Role of Conserved Transmembrane Cationic Amino Acids in the Prostaglandin Transporter PGT†

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ABSTRACT: The prostaglandin transporter "PGT" interacts electrostatically with its anionic substrate, based on inhibition by the disulfonic stilbenes [Chan, B. S. (1998) *J. Biol. Chem. 273*, 6689−6697], inhibition by the thiol-reactive anion sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) [Chan, B. S. (1999) *J. Biol. Chem. 274*, 25564−25570], and the requirement for a negatively charged 1-position carboxyl on the substrate [Itoh, S. (1996) *Mol. Pharm. 50*, 736−742]. Here we found that modification of positively charged residues on wild-type PGT by arginine- and lysine-specific reagents significantly inhibited transport. We previously found that the binding site of PGT is formed, at least in part, by its membrane-spanning segments [Chan, B. S. (1999) *J. Biol. Chem. 274*, 25564−25570]. Three charged residues within predicted transmembrane spans (E78, R560, and K613) are conserved in PGT and in related transporters. Substitution of the anionic residue E78 (E78D and E78C) produced an essentially functional transporter, whereas substitution of the cationic residues with neutral residues (R560N and K613Q) resulted in poorly functional transporters. Immunoblotting revealed similar expression levels of wild-type and mutant transporters, and immunostaining indicated correct targeting. Conservative charge substitutions (R560K, K613R, and K613H) resulted in generally functional transporters. In contrast, R560N was nonfunctional, whereas the substrate affinity of K613G decreased greater than 50-fold. Conservative substitutions retaining the charge at position 613 (K613R and K613H) restored the substrate affinity, suggesting a direct role of K613 in substrate binding. Double-neutral mutants E78G/R560C and E78G/K613C were inactive, indicating that these residues are not simply charge-paired. Our results suggest that an arginine at position 560 is critical for maximal substrate translocation, and that a positively charged side chain at position 613 contributes to electrostatic binding of the anionic substrate.

The prostaglandin transporter "PGT" is a member of the organic anion transporter family, including oatp1 (*1*), oatp2 (*2*), oatp3 (*3*), and OAT-K1 (*4*). Secondary structure homologies indicate that these transporters may share a common transport mechanism. On the other hand, their different substrate specificities suggest that certain divergent domains exist to confer substrate affinity. By delineating the substrate binding site of PGT, we may begin to elucidate the substrate specificity regions of this family of transporters.

Using a cysteine-scanning approach, we previously found that the binding site of PGT is formed, at least in part, by its membrane-spanning segments (*5*). The binding site of PGT is also likely to contain positively charged amino acid side chains that interact with the negatively charged carboxyl group of the substrate. Therefore, to identify cationic residues that interact with the substrate, we considered charged amino acids within putative membrane spans. In the study presented here, we focused on R560 and K613, cationic residues that are conserved in all members of the organic anion transporter family (Figure 1). We have studied the functional consequences of chemical modification of these amino acids in wild-type PGT and of site-directed mutation of each of the conserved charged residues, to determine their importance in PGT for prostanoid uptake.

MATERIALS AND METHODS

Materials. Tritiated PGE_2 was from Dupont New England Nuclear (Boston, MA). Unlabeled PGE_2 was from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma (St. Louis, MO). 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 mutation (Table 1) and to simultaneously introduce either a unique silent restriction site or antibiotic resistance. Mutagenesis was performed according to the manufacturer's protocol (Chameleon Double Stranded Site Directed Mutagenesis Kit from Stratagene or Gene Editor from GIBCO). Mutations were identified by restriction digestion or growth in antibiotic selection media and confirmed by DNA sequencing.

Transient Expression in HeLa Cells. Wild-type and mutant rPGT cDNAs cloned in pGEM-3z with the coding strand

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                                                         1                                   2
PGT     MGLLLKPGAR QGSGTSSVPD RRCPRSVFSN IKVFVLCHGL LQLCQLLYSA YFKSSLTTIE KRFGLSSSSS GLISSLN E IS
oatp1   MEETEKKIAT QEG------- -RL----FSK MKVFLLSLTC ACLTKSLSGV YMNSMLTQIE RQFDISTSVA GLINGSF E IG
oatp3   MGETEKRVAT HEV------- -RC----FSK IKMFLLALTW AYVSQSLSGI YMNTMLTQIE RQFDIPISIV GFINGSF E IG
oatp2   MGKSEKRVAT HGV------- -RC----FAK IKMFLLALTC AYVSKSLSGT YMNSMLTQIE RQFGIPTSIV GLINGSF E IG
OAT-K1  MGDLEKGAAT HGA------- -GC----FAK IKVFLMALTC AYVSKSLSGT FMSSMLTQIE RQFGIPTAIV GFINGSF E IG

                           3
PGT     NATLIIFISY FGSRVNRPRM IGIGGLLLAA GAFVLTLPHF LSEPYQYTST TDGNRSSFQT DLCQKHFGAL PPSKCHSTVP
oatp1   NLFFIVFVSY FGTKLHRPVV IGIGCVIMGL GCLLMSLPHF FMGRYEYETT ISPTGNLSSN SFLCMENRTQ TLKPTQDPAE
oatp3   NFLLIIFVSY FGTKLHRPIM IGVGCVIMGL GCFLMSLPHF LMGRYEYETT ISPTSNLSSN SFLCMENRSQ TLKPTQDPAE
oatp2   NLLLIIFVSY FGTKLHRPIM IGVGCAVMGL GCFLISLPHF LMGQYEYETI L-PTSNVSSN SFFCVENRSQ TLNPTQDPSE
OAT-K1  NLLLIIFVSY FGMKLHRPIV IGVGCAVMGL GCFIISLPHF LMGRYEYETT ILPTSNLSSN SFLCMENQTQ TLNPAQDPAE

                   4                                     5
PGT     DTHKETSSLW GLMVVAQLLA GIGTVPIQPF GISYVDDFAE PTNSPLYISI LFAIAVFGPA FGYLLGSVML RIFVDYGRVD
oatp1   CVKEMKSLMW ICVMVGNIIR GIGETPIVPL GISYIEDFAK SENSPLYIGI LEMGKVAGPI FGLLLGSYCA QIYVDIGSVN
oatp3   CIKEMKSLMW IYVLVGNIIR GIGETPIMPL GISYIEDFAK SENSPLYIGI LETGKVFGPI VGLLLGSFCA SIYVDTGSVN
OAT-K1  CVKEVKSLMW IYVLVGNIIR GIGETPIMPL GVSYIENFAK SENSPLYIGI LETGKMIGPI FGLLLGSFCA SIYVDTGSVN

                           6
PGT     TATVNLSPGD PRWIGAWWLG LLISSGFLIV TSLPFFFFPR AMSR-GAERS V--TAEETMQ TEEDKSRGSL MDFIKRFPRI
oatp1   TDDLTITPSD TRWVGAWWIG FLVCAGVNIL TSIPFFFLPK ALPKKGQQEN VAVTKDGKVE KYGGQAREEN LGITKDFLTF
oatp3   TDDLTITPTD TRWVGAWWIG FLICAGVNIL SSIPFFFFPK TLPKEGLQDD VDGTNNDKEE KHREKAKEEN RGITKDFLPF
oatp2   TDDLTITPTD TRWVGAWWIG FLVCAGVNIL TSFPFFFFPK TLPKEGLQEN VDGTENAKEK KHRKKAKEEK RGITKDFFVF
OAT-K1  TDDLTITPTD IRWVGAWWIG FLVCAGVNIL ISIPFFFFPK TLPKEGLQEN VDGTENAKEE STEKRPRKKN RGITKDFFPF

                  7                                      8
PGT     FLRLLMNPLF MLVVLSQCTF SSVIAGLSTF LNKFLEKQYG ATAAYANFLI GAVNLPAAAL GMLFGGILMK RFVFPLQTIP
oatp1   MKRLFCNPIY MLFILTSVLQ VNGFINKFTF LPKYLEQQYG KSTAEAIFLI GVYSLPPICL GYLIGGFIMK KFKITVKKAA
oatp3   MKSLSCNPIY MLLILTSVLQ INAFINMFTF LPKYLEQQYG KSTAEVVLLI GVYNLPPICI GYLLIGFIMK KFKITVKKAA
oatp2   MKSLSCNPIY MLFILISVLQ FNAFINSFTF MPKYLEQQYG KSTAEVVFLM GLYMLPPICL GYLIGGLIMK KFKVTVKKAA
OAT-K1  LKSPVLQPDL HAVHPYKVLQ VNAFNIYFSF LPKYLENQYG KSTAEVIFLM GVYNLPAICI GYLIAGFMMK KFKITVKTAA

                  9
PGT     RVAATIITIS MILCVPLFFM GCSTSAVAEV ---YPPSTSS -SIHPQQPPA CRRDCSCPDS FFHPVCGDNG VEYVSPCHAG
oatp1   YLAFCLSVFE YLLFLCHFML TCDNAAVAGL TTSYKGVQHQ LHVESKVLAD CNTRCSCSTN TWDPVCGDNG VAYMSACLAG
oatp3   YMAFCLSLFE YLLYFLHFMI TCDNFPVAGL TALYEGVHHP LYVENKVLAD CNRGCSCSTN SWDPVCGDNG LAYMSACLAG
oatp2   HLAFWLCLSE YLLSFLSYVM TCDNFPVAGL TTSYEGVQHQ LYVENKVLAD CNTRCNCSTN TWDPVCGDNG LAYMSACLAG
OAT-K1  FLRFCLSLSE YSFGFCNFLI TCDNVPVAGL TNSYERDQKP LYLENNVLAD CNTRCSCLTK TWDPVCGDNG LAYMSACLAG

                                                      10
PGT     CSSTNTSSEA SKEPIYLNCS CVSGGSASAK TGSCPTSCAQ LLLPSIFLIS FAALIACISH NPLYMMVLRV VNQDEKSFAI
oatp1   CKKFVGTGTN MVFQDCSCIQ SLGNSSAVLG LCKKGPECAN RLQYFLILTI IISFIYSLTA IPGYMVFLRC VKSEEKSLGV
oatp3   CKKSVGTGTN MVFQNCSCIR SSGNSSAVLG LCKKGPECAN KLQYFLIMSV IGSFIYSITA IPGYMVLLRC IKPEKKSLGI
oatp2   CEKSVGTGTN MVFQNCSCIQ SSGNSSAVLG LCNKGPDCAN KLQYFLIIAI FGCFIYSLAG IPGYMVLLRC IKSEEKSLGV
OAT-K1  CEKSVGTGTN MVFHNCSCIQ SPGNSSAVLG LCNKGPECTN KLQYLLILSG FLSILYSFAA IPGYMVFLRC IKSEEKSLGI

              11                                         12
PGT     GVQFLLM R LL AWLPAPSLYG LLIDSSCVRW NYLCSGRRGA CAYYDNDALR NRYLGLQMVY K ALGTLLLFF ISWRMKK---
oatp1   GLHTFCI R VF AGIPAPVYFG ALIDRTCLHW GTLKCGQRGA CRMYDINSFR HIYLGLPIAL R GSSYLPAFF ILILMRKFQF
oatp3   GLHAFCT R VF AGIPAPIYFG ALIDRTCLHW GTLKCGEPGA CRMYNINNFR RIYLVLPAAL R GSSYLPALF ILILMRKFQF
oatp2   GLHAFCI R IL AGIPAPIYFG ALIDRTCLHW GTLKCGEPGA CRMYDINSFR RLYLGLPAAL R GASFVPAFF ILRLTRTFQF
OAT-K1  GIHAFCI R VF AGIPAPIYFG ALIDRTCLHW GTQKCGAPGR -RMYDINSFR RIYLGMSAAL R GSSYLPAFV IVILTRKFSL

PGT     ---------- --NREYSLQE NTSGLI---- ---------- ---
oatp1   PGDIDSSATD HTEMMLGEKE SEHTDVHGSP QVENDGELKT KL-
oatp3   PGEIDSSETE LAEMKITVKK SECTDVHGSP QVENDGELKT RLL
oatp2   PGDIESSKTD HAEMKLTLKE SECTEVLRS- KVTED----- ---
OAT-K1  PGKINSSEME IAEMKLTEKE SQCTDVHRNP KFKNDGELKT KL-
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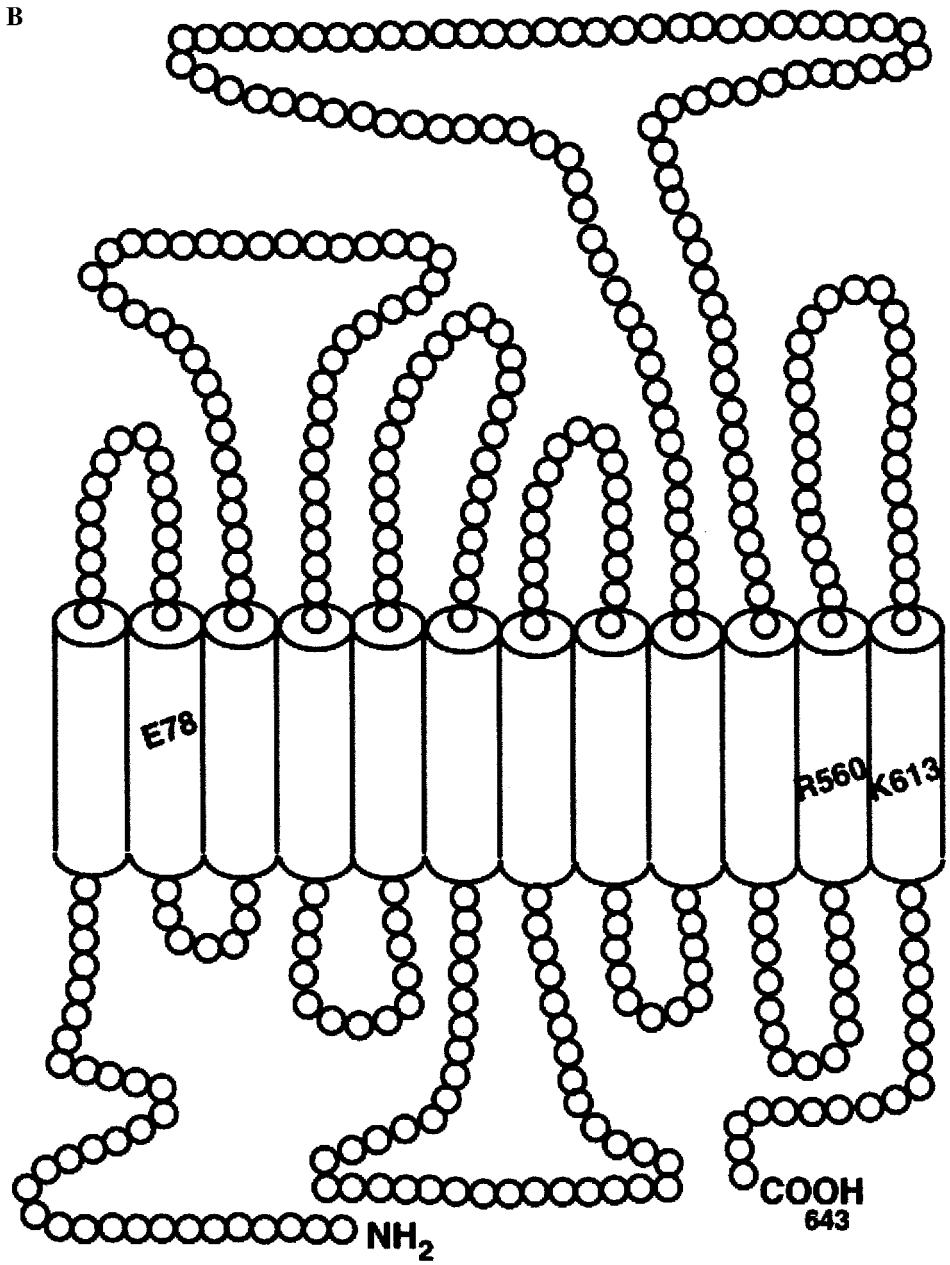

FIGURE 1: (A) Amino acid comparison of rat PGT, oatp1, oatp2, oatp3, and OAT-K1. The 12 putative transmembrane segments (1−12) were assigned on the basis of hydropathy analysis. The three conserved transmembrane charges corresponding to E78, R560, and K613 in PGT are shown in bold. (B) Secondary structure model of PGT based on hydropathy analysis. The three transmembrane charges E78, R560, and K613 are conserved within putative transmembrane spans 2, 11, and 12, respectively, in all related organic anion transporters.

downstream of the T7 promoter were transfected into HeLa cells, and expression was driven by vaccinia vtf7-3 as previously described (*8*). [^{3}H]PGE_2 uptake was assayed 18−22 h after transfection.

Assay of [^{3}H]PGE_2 Transport. Influx measurements were initiated by the addition of tritiated PGE_2 (Dupont New England Nuclear) to a balanced salt solution (BSS)[1] (135 mM NaCl, 13 mM H-Hepes, 13 mM Na-Hepes, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM $MgSO_4$, 5 mM KCl, and 1 mM D-glucose). Influx measurements were carried out at room temperature over the course of 2−10 min. Isotopic influx experiments were terminated by aspiration of the incubation medium followed by two rapid washings with ice-cold 5% bovine serum albumin (BSA) in BSS and two additional washings with ice-cold BSS. HeLa cells were scraped into 1 mL of saline, then mixed with liquid scintillation cocktail (National Diagnostics, Atlanta, GA), and analyzed by liquid scintillation counting.

Treatment with Amino Acid-Modifying Reagents. HeLa cell monolayers expressing wild-type rPGT were preincubated at room temperature with either phenylglyoxal (PGO) (*7*, *8*) (1 mM) or trinitrobenzenesulfonic acid (TNBS) (1 mM) (*9*, *10*) in BSS for 30 min, washed twice, and then sub-

[1] Abbreviations: MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSEA, MTS-ethylammonium; MTSEA, MTS-ethylammonium; BSS, balanced salt solution; BSA, bovine serum albumin; PGO, phenylglyoxal; TNBS, trinitrobenzenesulfonic acid; WRK, Woodward's reagent K; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; FITC, fluorescein 5′-isothiocyanate; SE, standard error.

Table 1: Mutations Introduced into PGT[a]

	mutagenic primer
R560N	5′-CAGTTCTTGTTGATGAACTTGCTGGCCTGGCTG
R560K	5′-CAGTTCTTGTTGATGAAATTGCTGGCCTGGCTG
R560H	5′-CAGTTCTTGTTGATGCACTTGCTGGCCTGGCTG
K613G	5′-CTACAGATGGTCTACGGAGCCTTGGGCACACTG
K613R	5′-CTACAGATGGTCTACCGGGCCTTGGGCACACTG
K613H	5′-CTACAGATGGTCTACCACGCCTTGGGCACACTG
E78N/R560C	5′-CCAGTTTGAATCAGATCAGCAACGCTACC
	5′-CAGTTCTTGTTGATGTGCTTGCTGGCCTGGCTG
E78N/K613C	5′-CCAGTTTGAATCAGATCAGCAACGCTACC
	5′-CTACAGATGGTCTACTGCGCCTTGGGCACACTG
E78D	5′-CCAGTTTGAATGACATCAGCAACGCTACC
E78C	5′-CCAGTTTGAATTGCATCAGCAACGCTACC

[a] The mutagenic primer is in the sense orientation. The affected codon is underlined.

Table 2: Kinetics of [^{3}H]PGE_2 Uptake in PGT Mutants[a]

	K_1 (nM)	$uptake_{mut}/uptake_{wt}$ (%)
wild type	17 ± 16	100
R560N	−	
R560K	20 ± 20	14 ± 6
K613G	>1000	7 ± 2
K613R	15 ± 16	9 ± 2
E78G/R560C	−	0
E78G/K613C	−	0
E78D	61 ± 21	46 ± 8
E78C	−	34 ± 8

[a] The means ± SE from two to three independent experiments are shown. $uptake_{mut}/uptake_{wt}$ was obtained by dividing each [^{3}H]PGE_2 uptake value by the [^{3}H]PGE_2 uptake value of the wild-type transporter.

jected to isotopic PGE_2 influx measurements. In some experiments, cells were preincubated with reagents in the presence of 1 μM unlabeled PGE_2 prior to the [^{3}H]PGE_2 transport assay.

Inhibitory Constants. Inhibitors were added at various concentrations during [^{3}H]PGE_2 uptake. $K_{1/2}$ was determined by relating the uninhibited uptake rate to the inhibited uptake rate calculated from the relation (*11*)

$$K_{1/2} = [v(i)/v - v(i)]K_m([i]/[S]) + K_m$$

where v is the rate of uptake without inhibitor, $v(i)$ is the rate of uptake with inhibitor, and [i] is the inhibitor concentration. Since the [^{3}H]PGE_2 concentration was much less than K_m (*12*), the equation may be simplified to $K_{1/2} = [v(i)/v - v(i)][i]$.

Protein Immunoblotting and Immunohistochemistry. Antipeptide monoclonal antibodies directed against deduced amino acids 430−505 on putative exofacial loop 5 of rPGT, as described previously (*13*), were used for Western blot analysis and immunolocalization of wild-type and mutant rPGT. Wild-type and mutant rPGT-transfected HeLa cells and sham-transfected HeLa cell lysates were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to nitrocellulose membranes. Membranes were then incubated with rPGT-specific monoclonal antibody for 1 h at room temperature, and then subsequently with horseradish peroxidase-linked (HRP) goat anti-mouse secondary antibody (Boehringer Mannheim, Indianapolis, IN) at a dilution of 1:5000 for 1 h at room temperature. Detection of the transporter protein was performed using enhanced chemiluminescence (ECL) reagent (Dupont New England Nuclear).

For immunohistochemistry, wild-type rPGT, mutant rPGT, and sham-transfected HeLa cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) [138 mM NaCl, 2.7 mM KCl, and 10 mM Na_2HPO_4 (pH 7.4)] at room temperature for 15 min, washed three times with PBS, and blocked with 1% bovine serum albumin, 0.2% gelatin, and 0.1% Triton X-100 in PBS at room temperature for 1 h. Cells were then incubated with rPGT-specific monoclonal antibody overnight at 4 °C, washed three times with PBS, and incubated for 1 h at room temperature with either HRP-linked goat anti-mouse IgG at a 1:500 dilution or fluorescein 5′-isothiocyanate-conjugated (FITC) goat anti-mouse IgG at a 1:200 dilution. Cells incubated with HRP-linked secondary antibody were fixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, and stained with 4% uranyl acetate and lead citrate (0.01 g/10 mL). Samples were examined under a Philips 410 transmission electron microscope operated at 80 kV. Cells incubated with FITC-conjugated secondary antibody were washed three times with PBS, mounted onto slides with Vectashield (Vector Laboratories, Burlingame, CA), and examined with a Bio-Rad confocal fluorescence microscope.

RESULTS

Effect of Amino Acid-Modifying Reagents on Wild-Type PGT. We examined the effect of various chemical modifiers on the rate of [^{3}H]PGE_2 uptake by HeLa cells transiently expressing wild-type PGT. The modifiers that were used were arginine-specific phenylglyoxal (PGO) and lysine-specific trinitrobenzenesulfonic acid (TNBS). As shown in Figure 2, modification with PGO or TNBS resulted in inhibition of PG transport (18 ± 9 and 55 ± 16% of that of wild-type PGT, respectively). These results suggest that arginine and lysine residue(s) may play a role in the transport mechanism of PGT. We examined the effect of PGE_2 on PGO and TNBS inhibition by co-incubating PGE_2 and chemical modifiers prior to the uptake assay. No protective effect of substrate was observed, as indicated by the similar degrees of inhibition in the presence and absence of PGE_2 (data not shown).

Western Blot and Immunolocalization of PGT Mutants. To explore further the role of transmembrane cationic residues, we neutralized R560 and K613 and made conservative mutations retaining the charge. We also considered if these residues may be paired with a transmembrane negatively charged amino acid residue as a salt bridge and, therefore, made double-neutral replacement mutants with

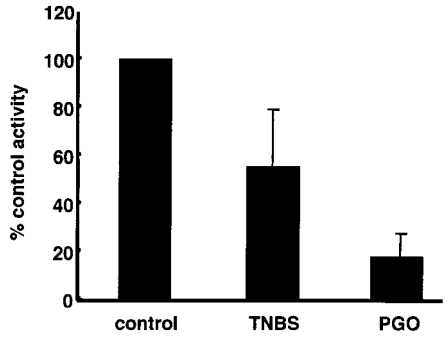

FIGURE 2: Effect of amino acid-modifying reagents on [^{3}H]PGE_2 uptake in PGT-expressing HeLa cells. Control influx (PGT) was performed under the usual conditions at 25 °C. Other cells were either preincubated with (1) 1 mM PGO, (2) 1 mM TNBS, or (3) 1 mM WRK and sodium borohydride prior to the uptake assay. Values represent means ± standard error of paired monolayers for three independent experiments.

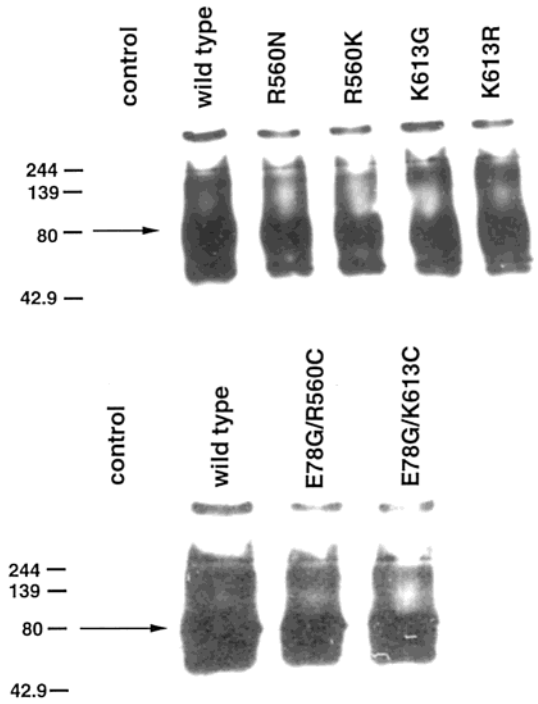

FIGURE 3: Expression of PGT and mutant transporters as detected by immunoblot analysis. HeLa cells were transfected with either wild-type PGT cDNA or mutant PGT cDNA and homogenized 18−22 h later. The protein (10 μg) from each homogenate was subjected to immunoblot analysis with antibodies to putative exofacial loop 5 of rat PGT and visualized using HRP-conjugated secondary antibody. The 80 kDa recombinant transporters were visualized with the ECL detection system and autoradiography.

conserved transmembrane E78. We performed Western blots to determine whether the amino acid substitutions caused any effect on the expression efficiency or stability of the proteins, and we also immunolocalized the expressed proteins to determine whether the mutations prevented trafficking of the proteins to the plasma membrane.

As shown in Figure 3, both wild-type and mutated PGT cDNAs were translated into proteins with a molecular mass of 80 kDa. Moreover, the expression rates of wild-type and mutant transporters were comparable, indicating that the mutations did not alter the expression efficiency. Although the biosynthesis of the transporters appeared to be normal, the mutations may have caused misfolding of the proteins

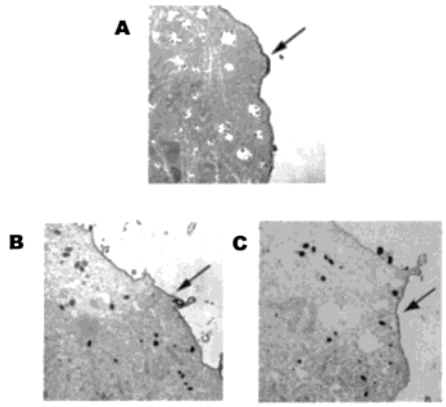

FIGURE 4: Wild-type and mutant PGTs are expressed at the plasma membrane. Electron micrographs of HeLa cells expressing (A) wild-type PGT, (B) R560N, and (C) K613G demonstrated cell surface expression with similar intensities (arrows).

such that they would not be properly sorted to the plasma membrane. However, as shown in Figure 4, sections through HeLa cell monolayers expressing wild-type, R560N, or K613G mutants showed that they expressed protein at the plasma membrane in similar amounts. In addition, all the mutants expressing single- and double-amino acid mutations were analyzed by confocal immunofluorescent microscopy and were found to have similar levels of surface expression (data not shown).

Functional Characterization of PGT Mutants. The effect of the mutations on PGT was analyzed in HeLa cells expressing wild-type and mutant transporters by assessing PGE_2 uptake and substrate affinity. The kinetics of transport by wild-type PGT and its mutants are summarized in Table 1. Neutralization of R560 completely abolished the function of the transporter. Although it is possible that R560 contributes to electrostatic binding of the anionic substrate, we were unable to measure the K_m because the R560N mutant was nonfunctional. Replacement of arginine with lysine restored transport activity to ~14% of wild-type activity. R560K had reduced transport activity but exhibited no reduction in K_m (20 nM). To explore further the importance of a positively charged residue at position 560, we generated a R560H mutant and determined the pH dependence of transport. In a pH range of 6−8, the R560H mutant was nonfunctional (data not shown), indicating either that protonated histidine at that position was unable to restore function or that R560H was not titratable in this pH range.

Neutralization of K613 caused a significant decrease in transport activity to ~7% of wild-type activity. Importantly, K613G demonstrated a greater than 50-fold increase in K_m. Replacement of K613 with the conservative charge mutation arginine restored substrate binding as indicated by the restoration of wild-type K_m; however, transport activity was still significantly decreased to only 9% of that of wild-type PGT. The kinetic data for these three K613 substitutions are shown in Figure 5.

We determined further the importance of a positively charged residue at position 613 by assessing the effect of pH on K613H. As shown in Figure 6, the transport rate of K613H increased as the external pH was lowered from 8.0 to 6.0. In contrast, transport by wild-type PGT was not affected by an external pH change in the range of 6.0−8.0. These data are consistent with an effect of pH by titrating

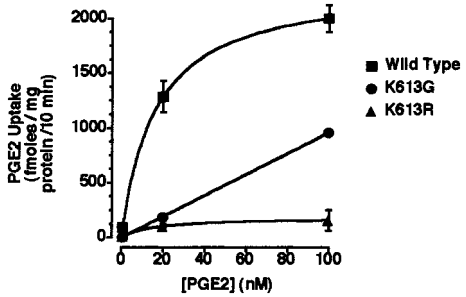

FIGURE 5: Kinetics of [^{3}H]PGE_2 transport by wild-type, K613G, and K613R PGT. Data are expressed as the means ± SE of three independent experiments.

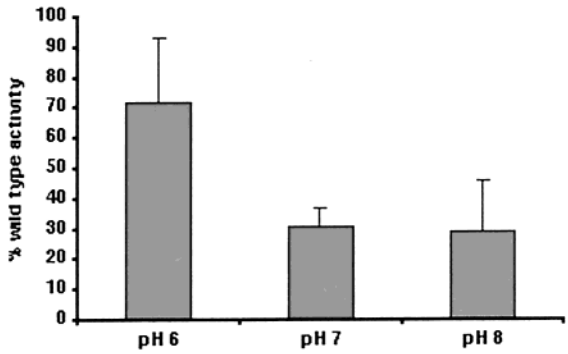

FIGURE 6: pH dependence of [^{3}H]PGE_2 transport by K613H. [^{3}H]-PGE_2 transport was assessed at pH 6.0, 7.0, and 8.0. Data are expressed as means ± SE of three independent experiments, normalized to that of the wild-type transporter.

the histidine substituted at position 613 to produce an electrostatic interaction with the substrate.

Substitution of E78 with the conservative mutation E78D resulted in retention of ~46% of PGT function (Table 1). However, the K_m was increased approximately 3.5-fold. The neutral substitution E78C retained 34% of the function. Both E78D and E78C were well-expressed at the plasma membrane (data not shown).

Because the presence of charged residues E78, R560, and K613 in the dielectric environment of the membrane is predicted to be energetically unfavorable (*14*, *15*), we considered whether these residues might be interacting to form self-neutralizing ion pairs. To test if E78 might be charge paired with either R560 or K613, we neutralized both E78 and R560, or E78 and K613. The prediction is that, if a pair of residues were forming a salt bridge, then a neutral replacement of one of those residues would lead to a decrease in activity, whereas double-neutral replacements of the opposing charges would restore activity (*16*, *17*). As shown in Table 1, both double-neutral replacement mutants E78N/R560N and E78N/K613G were essentially inactive.

DISCUSSION

We previously proposed that the substrate binding site of PGT lies within transmembrane spans, that residues facing the water-accessible surface on the cytoplasmic end of transmembrane helix 10 contact the substrate, and that the substrate binding site likely contains positively charged amino acid residues that interact with the anionic substrate (*5*). In the study presented here, we focused on cationic residues within putative transmembrane spans, Arg560 and Lys613, that are conserved in PGT and in related organic anion transporters (*1*−*4*). Substitution with uncharged residues (R560N and K613G) resulted in properly sorted, but poorly functional, transporters when they were expressed transiently in HeLa cell monolayers (Figure 4 and Table 1). Conservative mutations retaining the charge restored function in K613H and modestly in R560K and K613R, but not in R560H (Table 1 and Figure 5). Although mutation of R560 to asparagine rendered the transporter nonfunctional, making K_m unmeasureable, the K613G mutation exhibited a greater than 50-fold increase in K_m, consistent with a direct role of K613 in substrate binding.

On the basis of the structures of PGT substrates, we have postulated that the substrate binding site consists of two major domains: (1) a positively charged site to accommodate the charged carboxyl end of PG and (2) a more hydrophobic site that confers specificity to the remaining regions of the substrate. Three lines of evidence support a role for electrostatic forces in PGT substrate binding. First, transport is inhibited by several classic anion transport inhibitors (*6*). Second, the anionic, thiol reactive sodium (2-sulfonatoethyl)-methanethiosulfonate (MTSES) inhibits function, but the cationic version (MTSEA) does not, evidence for a translocation pathway that is anion selective (*5*). Third, removal of the negatively charged 1-position carboxyl group abolishes substrate affinity (*12*).

Consistent with a positively charged binding site, our data indicate that modification of positively charged residues on wild-type PGT using arginine-specific PGO and lysine-specific TNBS (Figure 2) inhibits transport. Inhibition by these agents may result from one or more of the following. First, the reagent(s) may neutralize cationic residue(s) in the binding site, reducing the extent of substrate binding. Second, the reagents may react with residues away from the binding site but inhibit transport by steric blockade (*18*). Third, the reagent(s) may cause a nonspecific long-range conformational change in the protein, rendering it nonfunctional.

Because our data from cysteine-scanning mutagenesis indicate that the substrate binding region lies, at least in part, within transmembrane spans (*5*), transmembrane cationic residues would likely contribute to the positively charged binding site. Our model of PGT predicts that E78, R560, and K613 (*19*) lie within membrane-spanning domains. The conservation of these three residues in corresponding positions in related organic anion transporters suggests a fundamental role, either in substrate binding or in translocation, or as self-neutralizing ion pairs involved in stabilizing the protein structure.

When we neutralized K613 and R560, K613G was able to transport PGE_2 at only 7% of the rate of the wild type, whereas R560N was nonfunctional (Table 1). We considered four possible explanations for these results. (1) The mutations cause a disruption in protein synthesis. (2) The mutations disrupt targeting to the plasma membrane. (3) The mutant protein is properly sorted to the plasma membrane but is unable to bind the substrate. (4) The mutant protein is properly sorted and is able to bind, but not translocate, the substrate. We eliminated the first two possibilities by Western blot analysis (Figure 3) and immunocytochemical localization (Figure 4). Therefore, these residues are likely to be participating in the transport mechanism of PGT, in either the binding or translocation of the substrate.

An important result of this study is that substitution of K613 with a neutral residue dramatically increased K_m, suggesting that this residue contributes directly to PG binding. Consistent with this hypothesis, the mutation K613R completely restored substrate affinity (Table 1). Although it is possible that the K613R mutation alters K_m from a distance, the simplest interpretation of our data is that there is an electrostatic interaction of K613 with the substrate. Of interest, whereas K613R exhibited only 9% of wild-type transport activity, the mutant K613H at pH 6.0 (Figure 5) retained nearly full transport activity. Under pH 6.0 conditions, the main difference between the three amino acids is the length of the side chain (R > K > H). Because substitution with arginine, which is ≈1.5 Å longer than lysine, significantly decreased the rate of transport, it appears that the size of the side chain at position 613 is also important.

Substitution of R560 with a neutral residue abolished transport, whereas substitution with lysine restored function to 14% of that of the wild type. Because R560N was nonfunctional, we were unable to measure its K_m. However, the K_m of R560K was comparable to that of the wild type. Because R560N is sorted to the plasma membrane, these data indicate that a positive charge in this location is critical for substrate translocation. In contrast to K613H, R560H, even at pH 6.0, remained nonfunctional. Thus, as the side chain at amino acid position 560 becomes shorter, the rate of transport progressively diminishes. One explanation for these findings is that R560 plays a role in transport via short-range electrostatic interactions, and that progressive shortening of the side chain disrupts these forces. Alternatively, histidine may be in a nonpolar environment at this position such that its p*K* is not titratable at pH 6 (*20*, *21*).

The conservative substitution E78D resulted in a modest decline of function and affinity. Complete removal of the negative charge at this position (E78C) still allowed about one-third of normal function. Thus, a negative charge at position 78 appears to be important, though not essential. We considered that E78 might form an interacting ion pair with either R560 or K613. To test this hypothesis, we constructed the double mutants E78N/R560C and E78N/K613C, which introduce neutral residues at the two putative opposite charge positions. In lac permease, for example, neutralizing both partners of an ion pair rescues function (*22*, *23*). However, both the E78N/R560C and E78N/K613C double mutants were nonfunctional, suggesting that these residues are not simply charge-paired. If our prediction is correct that K613 is critical for substrate binding, then we would not expect that the E78N/K613C mutation would restore function. It remains possible that R560 is ion paired with E78 but that neutralization of charge alone was inadequate to rescue function. R560 has one guanidino group; one NH_2 group of this guanidino group might interact with E78 to form a salt bridge, and the other NH_2 group might form a functionally important hydrogen bond. Such a model has been proposed in lac permease, in which the irreplaceable residue R144 interacts with the substrate via hydrogen bonding and is also ion paired with E126 (*24*).

In summary, these results demonstrate that R560 and K613 of rat PGT are critical for substrate transport. The residue at position 613 must be positively charged for maximal transport function and likely contributes to electrostatic binding of the anionic substrate. Although R560 is irreplaceable for full transport function, it is unclear whether it plays a role in substrate translocation and/or direct substrate binding. Using a cross-species chimera approach, we previously reported that a region within the carboxy-terminal portion of the protein imparts the difference in PGE_2 affinity between mice and humans (*25*). Both R560 and K613 lie within this domain and, therefore, may interact directly with the substrate. Application of cysteine-scanning mutagenesis to residues flanking R560 and K613 will help determine whether neighboring residues on these membrane spans also contact the substrate.

REFERENCES

1. Jacquemin, E., Hagenbuch, B., Stieger, B., and Meir, P. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 133.
2. Noe, B., Hagenbuch, B., Stieger, B., and Meir, P. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10346.
3. Abe, T., Kakyo, M., Sakagami, H., Tokui, T., Nishio, T., Tanemoto, M., Nomura, H., Hebert, S. C., Matsuno, S., Kondo, H., and Yawo, H. (1998) *J. Biol. Chem. 273*, 22395.
4. Saito, H., Matsuda, S., and Inui, K. (1996) *J. Biol. Chem. 271*, 20719.
5. Chan, B. S., Satriano, J. A., and Schuster, V. L. (1999) *J. Biol. Chem. 274*, 25564.
6. Chan, B. S., Satriano, J. A., Pucci, M., and Schuster, V. L. (1998) *J. Biol. Chem. 273*, 6689.
7. Zaki, L., Bohm, R., and Merckel, M. (1996) *Circ. Res. 42*, 1053.
8. Communi, D., Lecocq, R., Vanweyenberg, V., and Erneux, C. (1995) *Biochem. J. 310*, 109.
9. Chang, L. S. (1996) *J. Protein Chem. 15*, 321.
10. Hanau, S., Dallocchio, F., and Rippa, M. (1993) *Arch. Biochem. Biophys. 302*, 218.
11. Neame, K. D., and Richards, T. G. (1972) *Elementary Kinetics of Membrane Carrier Transport*, pp 56−79, John Wiley and Sons, New York.
12. Itoh, S., Lu, R., Bao, Y., Morrow, J. D., Roberts, L. J., and Schuster, V. L. (1996) *Mol. Pharmacol. 50*, 738.
13. Bao, Y., Chan, B. S., Lu, R., and Schuster, V. L. (1996) *J. Am. Soc. Nephrol. 7*, 1644.
14. Rashin, A. A., and Honig, B. (1984) *J. Mol. Biol. 173*, 515.
15. Honig, B. H., and Hubbell, W. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5412.
16. Dunten, R. L., Sahin-Toth, M., and Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547.
17. Sahin-Toth, M., Dunten, R. L., Gonzalez, A., and Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547.
18. Hancock, R. E., Schmidt, A., Bauer, K., and Benz, R. (1986) *Biochim. Biophys. Acta 860*, 263.
19. Schuster, V. L. (1998) *Annu. Rev. Physiol. 60*, 221.
20. Perez-Canadillas, J. M., Campos-Olivas, R., Lacadena, J., Del, P. A., Gavilanes, J. G., Santoro, J., Rico, M., and Bruix, M. (1998) *Biochemistry 37*, 15865.
21. Geierstanger, B., Jamin, M., Volkman, B. F., and Baldwin, R. L. (1998) *Biochemistry 37*, 4254.
22. Frillingos, S., and Kaback, H. R. (1996) *Biochemistry 35*, 13363.
23. King, S. C., Hansen, C. L., and Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177.
24. Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802.
25. Pucci, M. L., Bao, Y., Chan, B., Itoh, S., Lu, R., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Schuster, V. L. (1999) *Am. J. Physiol. 277*, R734.

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