Pathophysiology of functional mutations of the thiazide-sensitive Na-Cl cotransporter in Gitelman disease

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Sabath, Ernesto, Patricia Meade, Jennifer Berkman, Paola de los Heros, Erika Moreno, Norma A. Bobadilla, Norma Vázquez, David H. Ellison, and Gerardo Gamba. Pathophysiology of functional mutations of the thiazide-sensitive Na−Cl− cotransporter in Gitelman disease. Am J Physiol Renal Physiol 287: F195–F203, 2004. First published April 6, 2004; 10.1152/ajprenal.00044.2004.—Most of the missense mutations that have been described in the human SLC12A3 gene encoding the thiazide-sensitive Na+−Cl− cotransporter (TSC, NCC, or NCCT), as the cause of Gitelman disease, block TSC function by interfering with normal protein processing and glycosylation. However, some mutations exhibit considerable activity. To investigate the pathogenesis of Gitelman disease mediated by such mutations and to gain insights into structure-function relationships on the cotransporter, five functional disease mutations were introduced into mouse TSC cDNA, and their expression was determined in Xenopus laevis oocytes. Western blot analysis revealed immunoreactive bands in all mutant TSCs that were undistinguishable from wild-type TSC. The activity profile was: wild-type TSC (100%) > G627V (66%) > R935Q (36%) > V995M (32%) > G610S (12%) > A585V (6%). Ion transport kinetics in all mutant clones were similar to wild-type TSC, except in G627V, in which a small but significant increase in affinity for extracellular Na+ or Cl− was observed. In addition, G627V and G610S exhibited a small increase in metolazone affinity. The surface expression of wild-type and mutant TSCs was performed by laser-scanning confocal microscopy. All mutants exhibited a significant reduction in surface expression compared with wild-type TSC, with a profile similar to that observed in functional expression analysis. Our data show that biochemical and functional properties of the mutant TSCs are similar to wild-type TSC but that the surface expression is reduced, suggesting that these mutations impair the insertion of a functional protein into the plasma membrane. The small increase in Cl− and thiazide affinity in G610S and G627V suggests that the beginning of the COOH-terminal domain could be implicated in defining kinetic properties.

distal tubule; salt reabsorption; structure; Na-Cl cotransporter; diuretics

GITELMAN DISEASE IS AN AUTOSOMAL recessive hereditary disorder characterized by hypokalemic metabolic alkalosis, hypomagnesemia, salt wasting, low blood pressure, and hypocalciuria (23) (OMIM 600968), which is caused by inactivating mutations in the SLC12A3 gene that encode the thiazide-sensitive Na+−Cl− cotransporter (TSC) (31). To date, >100 different mutations in this gene, including nonsense, splice site, and missense mutations, have been described to be linked with Gitelman disease (25, 31). One of the major features of Gitelman disease is a reduction in arterial blood pressure, resulting, at least in part, from a decrease in extracellular fluid volume (8). Cruz et al. (8) observed in a large family with Gitelman disease that heterozygous subjects are able to maintain normal blood pressure by increasing dietary salt intake. In addition, loss of TSC downregulation that is normally induced by WNK1 and WNK4 kinases has been suggested to be involved in the pathogenesis of a salt-dependent form of human hypertension known as pseudohypoaldosteronism type II (39, 40). Thus TSC is one of the genes that are involved in setting arterial blood pressure levels and, in doing so, is a candidate gene in the genesis of primary hypertension.

TSC represents the major NaCl transport pathway in the apical membrane of the mammalian distal convoluted tubule (7, 14, 21, 29, 37) and also serves as the receptor for thiazide-type diuretics, which are the first line pharmacological therapy for hypertension (6). Despite the important role of TSC in cardiovascular and renal physiology, pharmacology, and pathophysiology, little is currently known about the structure-function relationships in this cotransporter. In this regard, naturally occurring mutations in Gitelman patients can help to reveal amino acid residues playing a key role in defining functional characteristics. Analysis at the physiological level has demonstrated that most of the point mutations occurring in patients with Gitelman disease result in a complete block of cotransporter activity (9, 22). Thus the contribution of these amino acid residues to define the functional properties of the cotransporter cannot be obtained because the protein is not expressed. During the course of our previous study (22), however, we noticed that some of the missense mutations exhibit partial function. De Jong et al. (9) also observed that some point mutations result in TSC proteins that are glycosylated and exhibit partial activity as metolazone-sensitive 22Na+ uptake. Immunocytochemical analysis revealed that proteins harboring the functional mutations were equally present in the cytoplasm and plasma membrane. In the present study, we have now extended the functional and molecular characterization of TSC harboring “functional” mutations by performing a quantitative analysis comparing plasma membrane expression of wild-type and mutant TSC, as well as by defining the functional kinetic properties for ion transport and thiazide inhibition. Our observations expand the spectrum of mechanisms underlying Gitelman disease and suggest that the begin-
ning of the COOH-terminal domain could be implicated in defining affinity for extracellular Cl⁻ and thiazides.

**METHODS**

*Helix laevis* oocyte preparation. Oocytes were surgically harvested from anesthetized adult female *X. laevis* frogs under 0.17% tricaine and incubated for 1 h under vigorous shaking in frog Ringer-ND96 (mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES/Tris, pH 7.4), supplemented with collagenase B (2 mg/ml). Then, oocytes were manually defolliculated and incubated overnight in ND96 at 18°C containing 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. The next day, mature oocytes (13) were injected with 50 nl of water with or without cRNA from wild-type or mutant TSC, at a concentration of 0.5 µg/µl (i.e., 25 ng cRNA/oocyte). After injection, oocytes were incubated for 4 days in ND96 with sodium pyruvate and gentamicin, and the day before the uptake experiments were performed oocytes were switched to a Cl⁻-free ND96 (mM: 96 Na⁺, isethionate, 2 K⁺ gluconate, 1.8 Ca²⁺ gluconate, 1.0 Mg²⁺ gluconate, 5 HEPES, and 2.5 sodium pyruvate as well as 5 mg/100 ml gentamicin, pH 7.4) (17, 18).

*In vitro rTSC cRNA translation.* To prepare cRNA, all clones were digested at the 3' end using NotI (Invitrogen), and cRNA was transcribed in vitro using a T7 RNA polymerase mMESSAGE kit (Ambion). Transcription product integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA) and by densitometric analysis of the corresponding band in ethidium bromide-stained gels. cRNA was stored frozen in aliquots at −80°C until use.

Assessment of the Na⁺-Cl⁻ cotransporter function. Functional analysis of the Na⁺-Cl⁻ cotransporter was determined by assessing tracer ⁴²Na⁺ uptake (New England Nuclear) in groups of at least 15 oocytes. ⁴²Na⁺ uptake was measured using our usual protocol (27): a 30-min incubation period in an isotonic K⁺ - and Cl⁻-free medium (mM: 96 Na⁺ gluconate, 6.0 Ca²⁺ gluconate, 1.0 Mg²⁺ gluconate, 5 HEPES/Tris, pH 7.4) with 1 mM ouabain, 100 µM bumetanide, and 100 µM amiloride, followed by a 60-min uptake in a K⁺ -free isotonic medium. The isotonic medium contained (in mM) 40 NaCl, 56 Na-methyl-D -glucamine (NMDG)-Cl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4, and 1 ouabain as well as 100 µM bumetanide, 100 µM amiloride, and 2.5 µCi ⁴²Na⁺.

To determine ion transport kinetics, experiments were performed by varying concentrations of Na⁺ and Cl⁻ in the uptake medium (from 0 to 20 or 40 mM). To maintain osmolality and ionic strength, gluconate was used as a Cl⁻ substitute and NMDG as a Na⁺ substitute. All kinetic experiments were obtained in duplicate. The dose-response for metolazone was assessed by exposing groups of cRNA-injected oocytes to concentrations varying from 10⁻⁹ to 10⁻⁴ M. The desired concentration of the diuretic was present in both the incubation and uptake periods.

All uptakes were performed at 32°C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without the isotope to remove extracellular fluid tracer. After the oocytes were dissolved in 10% SDS, tracer activity was determined for each oocyte by β-scintillation counting.

Western blotting. Western blotting was used to compare wild-type and mutant TSC protein in cRNA-injected oocytes following our standard protocol (22, 26). In brief, groups of 15 oocytes injected with water or cRNA were homogenized in 2 µl/oocyte of homogenization buffer, centrifuged twice at 100 g for 10 min at 4°C, and the supernatant was re-collected. Oocyte protein (4 oocytes/lane) was heated in sample buffer containing 6% SDS, 15% glycerol, 0.3% bromophenol blue, 150 mM Tris, pH 7.6, and 2% β-mercaptoethanol and resolved by SDS-PAGE (7.5%). The proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) at 100 V for 140 min in a buffer containing 25 mM Tris, 190 mM glycine, 0.1% SDS, and 20% (vol/vol) methanol. Prestained Rainbow markers (Amersham) were used as molecular mass standards. Non-specific binding sites were blocked for 1 h at room temperature in TBS (pH = 7.5) containing 0.4% blocking grade blocker nonfat dry milk (Bio-Rad), exposed overnight at 4°C to rabbit polyclonal TSC primary antibody diluted 1:1,000 in blocking buffer, TTBS 0.2% [either an antibody previously generated by ourselves (3) or one kindly provided by Dr. Mark Knepper (38)]. Membranes were washed for 40 min with TTBS changed every 10 min, then incubated for 60 min at room temperature with alkaline phosphatase-conjugated second-ary (anti-rabbit) antibody (Bio-Rad) diluted 1:2,000 in blocking buffer and washed again. Bands were detected by using the Immun-Star Chemiluminescent Protein Detection System (Bio-Rad).

**RESULTS**

Functional expression of wild-type and mutant TSC. In preliminary experiments (2), 25 Gitelman mutations were introduced into the TSC by site-directed mutagenesis using the Quick Change kit from Stratagene. The products of site-directed mutagenesis were sequenced to confirm that additional mutations did not occur. As Fig. 1 shows, we observed in a Western blot from oocytes expressing wild-type and mutant TSCs, using a polyclonal antibody we generated previously (3), that some of the Gitelman mutants’ TSCs produce proteins that are not fully glycosylated, whereas others generate proteins in which glycosylation patterns appear indistinguishable from wild-type (as shown by treating with endoglycosidase F). When expressed in *X. laevis* oocytes, Na⁺ uptake rates relative to wild-type TSC were very different among the mutant clones. One of these mutations was chosen for further functional
Table 1. Functional properties of TSC with or without Gitelman-type mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Activity (%)</th>
<th>( K_m ) (Na(^+)) (mM)</th>
<th>( K_m ) (Cl(^-)) (mM)</th>
<th>Metolazone IC(50) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100%</td>
<td>7.23 ± 0.4</td>
<td>5.62 ± 0.5</td>
<td>2 \times 10^{-6}</td>
</tr>
<tr>
<td>G627V</td>
<td>66.5%</td>
<td>4.68 ± 2.2</td>
<td>2.53 ± 1.4*</td>
<td>1 \times 10^{-7}</td>
</tr>
<tr>
<td>R935Q</td>
<td>36.5%</td>
<td>3.26 ± 1.5</td>
<td>7.69 ± 3.5</td>
<td>5 \times 10^{-6}</td>
</tr>
<tr>
<td>V995M</td>
<td>32.5%</td>
<td>4.39 ± 2.3</td>
<td>5.22 ± 3.2</td>
<td>2 \times 10^{-6}</td>
</tr>
<tr>
<td>G610S</td>
<td>12.7%</td>
<td>10.4 ± 8.1</td>
<td>7.51 ± 6.1</td>
<td>7 \times 10^{-7}</td>
</tr>
<tr>
<td>A585V</td>
<td>6.2%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE. TSC, thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter; ND, not detectable. *P < 0.05 vs. wild-type TSC.
transport in all clones that exhibited enough expression as to measure uptakes with extracellular ion concentrations that will be below $K_m$ values. Results from this series of experiments are shown in Figs. 4 and 5 and Table 1. The ion transport kinetics observed in wild-type TSC from the mouse are very similar to those previously observed for rat TSC (27). The $\text{Na}^+$ transport kinetics in Fig. 4 revealed that $^{22}\text{Na}^+$ uptake in all clones exhibited saturation curves that are compatible with Michaelis-Menten behavior. In all cases, a plateau was reached with an extracellular $\text{Na}^+$ concentration $< 20 \text{ mM}$, and we observed no significant differences in $K_m$ values (Table 1). $\text{Cl}^-$ transport kinetics are depicted in Fig. 5. All curves reached the plateau with extracellular $\text{Cl}^-$ concentrations $< 10 \text{ mM}$, and $K_m$ values were very similar, except in TSC harboring the G627V mutation, in which $K_m$ was decreased to $2.5 \pm 1.4 \text{ mM}$, a value significantly lower than the values of all other clones, including wild-type TSC. These observations suggest that the affinity for the cotransported ions is similar to wild-type TSC in all clones, except in G627V, where we observed an increased affinity for $\text{Cl}^-$. Thus because there is no decrease in the affinity for ions in mutant TSCs, a change in the functional properties of the cotransporter does not explain the reduced $^{22}\text{Na}^+$ uptake observed in the mutant clones.

In addition to ion transport kinetic analysis, we also assessed the dose response to the thiazide-type diuretic metolazone to determine the effect of functional missense mutations on the kinetics of thiazide inhibition of the cotransporter. The results of these experiments are shown in Fig. 6 and Table 1. TSC harboring the R935Q and V995M mutations exhibited an IC$_{50}$ for metolazone inhibition of $^{22}\text{Na}^+$ uptake that was similar to that for wild-type TSC. In contrast, TSC with the G610S and G627V exhibited an IC$_{50}$ that was shifted to the left by one-half and one order of magnitude, respectively.

**Surface expression of wild-type and mutant EGFPTSC constructs in X. laevis oocytes.** Another potential mechanism that can account for a reduction in the activity of mutated clones is that mutations affect the insertion of normally functional protein into the plasma membrane. Thus, to quantitatively compare the plasma membrane expression of wild-type vs. mutant TSC, we assessed the surface expression by fluorescence under a confocal microscope using an EGFPTSC fusion construct, identical to the one we have previously used to assess surface expression of rat TSC (19, 39). This analysis is performed in real time using live oocytes, and only the EGFPTSC present in the plasma membrane generates fluorescence when EGF protein is stimulated with light (19). Figure 6 shows the results of these experiments.
Fig. 5. Kinetic analysis of the Cl− dependency of 22Na+ uptake in WT or mutant TSC-injected oocytes. A: WT. B: G627V. C: R935Q. D: V995M. E: G610S. Uptake was performed for 30 min with a fixed concentration of Na+ at 96 mM, with extracellular Cl− concentration ([Cl−]e) at 0, 2.0, 4.0, 6.0, 8.0, 10, 20, and/or 40 mM. Uptake was also assessed in water-injected oocytes (data not shown), and the mean values for water groups were subtracted in corresponding cRNA groups. Each point represents the mean ± SE of 15 oocytes.

Fig. 6. Dose-dependent inhibition of 22Na+ uptake by the thiazide-type diuretic metolazone in WT and mutant TSC-injected oocytes. Metolazone was tested at concentrations from 10−9 to 10−4 M. Injected cRNA was from WT (○), V995M (△), R935Q (□), G610S (●), and G627V (●). Uptake was performed for 60 min in uptake solution containing 96 mM NaCl. Uptake in the absence of metolazone was taken as 100%. Each point represents the mean ± SE of 15 oocytes.
tion of transport activity, except for G610S, where confocal analysis revealed a percentage of surface expression (~50%) higher than the percentage of functional activity (~12%). This suggests that, in this particular clone, where the mutation is located in a residue very near to the end of transmembrane domain 12, both intrinsic activity and arrival to the plasma membrane are affected.

**DISCUSSION**

The present work describes the functional analysis of activity, ion transport kinetics, diuretic dose-response kinetics, and quantitative surface expression analysis of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter harboring five different Gitelman-type mutations. The results revealed that these mutations result in TSC proteins that are similar to wild-type TSC by Western blot analysis and with similar functional transport properties but that exhibit different degrees of activity and arrival rate to the plasma membrane.

The functional characteristics of wild-type and mutant TSCs were assessed using the heterologous expression system in *X. laevis* oocytes. This expression system has shown to be an excellent tool for a robust and reproducible expression of TSC in our hands (17–19, 27, 36, 39) and other laboratories (9, 10, 22, 40), whereas TSC expression in transfected mammalian cells has not been successful in many laboratories, including our own. Thus previous studies using TSC cDNA in which Gitelman-type mutations were introduced have been performed using *X. laevis* oocytes (9, 22). Although expression in mammalian cells is preferred, the best results that have been obtained in stably transfected cells (Madin-Darby canine kidney cells) using wild-type TSC cDNA consisted of a small increase over background that was not >25% (11). This small increase in TSC expression would not be useful in determining the functional properties of mutant clones in which the activity is already lower than that using the wild-type cotransporter.

Studies by Kunchaparty et al. (22) were the first to analyze the functional consequences of several missense mutations reported along TSC protein in kindreds with Gitelman disease. All mutations studied were shown to be functionally inactive, when expressed in *X. laevis*, owing to defective protein processing. It was observed that proteins were synthesized, but they were not properly glycosylated and were not expressed at the plasma membrane. Thus it was concluded that Gitelman mutations impair the function of cotransporter protein by interfering with protein processing. These conclusions are supported by results from Hoover et al. (19), who demonstrated that TSC is a glycoprotein and that both N-linked glycosylation residues (N404 and N424; Fig. 2) are required for the adequate processing of the cotransporter, because single-glycosylation TSC mutants N404Q or N424Q exhibited a significant reduction in functional activity of ~50% compared with wild-type TSC, whereas the double-mutant N404, 424Q resulted in a reduction of expression of ~95%. Most of the reduction in activity was explained by a decrease in the surface expression of the cotransporter. Thus as has been shown with other membrane proteins (1, 15, 24), glycosylation of TSC seems to be required for the proper folding and trafficking of the cotransporter to the plasma membrane.

During the course of previous experiments (22), we noticed that, while most of the Gitelman-type mutations result in nonglycosylated cotransporters, some missense mutations resulted in proteins that were indistinguishable from wild-type TSC by Western blot analysis. Interestingly, some of these “glycosylated” TSC mutants were observed in patients exhibiting a mild form of disease (Lifton R, personal communication), suggesting that the mutant cotransporter could be partially active. De Jong et al. (9) performed a partial characterization of four such functional missense TSC mutations from patients with Gitelman disease. They observed that mutant proteins were glycosylated in the injected *X. laevis* oocytes and that immunocytotoxic analysis showed that mutants with some TSC activity exhibited immunostaining in both cytoplasm and the plasma membrane. The functional properties of the mutant TSCs, however, were not assessed. Thus they were
not able to define whether the studied missense mutations resulted in a defect of TSC insertion into the plasma membrane or in a defect in the functional properties of the cotransporter. In addition, all experiments by De Jong et al. were performed at room temperature, and thus a protein dysfunction associated with temperature was not ruled out. In the case of cystic fibrosis, for instance, the ΔF508 mutation leads to a temperature-dependent defect. When expressed in cells grown at 18°C, ΔF508 matures normally and is expressed at the plasma membrane. In contrast, when expressed in mammalian cells or in oocytes grown at higher temperatures, incomplete processing or insertion into the plasma membrane occurs (12).

In the present study, we have extended the analysis of functional missense mutations to include these possibilities. As shown in Fig. 8, there are at least five possible mechanisms by which mutations might reduce or abolish transporter activity. A mutation could 1) impair protein synthesis, 2) impair protein processing, 3) impair the insertion of an otherwise functional protein into the plasma membrane, 4) impair the functional properties of the cotransporter, and 5) accelerate protein removal or degradation. Although mutations that introduce stop codons in the initial part of the protein, or those in which splicing is abolished resulting in nonsense proteins, have not been studied at the functional level, it is highly likely that the mechanism in these mutations relates to the first possibility (Fig. 8), by which the synthesis of the complete protein is impaired (30, 33, 35). Our previous results are examples of mutations that belong to the second possibility (Fig. 8) because they impair protein processing (22). In the present work, we studied five Gitelman missense mutations that result in partial “functional” proteins. Western blot analysis using anti-TSC antibody revealed that all proteins were similar to wild-type TSC, suggesting that a defect in protein synthesis is not primarily responsible for the reduced activity in mutant TSCs. The functional analysis suggests that it is unlikely that a decrease in ion affinity is the mechanism responsible because Na⁺ and Cl⁻ affinities were either normal or increased in mutant clones. Closeness of fits were reasonable in all clones, except in the one with the lower activity (G610S; Figs. 4E and 5E). In this clone, the observed Kₘ for Na⁺ and Cl⁻ transport was slightly higher that in wild-type TSC, but the difference did not reach significance. However, we believe that, even in this clone, our results suggest that the reduced activity of the cotransporter in mutant TSCs is not due to a dramatic decrease in affinity for Na⁺ or Cl⁻ which prevents the cotransporter from reaching Vₘax when extracellular ions concentrations are >20 mM. These observations suggested that a reduction in the number of cotransporter proteins in the plasma membrane or a decrease in the intrinsic activity of the cotransporter could be the reason for the reduced activity in mutant TSCs. Thus we assessed the surface expression of the EGFP-tagged wild-type and mutant proteins. In this analysis, we tagged the NH₂-terminal domain of wild-type mouse TSC, as well as all mutant clones, with EGFP and assessed surface EGFP-TSC by measuring the fluorescence emission under the confocal microscope. This strategy has been successfully used by us to assess the surface expression of TSC (19, 39) and several isoforms of the apical renal-specific bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter (26, 28), as well as by others to assess the surface expression of membrane proteins in X. laevis oocytes (4, 5, 16).

The surface expression of all mutant proteins was significantly reduced (Fig. 7). In addition, the surface expression profile was similar to the functional profile, suggesting that reduction of transporter activity of mutant proteins is mainly caused by a decrease in the surface expression of the cotransporter. Thus our data suggest that mutations studied in the present work belong to the third possibility (Fig. 8), in which a missense mutation results in a cotransporter with normal functional properties but insertion into the plasma membrane is partially impaired. In this regard, we have recently shown (39, 40) that TSC insertion into the plasma membrane is an important regulatory control point, because mutations in a TSC-negative regulator, such as the WNK4 kinase in pseudohypopaldosteronism type II, result in a loss of inhibition of TSC activity due to uncontrolled insertion of TSC vesicles into the plasma membrane. Interestingly, missense mutations of other members of the electroneutral cotransporter family, such as the Na⁺-K⁺-2Cl⁻ cotransporter in Bartter syndrome type 1 (32) and the KCC3 K⁺-Cl⁻ cotransporter in the peripheral neuropathy associated with agenesis of the corpus callosum (Andersen’s syndrome) (20), behave like the fourth possibility (Fig. 8), because the mutated proteins are normally produced and inserted into the plasma membrane, implying a defect in the functional properties or intrinsic activity of the cotransporter. These data taken together, the first four mechanisms proposed (Fig. 8) have been shown to be implicated in the molecular pathophysiology of hereditary syndromes associated with mutations in members of the SLC12 family.

The observations in the present study are also important in terms of the functional characterization of TSC and in providing some insights into structure-function relationships. The present study shows for the first time the analysis of the kinetic transport properties of mouse TSC. We have previously reported the major functional properties of the rat (17, 27) and flounder (28) isoforms of TSC, which exhibit several differences in ion transport kinetics and dose-response inhibition to thiourea diuretics. Here, we show that Na⁺ and Cl⁻ transport and metolazone inhibitory kinetics in wild-type mouse TSC revealed Kₘ and IC₅₀ values that are undistinguishable from those observed in rat TSC (27) and significantly different from those of flounder TSC (28). In addition, we observed that missense mutations G610S and G627V, which are located at the very beginning of the long COOH-terminal domain, increase the affinity for Cl⁻ and/or metolazone. As discussed
above, this finding does not imply an important role for increased ion affinity in causing the clinical phenotype of Gitelman disease, because increased Cl\(^{-}\) affinity would not be expected to reduce TSC function in vivo. However, the results do suggest that this region of the protein is involved in defining the affinity for Cl\(^{-}\) and/or diuretics. Previous studies in which the binding of the tracer \([^{3}\text{H}]\text{metolazone}\) was used to assess the thiazide receptor suggested that thiazide diuretics and Cl\(^{-}\) competes for the same site on the cotransporter (34). In this regard, we have shown that the affinity for thiazides is shifted to the left when dose-response curves are performed under low-extracellular Cl\(^{-}\) concentrations (27, 36) and the prevention of glycosylation in rat TSC increases the affinity for both extracellular Cl\(^{-}\) and thiazides (19). In the present study, we observed that the mutation G627V produces a slight but significant increase in Cl\(^{-}\) affinity, together with an increase in the affinity modifying residue for Cl\(^{-}\) may also be involved in defining thiazide affinity. Further studies will be necessary to clarify this issue, but our results suggest that analysis of the functional properties of naturally occurring mutations may be useful in revealing the functional roles of individual amino acid residues, which will be helpful in beginning to understand the structure-function relationships among members of the cation Cl\(^{-}\) cotransporter gene family.

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DISCLOSURES

Part of this work was presented at the 2002 Annual Meeting of the American Society of Nephrology (Philadelphia, PA) and is published as an abstract (J Am Soc Nephrol 15: 75A, 2002).

REFERENCES

21. Defective processing and expression of thiazide-sensitive Na-CI cotransporter as a cause of Gitel-


