Mapping the Substrate Binding Site of the Prostaglandin Transporter PGT by Cysteine Scanning Mutagenesis*

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We have identified a cDNA, PGT, that encodes a widely expressed transporter for prostaglandin (PG) E₂, PGF₂α, PGD₂, 8-iso-PGF₂α, and thromboxane B₂. To begin to understand the molecular mechanisms of transporter function, we have initiated a structure-function analysis of PGT to identify its substrate-binding region. We have found that by introducing the small, water-soluble, thiol-reactive anion Na(2-sulfonatoethyl)methanethiosulfonate (MTSES) into the substrate pathway, we were able to cause inhibition of transport that could be reversed with dithiothreitol. Importantly, co-incubation with PGE₂ protected PGT from this inhibition, suggesting that MTSES gains access to the aqueous pore pathway of PGT to form a mixed disulfide near the substrate-binding site. To identify the susceptible cysteine, we mutated, one at a time, all six of the putative transmembrane cysteines to serine. Only the mutation of Cys-530 to serine within putative transmembrane 10 became resistant to inhibition by MTSES. Thus, Cys-530 is the substrate-protectable, MTSES-inhibitable residue. To identify other residues that may be lining the substrate-binding site, we initiated cysteine-scanning mutagenesis of transmembrane 10 using Cys-530 as an entry point. On a C530S, MTSES-resistant background, residues in the N- and C-terminal directions were individually mutated to cysteine (Ala-513 to His-536), one at a time, and then analyzed for MTSES inhibition. Of the 24 cysteine-substituted mutants generated, 6 were MTSES-sensitive and, among these, 4 were substrate-protectable. The pattern of sensitivity to MTSES places these residues on the same face of an α-helix. The results of cysteine-scanning mutagenesis and molecular modeling of putative transmembrane 10 indicate that the substrate binding of PGT is formed among its membrane-spanning segments, with 4 residues along the cytoplasmic end of helix 10 contributing to one surface of the binding site.

We recently identified a broadly expressed prostaglandin transporter called PGT that catalyzes high affinity transport of prostaglandin (PG) E₂, PGF₂α, PGD₂, 8-iso-PGF₂α, and thromboxane B₂ (1). Functional studies suggest that PGT transports its substrate as the charged anion and mediates substrate accumulation via electrogenic anion exchange (2). PGT is related to a family of polytopic membrane proteins that catalyze the transport of a variety of organic anions. These transporters include: oatp-1 (organic anion transporting polypeptide), known to transport bile acids and charged steroids (3); oatp-3, known to transport charged steroids and thyroid hormone (4); oatp-2, known to transport oatp3 substrates plus digoxin (5); and OAT-K1, known to transport methotrexate (6). All the members of this gene family are predicted to have a 12-membrane-spanning structure and, based on their primary and secondary structure homology, may share a common transport mechanism. Although transport by oatp-1 also has been suggested to also occur by anion exchange (7, 8), little is known about the molecular mechanism by which these organic substrates, bearing both hydrophilic and hydrophobic regions, are translocated across membranes.

Cysteine-scanning mutagenesis has been used extensively as a means to identify residues lining the substrate pathway of the nicotinic acetylcholine receptor (9–11), the GABA_γ-aminobutyrate receptor (12), the cystic fibrosis transmembrane conductance regulator (13), and the bacterial membrane transporter UhpT (14), and as a means to study the protein structures of lac permease (15–23) and bacteriorhodopsin (24, 25) among others.

Here we report a cysteine-scanning approach to transmembrane span 10 of PGT. Cysteine substitutions of six residues resulted in inhibition by the negatively charged reagent MTSES, and four of these residues were substrate-protected. The substrate binding region of PGT is likely formed, at least in part, by four residues along helix 10.

EXPERIMENTAL PROCEDURES

Materials—Na(2-sulfonatoethyl)methanethiosulfonate (MTSES) and MTSE-ethylammonium (MTSEE) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Tritated PGE₂ was from NEN Life Science Products. Unlabeled PGE₂ was from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma. All materials were reagent grade and obtained from commercial sources.

Oligonucleotide-directed Site-specific Mutagenesis—All mutants were prepared by oligonucleotide-directed site-specific mutagenesis of full-length rat PGT cDNA cloned into pGEM3z. Oligonucleotides were synthesized to generate the appropriate serine or cysteine mutation and to simultaneously introduce either a unique silent restriction site or antibiotic resistance. Mutagenesis was performed according to the manufacturers’ protocol (Chameleon double-stranded site-directed mutagenesis kit (Stratagene) or Gene Editor (Life Technologies, Inc.)). Mutations were identified by restriction digest or growth in antibiotic selection media and confirmed by DNA sequencing.

Oocyte Preparation and mRNA Injection—The intracellular mRNA transcription and the preparation and injection of Xenopus oocytes were performed as described previously (2). 30 ng of mRNA encoding full-length rat PGT were injected into individual oocytes prior to isotopic PGE₂ transport assays. Experiments were performed 2–5 days after mRNA injection.
**RESULTS**

**Effect of Sulfhydryl Reagents on Wild Type PGT—** Pretreatment of PGT-expressing oocytes with the negatively charged thiol-reactive agent MTSES caused a dose-dependent reduction in subsequent [3H]PGE2 uptake (Fig. 1). The addition of 1 mM dithiothreitol (DTT) during the MTSES exposure partially reversed the MTSES inhibition: 99% inhibition without DTT versus 45% inhibition with DTT. These findings indicate that MTSES inhibited transport by the formation of disulfide bonds. Moreover, the addition of 1 mM DTT alone caused a 30% inhibition of subsequent tracer PGE2 uptake, indicating that disulfide bonds may be important for full transport activity of wild type PGT.

The presence of PGE2 during the MTSES period, at a concentration 1000-fold lower than the MTSES, completely abrogated the MTSES effect. In contrast to the negatively charged MTSES, positively charged MTSEA was ineffective at inhibiting transport (Fig. 1). These findings support the following hypotheses: 1) the substrates of PGT are attracted into the substrate pathway as anions, and 2) a thiol-reactive, substrate-protectable cysteine(s) lies in the aqueous pore pathway within or near the substrate binding site.

**Identification of the MTSES-inhibitable Cysteine(s) in Wild Type PGT—** To identify the MTSES-inhibitable, substrate-protectable cysteine(s), we mutated, one at a time, each of six putative transmembrane cysteines as described previously (2). 

?H]PGE2 uptake was assayed 18–22 h after transfection.

**Transient Expression in HeLa Cells—** Wild type and mutant rPGT cDNAs cloned in pGEM-3z with coding strand downstream of the T7 promoter were transfected into HeLa cells, and expression was driven by vaccinia virus (3). Each of the six putative transmembrane cysteines represented here was individually mutated to serine, and each mutant was tested for its ability to transport [3H]PGE2 and for its susceptibility to MTSES inhibition.

**Substrate Binding and Transmembrane Span 10 in PGT**

![Figure 1](image1.png)

**FIG. 1.** Inhibition of PGT-mediated [3H]PGE2 influx by the water-soluble, thiol-reactive anion MTSES, but not by the cationic MTSEA. Oocytes injected with PGT cRNA were preincubated with MTSES (closed circles), MTSEA (closed squares), or MTSES plus 1 mM unlabeled PGE2 (open circle) at the indicated concentrations for 15 min and washed prior to the uptake assay. Pretreatment with MTSES resulted in a dose-dependent inhibition of subsequent [3H]PGE2 influx as compared with control. MTSES effect was blocked by co-incubation with PGE2. Pretreatment with MTSEA had no effect. Results are normalized to control oocytes lacking exposure to MTSES or unlabeled PGE2.

![Figure 2](image2.png)

**FIG. 2.** Secondary structure model of rat PGT based on hydrophathy analysis. There are 12 deduced regions of hydrophobicity that have been modeled as transmembrane helices. The six putative transmembrane cysteines are represented as dark circles. Each of the cysteines represented here was individually mutated to serine, and each mutant was tested for its ability to transport [3H]PGE2 and for its susceptibility to MTSES inhibition.
substrate-protectable cysteine in wild type PGT.

Construction and Transport Activity of Cysteine Substitution Mutants in Putative Transmembrane Domain 10—We used the MTSES-resistant mutant C530S as the starting point for further cysteine substitution mutagenesis. Starting with C530S, we mutated each residue, from Ala-513 through Leu-536 in transmembrane span 10, one at a time, to cysteine (Table I). We transiently expressed each of the 23 mutants in HeLa cell monolayers and determined its transport activity as compared with C530S. All of the cysteine-substituted mutants had transport activity ranging from 41% to 152% relative to that of C530S (Fig. 5). Thus, no residue from Ala-513 to Leu-536 within putative transmembrane span 10 was essential for transport.

Inhibition of the Cysteine Substitution Mutants with MTSES—We transiently expressed each of the mutants in HeLa cell monolayers and tested its susceptibility to MTSES inhibition. We assume that: 1) only residues on the water-accessible surface of the protein will react with the charged, water-soluble MTSES; 2) within a transmembrane span, cysteines that react with MTSES line the aqueous translocation pathway; and 3) cysteine-substituted side chains are in the same position as the wild type side chains. Based on these assumptions, our data indicate that, in addition to Cys-530, the following five residues are exposed to the aqueous pore pathway: Leu-515, Ile-522, Ala-526, Ala-529, and His-533 (Fig. 6).

Effect of PGE2 on MTSES Inhibition of Cysteine Substitution Mutants—To identify which of the MTSES-inhibitable cysteine substitutions could be in the substrate-binding site, we pretreated each mutant with MTSES in the presence and absence of 1 μM PGE2, washed, and assayed subsequent transport activity. Because the Km of PGT for PGE2 is 50–100 nM (26), this dose is saturating. We assume that residues located at or near the binding region would be protected from MTSES inhibition if the substrate PGE2 were present during MTSES pretreatment.

![Fig. 3](image3.png)

**Fig. 3.** [3H]PGE2 transport at 10 min by cysteine-to-serine mutants compared with wild type (WT) PGT. All influx values in these and subsequent graphs are from transfected HeLa cells and represent means ± S.E. of paired monolayers for two to eight independent experiments.

![Fig. 4](image4.png)

**Fig. 4.** Inhibition of [3H]PGE2 transport by cysteine-to-serine mutants using MTSES. Fractional inhibition was calculated as 1 (minus )transport after MTSES treatment/transport without MTSES). The solid bar indicates the mutant for which inhibition was significantly different (p < 0.05) from wild type (WT) by paired t test.

### Table I

**DNA sequence analysis of cysteine-substituted mutants**

| Mutant | Mutagenic oligonucleotide |
|--------|----------------------------|
| Q513C  | CCCAGGCTTGGGCTGCCGACTGCTGCTCCTGCCCTGTC   |
| L514C  | CGTCCTGCGGCGAGTCCCTGCTCCCTGGCTGCCCTG   |
| L515C  | CCTGCCGGCGCTGCACTGCTTCTGGCTCCCTGACATC   |
| L516C  | GCG GCCAGTACTGCTGCTCCGTCATTCCTCCGCT    |
| P517C  | GGCAGCTACGTCTGCCCTGCATCTTCCCTGCTCG   |
| S518C  | GACGCTACTGCTGCTTCCGTCATCTTCCCTGCTCC   |
| I519C  | GCTACTGCTGCCCTGGCTTCCTGCTATTCTCCGCT   |
| P520C  | GCTCCGCTTCACTGCTCAATTCTCCTGCTTCCGCT   |
| L521C  | GCTCCGCTTCACTGCTCAATTCTCCTGCTTCCGCT   |
| I522C  | CGTGCTCCATCTCCTCCTGCTCCGTCATTCCTCCGCT |
| S523C  | CCATCCTCTCCCTGCTTCCGTCATCTTCCCTGCTCC |
| F524C  | CTTGCTCCATCTCCTCCTGCTCCGTCATTCCTCCGCT |
| I525C  | GCTACTGCTGCCCTGGCTTCCGTCATTCCTCCGCT |
| A526C  | CTTACTTCTCCTCCTGCTCCGTCATTCCTCCGCT |
| L527C  | CTTGCTCCATCTCCTCCTGCTCCGTCATTCCTCCGCT |
| I528C  | CTTGCTCCATCTCCTCCTGCTCCGTCATTCCTCCGCT |
| A529C  | CGTCCTGCGGCGCTGCCGACTGCTGCTCCTGCCCTGTC |
| I531C  | GGCTCCTAGCGGCGCTGCCGCTCCGACAACCGGCGCT |
| S532C  | CGTCCTAGCGGCGCTGCCGCTCCGACAACCGGCGCT |
| H533C  | GCTCCGCTTCACTGCTCCGTCATTCCTCCGCTCTACATG |
| N534C  | GCTCCGCTTCACTGCTCCGTCATTCCTCCGCTCTACATG |
| P535C  | CCATCCTCCACAACTCGCCCTCTCACTAGTGG |
| L536C  | CCCACACCGCCTGCCACATGCTATGCCCTGCT |
ment. Of the six cysteine mutants that were inhibited by MT-SES, only A526C, A529C, H533C, and Cys-530 were significantly protected by PGE2 (Fig. 8).

**DISCUSSION**

In the present study, we have attempted to delineate the substrate-binding region of the prostaglandin transporter PGT by testing the susceptibility to covalent inhibition by the charged, hydrophilic, thiol-reactive reagents MTSES and MTSEA. We found that the anionic MTSES caused a dose-dependent reduction in subsequent tracer PGE2 uptake, whereas the cationic MTSEA had no effect. Importantly, co-incubation of MTSES with PGE2 protected PGT from this inhibition, suggesting that MTSES gains access to the aqueous pathway of PGT to form a mixed disulfide near the substrate binding site (Fig. 1). Mutagenesis revealed that Cys-530 is sensitive to MTSES in wild type PGT, that transmembrane span 10 (containing Cys-530) is α-helical, and that several residues of span 10, facing the aqueous pathway, are likely at or near the substrate-binding site.

As has been observed by others with a variety of transporters (27–30), mutagenesis of cysteines to other residues was generally well tolerated (Fig. 3). All the single cysteine mutants demonstrated significant tracer PGE2 uptake as compared with wild type PGT, except for C419S, which demonstrated 20% wild type activity. Of the six cysteines we mutated, only Cys-419 is conserved in the organic anion transporter family (oatp1 (3), oatp2 (5), and OAT-K1 (6). The other conserved cysteines all appear to be clustered within putative extracellular loops 5 and 6. Because we can remodel PGT and also place Cys-419 in putative extracellular loop 5, one explanation for the decrease in activity of the C419S mutant is that a structurally important extracellular disulfide pair was disrupted. Disulfide bonds have functional importance for PGT because treatment of wild type PGT with DTT results in a 30% decrease

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**FIG. 5.** Cysteine scanning of transmembrane span 10, no inhibitor. Figure shows [3H]PGE2 transport by HeLa cells expressing cysteine substitution mutants, normalized to that of the MTSES-resistant C530S mutant.

**FIG. 6.** Effect of MTSES pretreatment on [3H]PGE2 transport by HeLa cells expressing individual cysteine substitution mutants or wild type PGT containing Cys-530. Solid bars indicate mutants for which the MTSES inhibition was significantly different from 0 (p < 0.05).

**FIG. 7.** Helical wheel plot of residues in transmembrane span 10 of approximately 7 turns (24 residues). The putative helical span begins with Gln-513 (lower left) and proceeds counterclockwise through Leu-536 (right). Residues Leu-515, Ile-522, Ala-526, Ala-529, Cys-530, and His-533 lie along one face of a predicted α-helix.
Substrate Binding and Transmembrane Span 10 in PGT

![Substrate Binding and Transmembrane Span 10 in PGT](image)

Fig. 8. PGE₂ protection of cysteine substitution mutants. HeLa cells expressing the five-span 10 MTSES-sensitive cysteine substitution mutants or wild type (Cys-530) PGT were treated with 1 μM MTSES in the presence or absence of 1 μM unlabeled PGE₂ prior to the transport assay. Percentage of substrate protection was calculated as percentage of inhibition in the absence of PGE₂ minus percentage of inhibition in the presence of PGE₂. Solid bars indicate the residues for which protection by PGE₂ was significant (p < 0.05).

in transport. Alternatively, the poor function of C419C may be because serine at position 419 has the ability to hydrogen-bond to the backbone and disrupt structure.

Of the single cysteine-to-serine mutants generated, only C530S within putative transmembrane 10 was resistant to MTSES inhibition (Figs. 2 and 3). These findings support Cys-530 as the residue that reacts with MTSES to inhibit transport function. Using the C530S, MTSES-resistant mutant as a starting point, we applied the substituted cysteine accessibility method developed by Akabas et al. (31) to transmembrane 10 in order to identify residues exposed to the binding site region of PGT. On a C530S background, residues Ala-513 through Leu-536 were replaced, one by one, with cysteine and tested for MTSES-sensitivity. We found that cysteine at positions 515, 522, 526, 529, 530, and 533 made PGT sensitive to MTSES. The interpretation of the results is based on several assumptions: 1) that cysteine will generally be a well tolerated substitute for other residues, 2) that the hydrophilic MTSES will only react with residues exposed to the water-accessible surface of the protein, and 3) that the presence of substrate will prevent MTSES-induced inhibition of those residues at or near the binding site. Based on the assumptions above, we can infer from this pattern of inhibition that the secondary structure of transmembrane span 10 is that of an α-helix (Fig. 7). However, the mutation I519C, predicted to lie on the water exposed surface of helix 10, was not inhibited by MTSES, indicating that there may be a disruption in the helix, or that the mutation resulted in a distortion in the protein. Interestingly, there is a proline at position 517 that may be inducing this bend in the helix. In proline, the last atom of the side chain is bonded to the main chain N atom, preventing participation in hydrogen bonding and causing disruption of α-helical conformation. Proline-induced bends in helical domains have been described in the M3 membrane-spanning domain of the cystic fibrosis transmembrane conductance regulator (32) and the M7 transmembrane segment of the dopamine D₃ receptor (33).

Because we have experimental evidence using epitope tagging (data not shown) that the large hydrophilic region before transmembrane helix 10 is extracellular (putatively extracellular loop 5), we can correctly orient residue Ala-513 toward the extracellular face and Leu-536 toward the cytoplasm. We do not know whether Leu-536 is in helix 10 or within the cytoplasmic loop. Residues near the cytoplasmic end of a helix or in cytoplasmic loops may not react with the negatively charged MTSES because of repulsion by the inside-negative field across the plasma membrane or because of the reducing environment in the cell. Either of these may account for the lack of apparent reactivity of Leu-536.

Because classic anion transport inhibitors, including the stilbene disulfonates and niflumic acid, block PGT-mediated PG uptake (2), because the substrate must be anionic in order to bind (26), and because transport is inhibited by MTSES (anionic) but not MTSEA (cationic), PGT likely transports substrate as the anion. One interpretation of our data is that a positive charge near the external end of the pore screens out positively charged reagents and allows MTSES entry. MTSES, by virtue of covalent attachment to span 10 residues, would then block PGE₂ transport either by steric hindrance or by introducing a new negative charge that electrostatically repulses the carboxyl group of the substrate. In either case, because the inhibition by MTSES extends over 4 helical turns or >20 Å, MTSES could not be binding to a positive charge before its covalent reaction with the substituted cysteines. An alternative, and we think much less likely, possibility is that binding of substrate causes a structural change in the protein, disrupting its function and causing helix 10 to rotate, thus “burying” the previously MTSES-sensitive residues. In this model, none of the “MTSES-sensitive” residues would need to contact the substrate.

Substrate-protectability could occur for residues Ala-529, Cys-530, and His-533 if substrate bound external to them at residue 526 and blocked the accessibility of MTSES. In this model, only residue 526 would need to bind the substrate. Although this remains a possibility, when we constructed a molecular model of transmembrane span 10, we noted that the substrate-protectable residues, Ala-526 to His-533, span a length that corresponds to the length of the substrate (Fig. 9), consistent with the contention that these residues contact the substrate.

The sequence of rat PGT is related to the sequences of the other members of the oatp (organic anion transporting polypeptides) gene family, but the transporters exhibit no overlap in substrate specificity. Nonetheless, the deduced primary and putative secondary structure homology suggests that members of this gene family may share a common mechanism. By delineating the substrate-binding site of PGT, we may begin to understand which regions, and perhaps which residues, account for the differences in substrate specificities. The results presented here represent the first identification of specific residues that are associated with substrate binding in this gene family. Of the four MTSES-inhibitable, PGE₂-protectable residues identified in PGT, none is conserved among the other transporters, suggesting that these may be contributing to the specific binding of prostanoids. Domain swapping and chimera approaches will be required to address these issues more definitively.

Our data indicate that the substrate-protectable residues are near the cytoplasmic end of transmembrane span 10. In this regard, it is of interest to consider the general question of the location of substrate-protectable transporter residues relative to transmembrane spans. In the acetylcholine receptor channel α subunit M2 segment, Pascual and Karlin (34) reported partial or complete protection by organic transport inhibitors of several residues over several turns of shared helical faces. These substrate-protected residues are modeled near the cytoplasmic end of the predicted transmembrane spans. In P-glycoprotein, two cysteine-substituted residues, modeled in the middle of transmembrane span 12, and three modeled toward the extracellular end of transmembrane span 6 were substrate-protectable against reaction with the thiol-reactive agent dithiobismoiomane (35). In the serotonin transporter, the substrates 5-hydroxytryptamine and cocaine protected the...
FIG. 9. A model of PGE$_2$ binding along transmembrane span 10. Residues 513–536, based on the results of cysteine scanning mutagenesis of transmembrane span 10, were built as a standard $\alpha$-helix, and PGE$_2$ was built in an extended conformation. The geometries of carbons 12–20 (including the hydroxyl at C-15) undergo hydrogen bonding or hydrophobic interactions with the substrate-binding sites, may lie at any location along the length of transmembrane helical spans. The accumulation of more data on a variety of types of transporters (i.e., carriers, channels, pumps) should help to clarify this issue further.

In summary, our data strongly suggest that transmembrane span 10 of the prostaglandin transporter PGT is an $\alpha$-helix, and that four residues on a shared face of this helix (Ala-526, Ala-529, Cys-530, and His-533) are substrate-protectable. These residues likely form part of the substrate-binding region of PGT.

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