A Single Nucleotide Polymorphism Alters the Activity of the Renal Na\textsuperscript{+}:Cl\textsuperscript{-} Cotransporter and Reveals a Role for Transmembrane Segment 4 in Chloride and Thiazide Affinity

by

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Abstract

The thiazide-sensitive Na\textsuperscript{+}:Cl\textsuperscript{−} cotransporter is the major salt transport pathway in the distal convolute tubule of the kidney and a role of this cotransporter in blood pressure homeostasis has been defined by physiological studies on pressure natriuresis and by its involvement in monogenic diseases that feature arterial hypotension or hypertension. Database analysis revealed that 135 single nucleotide polymorphisms along the human SLC12A3 gene that encodes the Na\textsuperscript{+}:Cl\textsuperscript{−} cotransporter have been informed. Eight are located within the coding region and one result in a single amino acid change: the residue glycine at the position 264 is changed to alanine (G264A). This residue is located within the fourth transmembrane domain of the predicted structure. Because G264 is a highly conserved residue, we studied the functional properties of this polymorphism using \textit{in vitro} mutagenesis and the heterologous expression system in \textit{Xenopus laevis} oocytes. G264A resulted in a significant and reproducible reduction (~50\%) in $^{22}\text{Na}^+$ uptake when compared to the wild type cotransporter. The affinity for extracellular Cl\textsuperscript{−} and for thiazide diuretics was increased in G264A. Western blot analysis showed similar immunoreactive bands between the wild type and the G264A cotransporters and confocal images of oocytes injected with EGFP-tagged wild-type and G264A cotransporter showed no differences in the protein surface expression level. These observations suggest that the G264A polymorphism is associated with reduction in the substrate translocation rate of the
cotransporter, due to a decrease in the intrinsic activity. Our study also reveals a role of the transmembrane segment 4 in defining the affinity for extracellular Cl\textsuperscript{-} and thiazides diuretics.

Key words: thiazide, Na-Cl cotransporter, hypertension, single nucleotide polymorphism, diuretics, structure, osteoporosis.
Introduction

The thiazide-sensitive Na\textsuperscript{+}:Cl\textsuperscript{-} cotransporter (TSC, gene symbol: SLC12A3, Locus ID 6559) is the major NaCl transport pathway in the apical membrane of the mammalian distal convoluted tubule (DCT) and the teleost urinary bladder (1)(2)(3-7). The fundamental role of the Na\textsuperscript{+}:Cl\textsuperscript{-} cotransporter encoded by the SLC12A3 gene in preserving the extracellular fluid volume and divalent cation homeostasis has been firmly established by the identification of inactivating mutations of this gene as the cause of Gitelman’s disease (8-10), an inherited disorder featuring arterial hypotension, hypokalemic metabolic alkalosis with hypocalciuria, hypomagnesemia and renal salt wasting. TSC also serves as the target for the thiazide-type diuretics that are currently recommended as the drug of choice for treatment of hypertension (11). Finally, a defect in TSC regulation by the WNK1 and WNK4 kinases has been implicated in the pathogenesis of a salt-dependent form of human hypertension known as pseudohyopaldosteronism type II (PHAII) (12)(13), that features marked sensitivity to hydrochlorothiazide and a clinical picture that is a mirror image of Gitelman’s disease (hypertension, hyperkalemia, and metabolic acidosis) (14). Taken together, all these observations suggest that TSC molecular variants, resulting from single nucleotide polymorphisms (SNPs), could contribute to the normal variations in blood pressure in the population at large, to the inherited predisposition towards essential hypertension, and/or to the differential response to diuretic therapy.

Despite the important role of TSC in cardiovascular physiology, pharmacology, and
pathophysiology, little is currently known about the structure-function relationships in this cotransporter. Using \( [3H] \) metolazone binding to membrane preparations from rat renal cortex, Tran et al. (15) proposed that thiazides and Cl\(^-\) share the same binding site. Recent studies in which the functional properties of the cloned cotransporter were determined, however, provided evidence that metolazone competes with both Na\(^+\) and Cl\(^-\) ions (16), suggesting that thiazide binding site maybe shared by both ions and not only by Cl\(^-\), as suggested by Tran et al. (15). In addition, nothing is known regarding domains or amino acid residues defining the TSC ion transport kinetics or thiazide affinity. So far, within the family of electroneutral cotransporters, some aspects of the structure-function relationships have been investigated only in the two isoforms of the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter, BSC1/NKCC2 and BSC2/NKCC1. Results between both isoforms, however, have shown important differences suggesting that conclusions reached in one member of the family cannot be extended to the other members. For example, in BSC2/NKCC1, Isenring et al. (17)(18) have implicated transmembrane domains 4 and 7 in defining Cl\(^-\) transport affinity, while recent studies in BSC1/NKCC2 clearly showed that transmembrane domain 2 contains affinity modifier residues for extracellular Cl\(^-\) (19)(20)(21).

In the present study we show that a SNP that changes one amino acid residue in TSC results in a dramatic decrease in TSC function, apparently secondary to a decrease in the intrinsic activity of the cotransporter and reveals a role of transmembrane segment 4 in TSC affinity for
extracellular Cl⁻ and for thiazides diuretics.
Methods

An extensive search of genome databases (lpgws.nci.nih.gov/cgi-bin/GeneViewer.cgi; ncbi.nlm.nih.gov/SNP) was performed to find the SNPs that have been informed within the SLC12A3 gene. The SNPs within the coding regions that were considered as potentially important were incorporated into the rat TSC cDNA by using the Quickchange site directed mutagenesis system (Stratagene) following the manufacturer’s recommendations. Automatic DNA sequencing was used to corroborate all the mutations. All primers used for mutagenesis were custom made (Sigma).

Genotyping of the G264A polymorphism. A restriction fragment length polymorphism (RFLP) method was created for the G264A polymorphism to confirm it and to simplify its detection in 200 normal subjects. Total genomic DNA was extracted from whole blood according to standard procedures. PCR was conducted using 125 ng of genomic DNA using the primer pair sense 5´-AGACCGTGCGGGACCTGCTC-3´ and antisense 5´- CCTCCTCCATGGCCTCCTCACCTT-3´. PCR was conducted for 34 cycles with denaturation at 96°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. The G264A variant is recognized by RFLP by using Btg1 (New England Biolabs), the restriction fragments separated on 7.5% polyacrylamide gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. The polymorphism was confirmed by automatic sequencing (AbiPrism®) in all positive cases.
Assessment of the Na⁺:Cl⁻ cotransporter function. Oocytes were harvested from anesthetized adult female *Xenopus laevis* frogs, defolliculated, and prepared for microinjection following our standard procedure (16)(19). The next day mature oocytes were injected with 50 nl of water or cRNA transcribed *in vitro*, using the T7 RNA polymerase mMESSAGE kit (Ambion), at a concentration of 0.5 µg/µl. Oocytes were then incubated 3 days in ND96 with sodium pyruvate and gentamicin and one day in Cl⁻-free ND96 (16). The function of the Na⁺:Cl⁻ cotransporter was determined by assessing tracer $^{22}$Na⁺ uptake (New England Nuclear) in groups of at least 15 oocytes following our standard protocol (16): 30 min incubation in a Cl⁻-free ND96 medium containing 1 mM ouabain, 0.1 mM amiloride, and 0.1 mM bumetanide, followed by a 60-min uptake period in a K⁺-free, NaCl medium containing the same drugs plus 2 µCi of $^{22}$Na⁺ per ml. Gluconate was used as a Cl⁻ substitute and N-Methyl-D-Glucamine (NMDG) as a Na⁺ substitute. At the end of the uptake period tracer activity was determined for each dissolved oocyte by β-scintillation counting.

Western Blotting. Western blot analysis was used to compare WT with mutant protein in cRNA-injected oocytes following our standard protocol (22). In brief, groups of 15 oocytes injected with water or cRNA were homogenized in 2 µl/oocyte of homogenization buffer, centrifuged twice at 100 x g for 10 min at 4°C, and the supernatant was collected. Protein extracts from oocytes (four oocytes per lane) were heated in sample buffer containing 6% SDS, 15% glycerol, 0.3% bromophenol blue, 150 mM Tris pH 7.6 and 2% ²-mercaptoethanol, resolved by Laemmli SDS-
polyacrylamide (7.5%) gel electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane. For immunodetection we used a rabbit polyclonal anti rat TSC antibody (generously provided by Dr. Mark Knepper, NIH), diluted 1:1000 (23). The membrane was exposed to anti-TSC antibody diluted in blocking buffer (TTBS, 0.2% tween-20) overnight at 4°C, subsequently washed in TTBS and incubated for 60 min at room temperature with alkaline phosphatase-conjugated secondary (anti-rabbit) antibody (BIO-RAD) diluted 1:2000 in blocking buffer and washed again. Immunoreactive species were detected using Immun-Star Chemiluminescent Protein Detection Systems (BIO-RAD).

Assessment of the TSC expression at the oocytes plasma membrane. The surface expression of wild type or mutant TSC (see below) was determined by assessing the fluorescence in the *Xenopus* oocytes using a TSC fusion construct that we have previously validated (24)(12). In this construct, the enhanced green fluorescent protein (EGFP) was fused to the amino terminal domain of TSC. Then, *Xenopus* oocytes were microinjected with water as control or with EGFP-WT-TSC or EGFP-mutant-TSC cRNA. After four days of incubation, oocytes were monitored for EGFP fluorescence in the oocytes surface using a Zeiss laser scanning confocal microscope (objective lens x10, Nikon). Excitation and emission wavelengths used to visualize EGFP fluorescence were 488 nm 515-565 nm, respectively. We have shown previously that EGFP-TSC fluorescence in the oocytes surface co-localizes with the F-404 specific plasma membrane dye and that oocytes injected with EGFP-TSC exhibit significant thiazide-sensitive $^{22}\text{Na}^+$...
uptake, indicating the EGFP-TSC fluorescence is located in the plasma membrane (24). For densitometry analysis, the plasma membrane fluorescence was quantified by determining the pixel intensity around the entire oocytes circumference using SigmaScan Pro image analysis software.

**Statistical analysis.** Statistical significance is defined as two-tailed $p<0.05$ and the results are presented as mean ± SEM. The significance of the differences between means was tested with the Student’s t-test.
Results

Single nucleotide polymorphisms in the SLC12A3 gene. Up to 135 SNPs have been informed within the SLC12A3 gene. 127 SNPs are located within intronic and only eight are within exonic sequences. Figure 1 depicts the proposed TSC topology (25) and the localization of the eight SNPs within the coding sequence. Six SNPs result in no change of the amino acid sequence. These are the SNPs A122A, T465T, S628S, A714A, G875G, and I1017I, corresponding to the NCBI SNP cluster IDs rs2304479, rs5801, rs55802, rs5803, rs5804, and rs2289113, respectively. Two SNPs result in a single amino acid change. One is the R863K SNP (cluster ID rs8060046) that was considered as irrelevant because this SNP located within the carboxyl terminal domain results in a conserved substitution of the positively charged amino acid arginine, which is present in the human cotransporter (8), for the positively charged residue lysine, which is present in the TSC from rabbit, rat, mouse, and fish (26)(25)(27)(2). In contrast, the other SNP that alter the primary sequence of human TSC (ID number rs1529927) predicts a change of the nonpolar amino acid glycine at the position 264 for the residue alanine. The residue glycine is located within the fourth transmembrane domain and is a conserved amino acid residue, not only in the available TSC sequences from rat (25), mouse (27), rabbit (26), human (8), and flounder (2), but also in all members of SLC12 family that include two genes encoding Na⁺:K⁺:2Cl⁻ cotransporters (28)(25) and four genes encoding K⁺:Cl⁻ cotransporters (29)(30)(31). Thus, the G264A SNP was considered as potentially important and therefore was introduced into TSC by
Allele frequency of the G264A polymorphism. A RFLP strategy was used to assess the presence of G264A SNP by PCR. The PCR product (510 bp) contained a constant Btg1 restriction site and therefore, when digested, two bands of 461 bp and 49 bp, respectively, are observed in GG genotype (encoding glycine at position 264 in both alleles). In contrast, in GA genotype heterozygotes (that is, one allele encoding glycine and the other encoding alanine at position 264), a new specific Btg1 restriction site is used and then four bands of 461 bp, 390 bp, 71 bp and 49 bp are observed. To test the allele frequency of the G264A polymorphism, 200 Caucasian subjects were genotyped. The sample included 119 males and 81 females with the following characteristics (mean ± SD): age 52 ± 16 years, systolic blood pressure 117 ± 11 mmHg, diastolic blood pressure 69 ± 7 mmHg, and body mass index of 24 ± 4 kg/m². None of the subjects had present or past cardiovascular conditions including hypertension, coronary heart disease, stroke or diabetes. The frequency of the GA genotype was 2% in the sample studied. No subjects with AA phenotype were detected (that is, homozygotes encoding alanine at position 264 in both alleles). Shown in Figure 2A is a representative gel containing the Btg1 digested PCR fragment from a normal subject and one heterozygous for the GA genotype. Fig. 2B and C illustrate sequencing of this region in a GG and a GA phenotype (codons GGC and GCC, respectively). Thus, our results suggest that G264A is a true SNP.

Functional consequences of the TSC G264A polymorphism. The functional consequences of the
G264A SNP were assessed using a 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 (2)(25)(16)(32)(24)(12) and also in other laboratories (27)(13)(33)(34). In contrast, TSC expression in transfected eukaryotic cells has not been successful in many laboratories, including our own. The best expression so far observed in stably transfected eukaryotic cells (MDCK cells) with human TSC cDNA consist of small increase (~25%) over background (35). Thus, *X. laevis* oocytes were microinjected with cRNA transcribed from wild-type TSC (WT) or from TSC harboring the G264A SNP (G264A). As shown in figure 3, WT or G264A cRNA injection induced a significant increase in $^{22}$Na$^+$ uptake in *X. laevis* oocytes. However, the increase in G264A-injected oocytes was of a significantly lower magnitude that the increase observed in WT oocytes. Uptake in WT-injected oocytes was $3448 \pm 234$ pmol–oocyte$^{-1}$–h$^{-1}$, while in G264A-injected oocytes was $1712 \pm 366$ pmol–oocyte$^{-1}$–h$^{-1}$ ($p<0.01$, $n=20$). As shown in Figure 3, the uptake was due to the TSC activity since a complete inhibition of uptake was observed in the absence of extracellular chloride or in the presence of the thiazide-type diuretic, metolazone. A reduction of a similar magnitude in the G264A activity was consistently observed in every single experiment. In order to assure that the reduction in $^{22}$Na$^+$ uptake was not due to differences in the amount of cRNA injected or in the transcription rate of the protein, cRNA concentration was determined by densitometry of the corresponding bands in the ethidium bromide stained agarose gel and the transcribed protein was assessed by Western
blot analysis of the injected oocytes. No differences were found between WT and G264A in the amount of cRNA injected (Fig. 3B), as well as in the TSC protein produced by the oocytes (Fig. 3C).

**Surface expression analysis in EGFP-WT or EGFP-G264A.** Because X. laevis oocytes injected with WT or G264A exhibited similar immunoreactive proteins in the Western blot, we reasoned that potential explanations for the reduction in functional expression of the TSC containing the G264A could be a decrease in the amount of the transporter that reaches the plasma membrane, a decrease in the affinity for the cotransported ions, or a decrease in the intrinsic activity of the cotransporter. To study the first possibility, we assessed the surface expression of the WT and G264A proteins by injecting X. laevis oocytes with the cRNA encoding the WT or G264A cotransporters that has been previously tagged with the EGFP. To perform these experiments, we used the EGFP-tagged TSC construct that we have previously characterized (24) in which the EGFP was fused to the amino terminal domain of TSC. Then, the EGFP cDNA was subcloned into the G264A TSC. The cRNA encoding the EGFP-WT or EGFP-G264A was injected into X. laevis oocytes. As shown in Fig. 4A, densitometry of the corresponding cRNA bands in an ethidium bromide agarose gel assured that similar amount of cRNA was injected. Four days after injection, the surface expression was assessed by monitoring the EGFP fluorescence with a confocal microscope. After oocytes were analyzed in the microscope, half of them were used for protein extraction to assess the EGFP-tagged proteins by Western blot, using a rabbit polyclonal antibody against TSC, and the other half were use in a functional expression assay to determine
the thiazide-sensitive $^{22}\text{Na}^+$ uptake. Western blot revealed similar immunoreactive bands in
proteins extracted from EGFP-WT or EGFP-G264A injected oocytes (Fig. 4B) and surface
expression analysis revealed similar fluorescence intensity at the surface of oocytes injected with
EGFP-WT or EGFP-G264A, respectively (representative images are shown in Figures 4C and
4D). Figure 4E and 4F illustrate the plasma membrane fluorescence intensity analysis in 30
EGFP-WT or 30 EGFP-G264A-injected oocytes and the functional activity expressed as
$^{22}\text{Na}^+$ uptake in the same oocytes, respectively. While the surface expression of both clones was
comparable, the $^{22}\text{Na}^+$ uptake was reduced in G264A-injected oocytes. Thus, the G264A
substitution results in a reduction in the cotransporter activity, which does not appear to be due to
a decrease in the surface expression rate.

**Ion transport kinetics.** One potential source of reduction in the functional activity of a membrane
transporter is a reduction of the affinity for the transported ions or molecules, preventing that the
cotransporter reaches maximal transport capacity when incubated in regular uptake solutions.
Thus, we assessed the $\text{Na}^+$ and $\text{Cl}^-$ transport kinetics in *X. laevis* oocytes injected with WT or
G264A cRNA. Shown in Fig. 5A and 5C are the $\text{Na}^+$ transport kinetics analysis in WT and
G264A, respectively. The $\text{Km}$ values for $\text{Na}^+$ transport kinetics were $7.6 \pm 1.6$ and $5.7 \pm 1.1$ mM
in WT and G264A, respectively, with no significant difference between them. Shown in Fig. 5B
and 5D are the $\text{Cl}^-$ transport kinetic analysis. The apparent $\text{Km}$ value for extracellular $\text{Cl}^-$
uptake in WT (Fig. 5B) was 6.3 ± 1.1 mM. This value is similar to the previously reported for the wild-type TSC (16). In contrast, the apparent Km in G264A-injected oocytes was 0.89 ± 0.2 mM (p<0.001, N=3). We repeated the same analysis in triplicate using three different batches of oocytes and the results were similar. Thus, the G264A resulted in a significant increase in Cl⁻ affinity on the cotransporter. This increase in ion transport affinity, however, does not explain the reduction in the cotransporter activity.

**Diuretic inhibitory kinetics.** Studies in which the kinetic analysis of [³H]metolazone to renal cortex plasma membranes were assessed suggested that chloride and thiazides may compete for the same site in the cotransporter (15). Supporting this possibility we have shown that thiazide affinity in TSC is increased when oocytes are incubated in uptake solutions containing low Cl⁻ concentration (16). Because an increase in Cl⁻ affinity in the G264A cotransporter was observed, we assessed the dose-response simultaneously on TSC and G264A for the thiazide-type diuretic metolazone in order to determine the thiazide affinity on each cotransporter. As shown in figure 6, when uptakes where performed in 96 mM NaCl (closed symbols and continuous lines), the IC₅₀ for metolazone-induced reduction in ²²Na⁺ uptake was similar between WT and G264A. The IC₅₀ value in both was ~ 1 x 10⁻⁶ M, which is similar to the IC₅₀ that we have previous reported for rat TSC (16). However, because the Km for extracellular Cl⁻ in WT TSC is below 10 mM and in G264A below 1 mM, it is possible that competition between chloride and
thiazides does not become apparent using 96 mM of extracellular Cl\(^-\). Thus, we performed the metolazone dose-response inhibitory curve in the same experiment, but using another groups of oocytes in which uptake was done using an extracellular Cl\(^-\) concentration around the Km values for this ion, that is, \(~6\) mM for TSC and 1 mM for G264A (open symbols and discontinued lines). As we have shown before (16), the metolazone IC\(_{50}\) in the oocytes injected with WT-TSC changed from \(~1 \times 10^{-6}\) M to \(~3 \times 10^{-7}\) M, suggesting that metolazone binding to the cotransporter is enhanced when extracellular chloride is lower. In oocytes injected with G264A cRNA, the shift to the left was more dramatic than in WT-TSC since metolazone IC\(_{50}\) changed from \(~8 \times 10^{-7}\) M in the presence of 96 mM Cl, to \(~1 \times 10^{-8}\) M when extracellular Cl\(^-\) concentration was 1 mM. To test the possibility that the G264A SNP not only affects the affinity for thiazides, but also the diuretic inhibitory profile, we also assessed the dose-response to several thiazides. This experiment was performed simultaneously in WT and G264A-injected oocytes. Therefore, each of the thiazide dilutions that were used was the same for both WT and G264A oocytes. As shown in Figure 7, the thiazide inhibitory profile of polythiazide > bendroflumethiazide = trichloromethiazide = benzthiazide > hydrochlorothiazide = chlortalidone was similar between WT and G264A TSC cotransporters, suggesting that the G264A substitution increases the affinity of the cotransporter for thiazides, but does not affect the inhibitory profile.

**Role of the conserved residues in the transmembrane segment 4.** As shown in Figure 8, the alignment analysis of the transmembrane domain 4 in the electroneutral cotransporter family
members revealed that G264 is not the only residue that is conserved in all cotransporters. In addition to G264, the residues N258, R261, and G278 are also conserved in all members of the family. Thus we performed similar substitutions of these residues and analyzed the effects upon the functional properties of the cotransporter. As shown in figure 9, substitution of these residues resulted in different magnitudes of reduction in the TSC activity. The effect was similar between G264A and N258Q (~50% reduction). Further reduction in the activity was observed in the R261L transporter, while the G278A substitution resulted in a complete block of the cotransporter activity. To find out if the decrease in activity was associated or not with a decrease in the surface expression of the cotransporters, X. laevis oocytes were injected with similar amounts of cRNA transcribed from the EGFP-tagged wild type TSC or the EGFP-tagged TSC containing the substitutions and confocal microscopy analysis of the oocytes was performed four days later for assessing the fluorescence intensity at the surface. Interestingly, as shown in Figure 10, there were no significant differences among all groups. Thus, mutations in TSC on any of the conserved residues in the transmembrane segment 4 produce cotransporters in which the intrinsic activity is reduced. Finally, the ion transport kinetics analysis revealed that in TSC harbouring the N258Q and R261L substitutions, the Km values for extracellular Na⁺ were 7.3 ± 0.7 and 8.9 ± 2.3 mM, respectively, and for extracellular Cl⁻ were 3.2 ± 0.7 (p<0.05, N=4) and 3.2 ± 1.6 mM (p=NS, N=4), respectively. Thus, no change was observed in the affinity for extracellular Na⁺, while a slight increase was observed in the affinity for Cl⁻.
Discussion

One hundred and thirty five SNPs have been informed along the SLC12A3 gene that encodes the thiazide-sensitive Na\(^+\):Cl\(^-\) cotransporter of the renal distal convoluted tubule. Only eight of the 135 SNPs are located within exonic sequences and one of them, the SNP G264A, results in a significant change of a single amino acid residue. We showed that in our population the distribution of this SNP is 98% homozygous for G264G and 2% for heterozygous G264A. This frequency is different to the previously shown by Melander et al. (36) in Sweden to be ~91% for homozygous G264G, ~8% for heterozygous G264A, and ~1% for homozygous A264A. When expressed in the heterologous system of *X. laevis* oocytes, G264A exhibited a reduced maximal transport capacity to about 50% of that shown in simultaneous experiments with WT-TSC. As shown by Western blot analysis, the lower activity of the TSC harboring the G264A substitution does not appear to be due to reduced translation of the protein since densitometric analysis demonstrated no differences in the amount of TSC protein produced with or without the G264A substitution. The surface image analysis that was assessed by confocal microscopy in *X. laevis* oocytes injected with EGFP tagged WT or G264A cRNA revealed that reduced translocation of the cotransporter to the cell surface is not responsible for the lower activity in G264A, since surface expression in the plasma membrane was comparable between EGFP-WT and EGFP-G264A, while the \(^{22}\text{Na}^+\) uptake experiments performed with the EGFP-WT or EGFP-G264A injected oocytes revealed a significant reduction in TSC activity in the presence of
the G264A substitution. Thus, taking all these data together; we propose that the lower activity in TSC harboring the G264A substitution is due to a reduced ions translocation rate. That is, due to a decrease in the intrinsic activity of the cotransporter. A similar negative effect on the intrinsic activity or on the receptor signaling capacity has been documented for the SNP I89V and the SNP S268P that occur in the human high affinity choline transporter (37) and in the human \( \frac{1}{4} \)-opioid receptor (38), respectively.

The observation in the present study of one SNP in the renal \( \text{Na}^+:\text{Cl}^- \) cotransporter that results in reduction of the cotransporter intrinsic activity suggests a number of testable hypothesis. For example, this SNP may be probably less prevalent in hypertensive patients than in normal subjects or individuals harboring this SNP probably are less sensitive to treatment with thiazide drugs. In addition, it is well known that TSC activity inversely correlates with calcium reabsorption in the distal tubule (39) and that chronic thiazide treatment is associated with increased bone density (40) which is a protective factor against osteoporosis (41)(42). Therefore, another hypothesis could be that SNP G264A is less prevalent in patients with osteoporosis than in general population.

One study addressed the genotype frequency distribution on this G264A SNP in 264 normal and 292 hypertensive subjects from Sweden (36) and no difference was observed between normotensive and hypertensive subjects. It is possible, however, that more selected hypertensive patients will be necessary to be studied in order to reveal the association between hypertension
and certain SNPs. For example, Baker et al. (43) have shown that the T594M mutation in the β-subunit of the epithelial sodium channel correlates with the development of hypertension only in black patients with low renin hypertension and Zhu et al. (44) observed that in white patients, but not in black hypertensive subjects, there is a significant association between the intron 2 conversion allele of the aldosterone synthase gene and the development of essential hypertension.

Regardless of the potential role in the disease discussed above, the G264A SNP reveals a role of the transmembrane domain 4 in the affinity for extracellular Cl\(^-\) and also for thiazides. We observed that TSC harboring the G264A exhibits an increase in the affinity for extracellular Cl\(^-\), since the apparent Km value for extracellular Cl\(^-\) in G264A was almost 10 times lower than in WT. This increase in affinity for Cl\(^-\) was specific for this ion since no change was observed in the affinity for extracellular Na\(^+\). Glycine is a non-hydrophobic residue at the position 264, which is predicted to be located in the TSC putative transmembrane domain 4 and is conserved in all species in which TSC has been sequenced, including human (8), rat (25), mouse (27), rabbit (26), and flounder (2), as well as, among all members of the SLC12 family including TSC, two Na\(^+\):K\(^+\):2Cl\(^-\) and four K\(^+\):Cl\(^-\) cotransporter isoforms. Previous studies in the basolateral isoform of the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter (45)(18) indicated that transmembrane helix 4 contains affinity-modifying residues for Cl\(^-\) translocation. In addition to G264, there are three other residues within the transmembrane domain 4 that are conserved among all members of the
SLC12 family (Fig. 8). We observed that substitution of each of these residues resulted in Na\(^+\):Cl\(^-\) cotransporters with reduced intrinsic activity, and one of them also exhibited increased affinity for extracellular Cl\(^-\). Thus, as has been suggested by Isenring et al. in the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter BSC2/NKCC1, it is possible that transmembrane segment 4 in TSC also play a role in defining the affinity for extracellular chloride. The G278A substitution resulted in a non-functional protein, without affecting the surface expression rate, suggesting that this glycine is completely necessary for the cotransporter to reach its functional conformation state.

The structural mechanisms by which glycine substitution in the TSC fourth transmembrane domain produces the observed changes in its functional properties are not clear, but some hypothesis can be proposed. Glycine is an amino acid residue that plays an important structural role, because this residue allows unusual main chain conformations in proteins. This is probably why glycine is one of the amino acid residues that show high proportion of conservation among homologous protein sequences (46). Although not yet studied in cotransporters, it has been shown in several enzymes that some glycine residues can be important to define the protein flexibility because this residue can be part of a hinge (47)(48)(49)(50) and the extent of rigidity or flexibility in a given hinge has been proposed to play a role in the affinity of the protein for its ligand (51). Thus, one possibility is that the presence of glycine at position 264 provides certain flexibility that in TSC might affect both, rates of transport and anion binding. Alternatively, because the four residues that we studied, conserved among all members of the SLC12 family,
are hydrophilic (two glycines, one asparagine and one arginine), it is possible that these amino acids mainly face the putative translocation pocket in the cotransporter and the conformational change that results when these residues were substituted could render the putative translocation pocket more accessible to Cl\(^-\) ions, but with reduced rates of transport. Further studies will be necessary to clarify these issues.

Previous studies using the binding of tracer \([^{3}\text{H}]\text{metolazone}\) suggested that Cl\(^-\) ions and thiazide diuretics compete for the same site on the cotransporter (15). Supporting this hypothesis, we have shown that in TSC the higher affinity for chloride is accompanied by a higher affinity for thiazide diuretics. On one hand, the affinity for thiazides is shifted to the left when dose-response curves are performed in low extracellular Cl\(^-\) concentration (16)(32) and on the other hand, the prevention of glycosylation in rat TSC increases the affinity for both extracellular Cl\(^-\) and thiazides (24). In the present study, we observed that G264A substitution produce a dramatic increase in Cl\(^-\) affinity, together with an increase in the affinity for the thiazide-type diuretic metolazone. This observation also supports the hypothesis that in TSC, the affinity-modifying residues for Cl\(^-\) may also be involved in defining thiazide affinity, increasing the data supporting that anions and diuretics compete for the same site on the cotransporter. Interestingly, while a similar type of competence between Cl\(^-\) and loop diuretics was proposed for the Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter based on studies using \([^{3}\text{H}]\text{Bumetanide}\) (52), the functional analysis of chimeras
between the shark and human basolateral BSC2/NKCC1 revealed that changes in Cl⁻ transport kinetics are not accompanied by similar changes in bumetanide affinity (45).

In summary, we report here the functional characterization of a single nucleotide polymorphism in the SLC12A3 gene that encodes the thiazide-sensitive Na⁺:Cl⁻ cotransporter. This membrane protein has been implicated in human diseases such as arterial hypertension and osteoporosis and the pharmacological modulations of its function are currently used for treating or preventing these disorders. The studied SNP is a substitution of a glycine for alanine in the fourth transmembrane domain, which caused a significant reduction in the Na⁺:Cl⁻ transport rate, suggesting that people with this SNP could have an allele with reduced function of the cotransporter. In addition to the effect of the SNP upon the TSC activity, the G264A substitution produced an increase in the affinity of the cotransporter for extracellular Cl⁻, but nor for Na⁺, that was accompanied by an increase in the affinity for thiazide diuretics. Thus, our study represents the first detailed examination of genetic polymorphism in the SLC12A3 gene and reveals a role of the TSC transmembrane segment 4 in anion and thiazide affinity.
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**Figures**

**Figure 1.** Topological model of the thiazide-sensitive Na⁺:Cl⁻ cotransporter and the localization of the eight SNPs located within the coding region.

**Figure 2.** A) Example of a genotyping result for the Gly264Ala (G264A) polymorphism. The polymorphism consists in a G to C transversion at codon 264 that changed the glycine-encoding codon GGC to the alanine-encoding codon, GCC. The PCR products were digested with *Btg*I and resolved on SDS-PAGE: lane 1 molecular weight marker, lane 2 GA heterozygous (codon GCC); lane 3 GG homozygous (codon GCC), and lane 4 (undigested PCR product). B) Sequence of wild-type and C) polymorphic variant G264A PCR products were excised from the gel and fully sequenced.

**Figure 3.** Functional expression of WT and G264A cotransporters in *X. laevis* oocytes. A) ²²Na⁺ uptake in oocytes that were injected with water, with 25 ng of cRNA from WT, or from G264A. Uptake was assessed in control conditions (open bars), in the absence of extracellular Cl⁻ (hatched bars), or in the presence of 10⁻⁴ M of the inhibitor metolazone (closed bars). The absence of endogenous thiazide-inhibitable ²²Na⁺ uptake in *X. laevis* oocytes has been shown before (2)(25)(16). * p<0.01 vs. WT cRNA oocytes in control conditions. N=20 oocytes per bar.
B) Ethidium bromide stained agarose gel showing 2 ¼l of 0.5 ¼g/¼l of WT and G264A cRNA as stated. C) Autoradiograms of Western blot analysis of proteins extracted from WT or G264A cRNA-injected oocytes, as stated. The analysis was performed using rabbit polyclonal anti-TSC antibodies. Comparable immunoreactivities are observed in both lanes.

**Figure 4.** Surface and functional expression of EGFP-WT and EGFP-G264A cotransporters. A) Ethidium bromide agarose gel showing 2 ¼l of 0.5 ¼g/¼l of EGFP-WT and EGFP-G264A cRNAs as stated. B) Autoradiography of a Western blot analysis of proteins extracted from EGFP-WT or EGFP-G264A cRNA-injected oocytes, as stated. C and D) representative examples of surface fluorescence in *X. laevis* oocytes expressing EGFP-WT or EGFP-G264A, as stated. E) Mean ± SEM of the surface expression analysis in arbitrary unit of oocytes injected with EGFP-WT cRNA or EGFP-G264A cRNA, as stated (N=30 oocytes per bar). F) Four hours after the confocal microscopy analysis was performed, the $^{22}\text{Na}^+$ uptake was assessed using the same oocytes from Fig. 4E, in the absence (open bars) or presence (hatched bars) of $10^{-4}$ M metolazone. Thus, each bar represents the mean ± SEM of 15 oocytes. Uptake in the water control group was $189 ± 21 \text{ pmol-oocyte}^{-1}\cdot\text{h}^{-1}$. *p<0.05 vs uptake in EGFP-WT in control conditions.

**Figure 5.** Kinetic analysis of $^{22}\text{Na}^+$ uptake in oocytes injected with cRNA from WT (panel A
and B) or G264A (panels C and D). A) and C): Na+ dependency of 22Na+ uptake. B) and D): Cl- dependency of 22Na+ uptake. Uptakes were performed with Na+ or Cl- fixed at 96 mM, varying the concentration of the appropriate counterion from 0 to 40 mM, as indicated. Uptakes were also measured in water-injected oocytes (data not shown) and the mean values for the corresponding water groups were subtracted to analyze only the 22Na+ uptake due to each injected cotransporter. Curve fitting was performed using the Michaelis-Menten equation. Data are expressed as uptakes in pmol–oocyte⁻¹–h⁻¹, each point represents the mean of at least 15 oocytes.

**Figure 6.** Concentration-response for inhibition of WT (squares) and G264A (circles) by metolazone. Groups of 15 oocytes microinjected with WT or G264A were exposed to increased concentrations of metolazone in the preincubation and uptake mediums, from 10⁻⁹ to 10⁻⁴ M. Uptakes were performed in the presence of 96 mM of extracellular Cl- in both WT and G264A (continuous lines and closed symbols) or in the presence of 6 mM of extracellular Cl- for WT (open squares and discontinuous line), or 1 mM of extracellular Cl- for G264A (open circles and discontinuous line). Data were normalized as the percentage of influx, taking 100% as the value observed in oocytes in which uptake was performed in the absence of metolazone. Each point represents the mean ± SEM of at least 15 oocytes.
Figure 7. Kinetic analyses of the inhibition of WT (upper panel) or G264A (lower panel) function by several thiazide-type diuretics. All Na⁺ uptakes were preformed during 60 minutes with thiazides tested at concentrations from 10⁻⁸ M to 10⁻⁴ M in uptake solution containing 40 mM Na⁺ and 96 mM Cl⁻. Each point represents the mean ± SEM of at least 15 oocytes. The inhibitory profile polythiazide (s) > metolazone (not shown) > bendroflumethiazide (,) = trichloromethiazide (r) = benzthiazide ( ) > hydrochlorothiazide (0) = chlortalidone (TM) is similar between WT and G264A.

Figure 8. Alignment of the amino acid residues that in the electroneutral cation-chloride cotransporters correspond to the transmembrane domain 4, according with the topology analysis that has been performed for BSC2 (53). Amino acid numbers correspond to the TSC sequence.

Figure 9. Functional expression of WT and TSC harboring the substitutions G264A, N258Q, R261L, or G278A. The ²²Na⁺ uptake was determined in groups of oocytes that were injected with water or with 25 ng of cRNA from the WT or substituted mutants (as stated). Uptake was assessed in control conditions (open bars) or in the presence of 10⁻⁴ M of the inhibitor metolazone (closed bars). * p<0.01 vs. WT cRNA oocytes in control conditions. N=20 oocytes per bar.

Figure 10. Surface abundance of EGFP-tagged WT, or EGFP-tagged TSC harbouring the
substitutions G264A, N258Q, R261L, or G278A, as stated. Oocytes were injected with corresponding cRNA and green fluorescence was assessed by confocal microscopy as described in Methods. The mean and standard error of fluorescence is shown for each set of eight oocytes per injection in arbitrary units.
A single nucleotide polymorphism alters the activity of the renal Na+:Cl-cotransporter and reveals a role for transmembrane segment 4 in chloride and thiazide affinity
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