve as an ER retention signal (18). In our experiments, we saw no obvious effect of this sequence on the distribution of PGHS-GFP fusion proteins. Proteins containing the membrane binding domains were localized to the ER and nuclear particulate fractions in a manner identical to the native proteins independent of whether they contained an STEL sequence. We did examine whether PGHS-GFP fusion proteins that did not contain either MBD and STEL sequences would be secreted from transfected 3T3 cells, but no significant secretion of these fusion proteins was detected.3

Other proteins have been shown to use amphipathic α-helices to transiently bind to the membranes. One example is the CTP-phosphocholine cytidylyltransferase (24), whose activity has been shown to be regulated by reversible binding to the ER and nuclear membranes. The PGHS isozymes are, however, the first proteins identified that use amphipathic α-helices for stable membrane association. Recently, the structure of squalene cyclase has been determined (25), and amphipathic helices on this protein have been postulated to serve as a membrane binding domains for this enzyme also. The amphipathic helices of the squalene cyclase and the PGHS have another potential functional similarity in that they also comprise the opening to the active sites of these enzymes. Since both enzymes are predicted to be positioned so that their membrane binding sequences rest in the lipid bilayer, substrate and products must enter and exit the enzymes through the hydrophobic interior of the membrane. Thus, this unique mode of membrane interaction probably has a functional significance and may be used by other enzymes that have hydrophobic substrates and products. The fact that the membrane binding domains of the PGHS-1 and PGHS-2 are only 38% similar compared with the over 75% similarity between the catalytic domains of these two isoforms suggests that differences in membrane association could also play some role in regulating the differential activities and/or signaling of these enzymes.

Although squalene cyclase and PGHS apparently share structurally similar membrane binding motifs, the amphipathic helices of these proteins have little or no sequence similarity. Indeed simple sequence analysis was not sufficient to identify the PGHS MBD sequences; their discovery depended on analysis of the crystal structures. It is likely that sequence homology to PGHS or squalene synthase will be insufficient to identify similar membrane binding domains in other proteins either. Thus, while a large family of integral membrane proteins anchored by amphipathic helices may exist, their discovery now depends on accumulation of additional structural information. Further study of the functional organization of the membrane binding helices of the PGHSs or the squalene synthase, however, may provide information in the future that can be used to predict the existence of these motifs from their primary sequences.

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3 3T3 cells were transfected with the plasmids pEGFP, PGHS-2-EGF-MBD-STEL, PGHS-2-EGF-STEL and PGHS-2-EGF as described under “Materials and Methods.” Forty-eight hours following transfection, the fluorescent intensity (509 nm) of the culture media was determined using an Perkin-Elmer 640-40 fluorescent spectrophotometer set with an excitation frequency of 395 nm. No significant fluorescence was observed in the media from any of the transfectants.
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