Amino acid residues in transmembrane segment IX of the Na\(^{+}/\)I\(^{-}\) symporter (NIS) play a role in its Na\(^{+}\) dependence and are critical for transport activity

Antonio De la Vieja\#, Mia D. Reed, Christopher S. Ginter\&, and Nancy Carrasco*
Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461

#Present address: Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC), C/ Arturo Duperier, 4, 28029 Madrid (Spain) 28029.\&Present address: Department of Physiology and Biophysics, Cornell Weill Medical College, New York NY 10021, USA. *Address correspondence to: Nancy Carrasco, Albert Einstein College of Medicine, Department of Molecular Pharmacology, 1300 Morris Park Ave., Bronx, NY 10461, Tel. 718-430-3523, Fax. 718-430-8922; e-mail: carrasco@aecom.yu.edu

Running title: Functional role of residues in the NIS TMS IX.

ABSTRACT

The Na\(^{+}/\)I\(^{-}\) symporter (NIS) is a key plasma membrane glycoprotein that mediates Na\(^{+}\)-dependent active I\(^{-}\) transport in the thyroid, lactating breast, and other tissues. The OH group of the side chain at position 354 in transmembrane segment (TMS) IX of NIS has been demonstrated to be essential for NIS function, as revealed by the study of the congenital I\(^{-}\) transport defect (ITD)-causing T354P NIS mutation. TMS IX has the most \(\beta\)-OH-group-containing amino acids (Ser and Thr) of any TMS in NIS. We have thoroughly characterized the functional significance of all Ser and Thr in TMS IX in NIS, as well as of other residues in TMS IX that are highly conserved in other transporters of the SLC5A protein family. Here we show that five \(\beta\)-OH-group-containing residues (T351, S353, T354, S356, and T357) and N360, all of which putatively face the same side of the helix in TMS IX, plus D369, located in the membrane/cytosol interface, play key roles in NIS function and seem to be involved in Na\(^{+}\) binding/translocation.

INTRODUCTION

The Na\(^{+}/\)I\(^{-}\) symporter (NIS) is a plasma membrane glycoprotein that mediates active I\(^{-}\) uptake in the thyroid and other tissues, such as salivary glands, gastric mucosa, and lactating mammary gland (1-3). In the thyroid, I\(^{-}\) uptake is the first step in the biosynthesis of the iodine-containing hormones triiodothyronine (T\(_3\)) and tetraiodothyronine or thyroxine (T\(_4\)). The pathophysiological and medical significance of NIS in the thyroid is difficult to overstate, for NIS is the molecular basis for the widespread and highly successful use of radioiodide in the diagnosis and treatment of major thyroid diseases, including thyroid cancer and its metastases (4).

NIS is a member of the SLC5A transporter family (5), which includes the high- and low-affinity Na\(^{+}/\)glucose cotransporters (SGLT1 and SGLT2), the Na\(^{+}/\)myoinositol transporter (SMIT), the Na\(^{+}/\)multivitamin transporter (SMVT), and the Na\(^{+}/\)monocarboxylate transporter (SMCT), among others. All these proteins couple the inward transport of Na\(^{+}\), which occurs in favor of its Na\(^{+}/\)K\(^{-}\)-ATPase-generated electrochemical gradient (6), to the simultaneous inward translocation of the corresponding solute against its gradient (5,6). NIS activity is electrogenic with a 2Na\(^{+}\) to 1I\(^{-}\) stoichiometry. The current NIS secondary structure model (Fig. 1A) depicts NIS as a protein with 13 TMS, the amino terminus facing the extracellular milieu and the carboxyl terminus facing the cytosol; the location of both termini has been confirmed experimentally (7). The 13-TMS model has been generally regarded as a typical pattern for all members of the SCL5A family, with one extra TMS in some members (8). Although several common structural and functional patterns (such as Na\(^{+}\) dependence and conformational changes in the transporter after binding of the substrates) and high homology (identity ranges from 17.5% between NIS and SMIT to 55.5% between NIS and SMCT) exist among these proteins, little is known about the regions involved in the Na\(^{+}\) or solute translocation pathways.

Eleven cases of congenital I\(^{-}\) transport defect (ITD), a condition that leads to hypothyroidism when untreated, have been reported as a direct cause of NIS mutations (2,3). We have characterized the ITD-causing T354P NIS mutation at the molecular level
(9). Although inactive when transiently transfected into COS-7 cells, the T354P NIS protein is normally expressed, posttranslationally processed, and correctly targeted to the plasma membrane. We showed the β-carbon OH at residue 354 to be essential for NIS function (9). T354 is located in NIS TMS IX, which has the most β-OH-group-containing amino acids (Ser and Thr) of any NIS TMS (Fig. 1). We have investigated the role of these and other residues within TMS IX. We found that some of them are involved in Na+ coupling and/or translocation. Our data suggest that the equivalent amino acids may have a similar function in other members of the SLC5A family of transporters.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis - Site-directed mutagenesis was carried out as previously described (9). The oligonucleotides used for mutagenesis contained mutations for changing the codon to a selected amino acid codon. Final PCR products were subcloned into WT rNIS cDNA (pSVSport-rNIS) and sequenced to verify the substitutions.

Transient transfections - COS-7 cells were transfected using the Lipofectamine Reagent (Invitrogen) enhanced with Plus Reagent (Invitrogen) according to the manufacturer's instructions. Flow cytometry was performed to determine transfection efficiency. After two days, transfected cells were assayed for I− uptake, cell surface biotinylation, immunoblot analysis, and immunofluorescence.

Iodide transport - Cells transiently transfected with WT or mutant NIS cDNAs were assayed as described (10). Briefly, cells were washed twice with 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.4 mM MgSO4, 7H2O, 0.5 mM MgCl2, 0.4 mM NaHPO4, 7H2O, 0.44 mM KH2PO4, and 5.55 mM glucose in 10 mM Hepes at pH 7.5 (HBSS). Cells were incubated in HBSS containing 20 μM KI supplemented with 1 μCi carrier-free Na125I to yield a specific activity of 100 μCi/mmol. For steady-state experiments, incubations proceeded for 1 hour at 37°C in a humidified atmosphere and were terminated by aspirating the radioactive medium and washing twice with 1 ml ice-cold HBSS.

To determine the amount of 125I accumulated in the cells, 500 μl cold-ethanol was added for 20 minutes at 4°C and radioactivity was quantified in a γ-counter. DNA was determined by the diphenylamine method after TCA precipitation. Uptake was expressed as pmoles of I− per μg of DNA. Results are the average of at least five different experiments performed in triplicate or sextuplicate. Data were analyzed with GraphPad Prism (Intuitive Software for Science, San Diego, CA). I− uptake values are the mean ± SEM. Statistical significance was determined by t-test analysis (two-tailed), and differences were considered significant at p < 0.05.

For I−-dependent kinetic analysis, cells were incubated with the indicated concentrations of I− (2.5-300 μM) and 140 mM NaCl for 4 min. Initial-rate data were analyzed by a nonlinear regression using the following equation for I−-dependent I− uptake: v([I−])=(Vmax*[I−])/(Km+[I−])+0.06*[I−]+0.60. The terms 0.06*[I−]+0.60 correspond to background adjusted by least squares of the data obtained with nontransfected cells. For Na+−dependent kinetic analysis, cells were incubated with the indicated concentrations of Na+ (0-280 mM) and 20 μM I− for 4 min. Osmolarity was kept constant with choline. Initial-rate data were analyzed by a nonlinear regression using the following equation for Na+−dependent I− uptake: v([Na+]−)=Vmax*[Na+]/(Km+[Na+])+0.001*[Na+]+0.87. The terms 0.001*[Na+]+0.87 correspond to background adjusted by least squares of the data obtained with nontransfected cells. Results were analyzed by nonlinear regression in GraphPad Prism software. Km and Vmax values are the average of at least 3 experiments and are expressed as mean ± SEM.

Immunoblot - SDS PAGE and electrophoretic transfer to nitrocellulose were performed as previously described (7,11). All samples were diluted 1:2 with loading buffer and heated at 37°C for 30 min prior to electrophoresis. Immunoblots were carried out with the corresponding amount of 2 nM anti-Ct-rNIS Ab, and a 1:3,000 dilution of a HRP-linked sheep anti-rabbit IgG (Chemicon International). Both incubations were performed for 1 h. Polypeptides were visualized by enhanced chemiluminescence (ECL) Western blot detection system (Amersham). Nitrocellulose membranes were stripped and reprobed with anti-tubulin (total protein blots) or anti-Na+−/K+− ATPase α-subunit (biotinylated protein blots) Ab (not shown).

Immunofluorescence and confocal microscopy - Cells were grown on glass coverslips, washed 2 times with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS for 20 min, quenched with 50 mM NH4Cl for 10 min and washed three times...
with PBS and then permeabilized with 0.2% (v/v) Triton X-100 in PBS containing 0.5% (w/v) bovine serum albumin (BSA) (Sigma), 0.1 mM CaCl$_2$, and 0.1 mM MgCl$_2$ (PBSACMT) for 5 min. Coversons were incubated with 4 nM anti-Ct-rNIS Ab PBSACMT for 1 hr, washed twice with PBS, and incubated with fluorescein-conjugated goat anti-rabbit (Vector Laboratories) PBSACMT for 1 h. Coversons were mounted and examined using a BioRad Radiance 2000 laser-scanning confocal microscope, using excitation wavelengths of 488 and 568 nm.

**Biotinylation** - Cell surface proteins were labeled with the membrane-impermeable biotinylation reagent Sulfo-NHS-SS-biotin (Pierce Biotechnology, Inc.) as described (12). Transiently transfected cells were rinsed twice with PBS containing 1 mM MgCl$_2$ and 0.1 mM CaCl$_2$ at pH 7.4 (PBSACMT) at 4°C. Cells were incubated with 1 mg/ml Sulfo-NHS-SS-biotin (Pierce) in a biotinylation medium containing 2 mM CaCl$_2$, 150 mM NaCl, and 20 mM Heps at pH 8.5 for 30 min at 4°C with gentle shaking. The reagent was quenched by washing twice with 100 mM glycine in PBSACMT for 10 minutes. Cells were lysed with a buffer containing 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1% (v/v) SDS, and protease inhibitors in 50 mM Tris-HCl at pH 7.5 (lysis buffer) for 15 minutes at 4°C. SDS was diluted 10 times in lysis buffer without SDS. Cell surface proteins were isolated from the cell extract with streptavidin-agarose beads (Pierce) incubated overnight with rotation at 4°C. Beads were rinsed 3 times with lysis buffer without SDS, twice with high salt buffer containing 5 mM EDTA, 0.1% (v/v) Triton X-100 and 500 mM NaCl in Tris-HCl pH 7.5, and once with 50 mM Tris-HCl at pH 7.5. The beads were eluted in SDS-PAGE sample buffer containing 200 mM DTT, heated for 5 minutes at 75°C, chilled for 10 minutes on ice and loaded onto the gel.

**Densitometrical Analysis** - Films were scanned and the optical density of the bands was measured using the image analysis software ImageQuant (Molecular Dynamics, Inc.). Only signals in the linear exposure range of the films were used. The amount of expressed protein (arbitrary units) was compared with WT NIS expression.

**RESULTS**

Five amino acid residues (T351, S353, T354, S356, and T357) in TMS IX are critical for NIS function on account of their β-OH groups. In a previous report on the detailed analysis of the ITD-

causing T354P NIS mutant, we showed that the presence of a β-carbon OH group at residue 354, located in TMS IX, is essential for NIS function (9), suggesting that TMS IX is, in turn, a potentially significant region for NIS activity. As we noted that TMS IX has the most β-OH-group-containing amino acids (Ser and Thr) of any TMS in NIS (Fig. 1), we undertook the characterization of the specific roles played by these amino acids (in addition to T354) in NIS function. We individually substituted each Thr and Ser in TMS IX with Ala, which lacks a OH group, and carried out transport assays in COS-7 cells expressing the resulting mutant NIS proteins under steady state conditions at 20 μM ι (a concentration close to the Km for ι) and a physiological concentration of Na+ (140 mM). Substitutions of T351 (Fig. 2A, lane 4), S353 (lane 5), S356 (lane 7), or T357 (lane 8) with Ala each caused a pronounced decrease in NIS activity to just ≤10% (background was 2.5%, lane 1) of that of WT NIS (111.4±3.9 pmols/µg DNA) (Fig. 2A, lane 2). Substitutions of T354 (Fig. 2A, lane 11), S356 (lane 12), and T357 (lane 13) with Pro impaired ι transport completely. Substitutions of S349 (lane 14), T354 (lane 15) and T357 (lane 16) with Cys caused a decrease in ι transport similar to that caused by replacing these residues with Ala. However, when we individually substituted S349 (lane 3), S358 (lane 9), or T366 (lane 10) with Ala, we observed that NIS function either decreased only modestly or actually increased (98%, 120%, and 78% of WT NIS activity, respectively). Similar results were obtained at supersaturating external ι concentrations (160 μM) (data not shown). These results suggest that S349 and T366, which according to our model are located close to the extracellular milieu and the cytosol, respectively (Fig. 1A), do not play a functional role comparable to that of T351, S353, T354, S356, and T357, all of which together form a pocket embedded in the membrane (Fig. 1B). The only exception to this trend was S358, a putative membrane-embedded residue whose substitution with Ala did not lead to loss of NIS activity (Fig. 2A, lane 9).

When in order to maintain the presence of OH groups at the β-carbons we substituted each Thr with a Ser and each Ser with a Thr in TMS IX, thus generating the mutants T351S (Fig. 2B, lane 1), S353T (lane 2), T354S (lane 3), S356T (lane 4), and T357S (lane 5), we observed that NIS activity ranged from 40 to 65% of that of WT NIS (Fig. 2A, lane 2). This was much higher than that obtained with NIS proteins with Ala/Pro/Cys substitutions at the same

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
positions, which was just 10% or less of WT NIS activity (Fig. 2A). Whereas these data suggest that the presence of OH groups at these positions is indeed necessary for NIS activity (compare Figs. 2A and B), they also demonstrate that such presence alone is not sufficient for full activity.

**Expression and plasma membrane targeting of mutant NIS proteins resulting from all TMS IX substitutions are similar to those of WT NIS.** We subjected lysates from cells expressing either WT or TMS IX mutant NIS to immunoblot analysis with an anti-carboxy terminus NIS Ab (Fig. 2C, Ala/Pro/Cys substitutions, and Fig. 2E Ser/Thr substitutions). Polypeptides corresponding to precursor and mature NIS species were detected for all mutant NIS proteins to an extent similar to that of WT NIS. Expression of WT and mutant NIS proteins was compared after normalization using either tubulin for total lysed blots or Na+/K+-ATPase α subunit for surface biotinylation blots (data not shown). We also observed virtually equal plasma membrane localization for mutant NIS proteins as compared to WT NIS by both cell surface biotinylation with a membrane impermeable reagent (Fig. 2D and F) and confocal immunofluorescence microscopy (Fig. 2G). These results demonstrate that the decrease in activity observed in the described NIS mutants was not due to either lower expression or a plasma membrane targeting defect.

**Substitutions in TMS IX do not alter the NIS K_m values for I^-**. Given the above results, we sought to determine by kinetic analysis whether the partial impairment of function in the mutant NIS proteins was attributable to increased K_m values for I^- We analyzed the kinetic properties of I^- uptake in COS-7 cells expressing all the described TMS IX NIS mutants as compared to WT NIS (Fig. 3A- C). Initial rates were assessed by measuring I^- accumulation at 4-min time points over a range of I^- concentrations (2.5–200 µM), with a constant 140 mM Na^+ concentration. A kinetic analysis of T354A, T354G, T354P, T354Y, S356P and T357P NIS was not possible because I^- uptake mediated by these mutants was almost undetectable. No major differences in K_m for I^- were observed in cells expressing NIS mutants that displayed significant transport activity with respect to WT NIS (23.7± 2.1 µM) (Fig. 3A). In contrast, the maximal rate of I^- uptake (V_{max,I^-}) was different in the various TMS IX NIS mutants (Fig. 3A-C), and that variation was similar to that observed in I^- uptake at steady state (Figs. 2A-B). As the decreases in activity did not result from either lower expression or impaired targeting to the plasma membrane, the lower V_{max} values observed most probably reflect a lower turnover rate.

**An increase in the Na^+ concentration rescues I^- transport in Ala mutant NIS proteins that were inactive at the physiological concentration of Na^+.** To investigate the possible involvement of residues S349, T351, S353, T354, S356, T357, S358, and T366 in NIS Na^+ dependence, we carried out I^- transport assays under steady-state conditions at 20 µM I^- and 280 mM Na^+ compared the results with those obtained at 140 mM Na^+ (Fig. 2A and B), and expressed the difference as a ratio. Virtually no change in activity was observed at 280 mM Na^+ with respect to 140 mM Na^+ in cells expressing WT NIS (Fig. 4A, lane 1) or in substitutions that displayed I^- uptake activity comparable to that of WT NIS, namely S349A (Fig. 4A, lane 2), S358A (Fig. 4A, lane 8), and T366A NIS (Fig. 4A, lane 9). Interestingly, with the exception of T354A, T354P, and T354Y, all mutant NIS proteins that displayed very low or no I^- uptake at 140 mM Na^+ exhibited a significant increase in I^- transport at 280 mM Na^+, ranging from 1.4-fold in T357P to 2.1-fold in T357A NIS. Furthermore, in two mutants (S356P and T357P NIS) that did not accumulate I^- at 140 mM Na^+ (Fig. 2A), there was significant I^- transport at 280 mM Na^+. That the impairment of NIS function caused by the absence of OH groups at positions 351, 353, 356, and 357 was partly overcome by a higher Na^+ concentration suggests that these residues are involved in the Na^+ dependence of NIS.

The T351S, S353T, T354S, S356T, and T357S mutant NIS proteins [which, because of the presence of OH groups at these positions, exhibited 40 to 65% of WT NIS activity at 140 mM Na^+ (Fig. 2B)] also displayed increased I^- transport activity when assayed at 280 mM Na^+ (Fig. 4B). The increased activity at 280 mM Na^+ for most mutants was significantly more pronounced in the Ala/Cys than in the Thr/Ser mutants (compare Figs. 4A and 4B). Thus, the increase was 1.8-fold in S353A vs. 1.4-fold in S353T; 1.7-fold in S356A vs. no increase in S356T; and 2.1-fold in T357A vs. 1.6-fold in T357S. The exception again was T354. The T351S, T354S and T357S, but not S353T and S356T NIS mutants, recovered full activity when assayed at 280 mM Na^+, may reflect the specific properties of these amino acids (see discussion). Clearly, substitution of T351, S353, S356, and T357 with residues that are devoid of OH groups impairs the Na^+ dependence of NIS to a
greater extent than substitutions that maintain OH groups.

The $K_m$ for Na$^+$ is altered in TMS IX NIS mutant proteins. We carried out a kinetic analysis of Na$^+$-dependent I$^-$ transport in COS-7 cells expressing TMS IX NIS substitutions as compared to WT NIS. Initial rates were assessed by measuring I$^-$ accumulation at 4-min time points over a range of Na$^+$ concentrations of 0–300 mM, with a constant 20 µM I$^-$ concentration, and keeping an isotonic medium with choline chloride (Fig. 4C). Tellingly, significant variations in the $K_m$ for Na$^+$ with respect to WT NIS (64.7±2.8 mM) were observed in cells expressing the various TMS IX mutant NIS proteins. More pronounced increases in $K_m$ for Na$^+$ were measured in cases where lower steady-state I$^-$ transport was observed, reaching values that were even higher than the physiological extracellular Na$^+$ concentration (137-142 mM) (13)(Fig. 4C). Variations in the maximal rate of Na$^+$ dependent I$^-$ uptake ($V_{\text{max}}$) in the TMS IX NIS mutant proteins were quite similar to those observed in the steady-state analysis using 20 µM I$^-$ and 280 mM Na$^+$ (Fig. 4A and B). These results suggest that Ser and Thr in TMS IX may be involved in Na$^+$ dependent transport.

Several residues in NIS TMS IX are highly conserved in other Na$^+$-dependent transporters. It is interesting to note that TMS IX is one of the regions of NIS that exhibits the highest overall homology with other members of the Na$^+$-dependent transporter family (Fig. 5). OH-containing Thr and Ser residues (such as the above-described T351, S353, T354, S356, and T357), whose substitutions gave rise to mutant proteins with higher $K_m$ values for Na$^+$ and which are likely to be involved in Na$^+$ dependence, are among the conserved ones (Fig. 5A). This suggests that corresponding amino acids in other proteins in the family may also participate in Na$^+$ dependence. Given this notion, we set out to examine whether a high degree of conservation of certain NIS TMS IX residues, independently of whether or not they contain OH groups, may be an indicator of the residues' possible functional significance vis-à-vis Na$^+$ dependence. N360 and D369 are 100% conserved in all members of the Na$^+$-dependent transporter family (Fig. 5A). In contrast, C346 is only present in three other transporters (SMVT, SMCT, and SMCT2), and A364 only in SMCT2. The rest of the members have a Val at position 346 and a Thr at 364 (Fig. 5A).

We generated C346A, N360A, N360Q, N360E, A364T, A364G, E368A, D369A, and E368A/D369A mutant NIS cDNAs; transfected each one individually into COS cells; and assayed the cells for I$^-$ transport activity. All resulting NIS proteins were expressed and targeted to the plasma membrane similarly to WT NIS (Fig. 6A-B). However, all substitutions, except C346A and E368A, caused a decrease in I$^-$ transport activity (11-47% in N360 substitutions, 40-76% in A364 substitutions, 44% in D369, and only 8% in the ED368/369AA double mutant) (Fig. 6C). I$^-$ uptake at 280 mM Na$^+$ increased with respect to that at 140 mM Na$^+$ only in cells expressing NIS proteins with substitutions of the conserved N360 and D369 but not in those expressing NIS with substitutions of the nonconserved C346, A364 or E368 (Fig. 6D).

Furthermore, a kinetic analysis of Na$^+$-dependent I$^-$ transport demonstrated a higher $K_m$ for Na$^+$ in NIS molecules with replacements at positions 360 and 368 than in WT NIS, whereas the $K_m$ values for Na$^+$ in NIS with C346 and A364 substitutions were similar to those of WT NIS (Fig. 6F). Also, none of these replacements led to significantly different $K_m$ values for I$^-$ from those of WT NIS (Fig. 6E). These findings suggest that the highly conserved N360 and D369 may play a role in the Na$^+$ translocation pathway, whereas the less conserved C346 and A364 do not.

DISCUSSION

The study of NIS mutations that cause congenital iodide transport defect (ITD) has yielded extremely valuable structure/function information on NIS. Perhaps the most intriguing mutation is T354P, the analysis of which demonstrated that the presence of a β-OH group at position 354 is required for NIS to be functional (9). Several studies have shown that OH-containing amino acids may participate in ion translocation in other transporters, such as the Na$^+$/dicarboxylate cotransporter, the glucose transporter (GLUT), the Na$^+$/K$^+$ ATPase, the glutamate transporter (GLT-1), and the bacterial Aquifex aeolicus Na$^+$-dependent leucine transporter, whose high-resolution structure has recently been determined (14-18). Significantly, NIS TMS IX, where T354 is located, contains the highest number of amino acids with OH groups at the β-carbon of any TMS in the protein, and many of these residues are conserved in all the family’s transporters (Fig. 5A). In this study, we have analyzed the functional role of all OH-containing and conserved residues in NIS TMS IX (Fig. 1). Remarkably, all mutant NIS proteins we generated exhibited normal maturation, expression, and targeting to the plasma membrane (Figs. 2C-G,
and 6A-B), making it clear that the observed changes in transport properties are a direct consequence of local structural changes caused by the engineered amino acid substitutions.

The first key finding of this study is that the presence of five β-carbon-OH-containing residues (T351, S353, T354, S356, and T357) located in NIS TMS IX is critical for the protein to be functional. Activity was no higher than 10% of WT NIS when Ala, Pro or Cys were individually substituted into these positions (Fig. 2A). Only when we kept a OH group at the β-carbon by substituting Thr with Ser or Ser with Thr at these positions were we able to recover transport activity, which ranged from 40 to 65% of WT NIS (Fig. 2B). However, the presence of these OH groups alone was not enough for full activity. Ala substitutions at the positions of the other 3 OH-containing residues in NIS TMS IX (S349, S358, and T366) did not significantly alter NIS function and caused only a modest decrease or increase in activity (Fig. 2A). These results suggest that these residues do not play any functional role.

None of the substitutions significantly altered the I K_m as compared to WT NIS (15-35 µM) (Fig. 3). In some cases (i.e., T35A in Fig. 3A), we obtained a higher K_m,i with respect to WT NIS, although it generally occurred with substitutions that yielded proteins with low V_max,i values. This attribute made it very difficult to obtain accurate values for kinetic parameters for initial rates, a point reflected in the higher SE values obtained (Fig. 3). The observed V_max variations are in close relation to steady-state I uptake (Fig. 2A and B). Next, we investigated whether these residues are involved in the Na^+ dependence of I transport. Ala-substituted NIS proteins exhibited a statistically significant increase in I transport (1.5- to 2.2-fold) when the Na^+ concentration was raised from 140 to 280 mM (Fig. 4A). Two substitutions (S356P and T357P) that were inactive at 140 mM of Na^+ recovered partial activity when the Na^+ concentration was increased to 280 mM. With the sole exception of S356T, all replacements that preserved the β-OH group either recovered full activity (T351S, T354S, T357S) or increased I transport 1.5-fold (S353T) (Fig. 4B and C). That Ser can completely recover WT NIS activity at saturating Na^+ concentrations when placed at positions where the original amino acid was Thr, but not the other way around, may be because in the context of a helix, Thr and Ser have different properties: the side chain of Thr is constrained to a single rotamer, while the OH of Ser can adopt any of the three possible rotamers (g^+, g^−, and t). When the WT sequence has a Ser, it seems that interactions with surrounding residues restrict the position of the OH to the rotamer allowed for Thr, so substitution by Thr does not change the position of the OH. When the WT sequence has a Thr, it appears that the position of the OH is not determined by interactions with other residues but rather by the β-branched side-chain. The replacement of Thr with Ser, which can adopt other rotamers, would thus weaken the interaction with Na^+.

The Na^+ dependence of I transport in most NIS mutant proteins showed higher K_m,Na^+ values (90-164 mM) than WT NIS (Fig. 4C). These results suggest that the studied NIS TMS IX residues are involved in Na^+ binding and/or translocation during transport. Unlike the situation with the I K_m, and given that the physiological concentration of Na^+ is ~140 mM (13), the observed increases in the Na^+ K_m are highly significant, because some of these mutants are essentially inactive at 140 mM Na^+ (i.e., T354C, S356P, T357P). This is so not only because of the apparent decrease in Na^+ affinity but also because the partial or total impairment of the Na^+ coordination may ultimately impair substrate coupling. Kanner et al (19) have similarly shown that amino acid substitutions in TMS VIII of the eukaryotic GABA transporter GAT1 have an effect on cation specificity and cause a 2-3-fold increase in the Na^+ K_m and a 2.5-8-fold increase in the GABA K_m, even though TMS VIII residues do not interact with GABA.

Finally, we examined whether our results were applicable to other proteins in the SLC5A family. NIS TMS IX alignment with the corresponding TMS in other transporters exhibits a high overall homology (Fig. 5A). All proteins contain a very high number of OH-containing residues, and most of the functionally critical OH-containing residues in NIS are conserved in the other transporters. We analyzed other residues in NIS TMS IX as indicators of their possible functional role in Na^+ transport. Interestingly, substitutions of the NIS residues N360 and D369, which are 100% conserved in all family members, increased the K_m of Na^+, even though transport activity was less than half of that in WT NIS under normal conditions. However, replacement of C346 with A (a site where other family members have a V or G), A364 for T or G (where most members have a T), and E368A (where other members have M, I or L) (Fig. 5A), yielded K_m values for Na^+ that were no different from those of WT NIS (Fig. 4C). These data indicate that the highly conserved N360 and D369...
may also influence Na\(^+\) dependence, whereas the less conserved C346, A364, and E368 do not. The single mutant D369A retained 50% of WT activity, even though the data indicate that D369 is critical for the Na\(^+\) pathway. This is most probably because the functional role of a negatively charged residue in that region may be compensated by the neighboring E368 residue. As predicted by this hypothesis, the double mutant E368A/D369A NIS barely transported I

Yamashita et al recently reported a high atomic resolution structure of a Na\(^+\)-driven transporter (20), the *Aquifex aeolicus* Na\(^+\)-dependent leucine transporter (LeuTAA), a bacterial homologue of eukaryotic Na\(^+\)-dependent neurotransmitter transporters. In this high-resolution structure (1.65 Å), the substrates (2 Na\(^+\) ions and 1 Leu) are bound to the transporter. Remarkably, some of the residues (T354 and S355) that are in contact with the second Na\(^+\) site in this protein’s TMS VIII (20) correspond exactly to the S353 and T354 residues of NIS TMS IX (Fig. 5B). Also, LeuTAA has a Q residue at position 361 that aligns with NIS N360 (Fig. 5B). Furthermore, LeuTAA has a pair of negatively charged residues after TMS VIII at positions 369 and 370 that align with NIS E368 and D369 (Fig. 5B). These LeuTAA residues are strictly conserved among NSS family members (to which LeuTAA, the serotonin (SERT) and the dopamine (DAT) transporters belong), and have been proposed to be implicated in the salt bridge that forms an intracellular gate that controls the access of Na\(^+\) and substrates (20). Substitutions of the equivalent residues in DAT (D436 and E437) impair dopamine transport (21).

Strikingly, there is more similarity between LeuTAA TMS VIII, which contains the residues that coordinate the second Na\(^+\), and NIS TMS IX, than between LeuTAA TMS VIII and TMS VIII of the eukaryotic neurotransmitter transporters of the SLC6 family, even though LeuTAA is a bacterial homologue of this family (Fig. 5C). Although these could be merely coincidental similarities, they also raise the possibility that the Na\(^+\) transport and coupling mechanisms may be conserved among several Na\(^+\)- driven cotransporters of different families. We analyzed a pattern from NIS TMS IX derived from our results, [S/A – (X)\(_3\) – S/A – (X)\(_3\) – S/T – S/T – X – S/T/A - S/T – (X)\(_2\) - N/Q – (X)\(_2\) -S/T/A -S/T/A – (X)\(_4\) – D/E] (Fig. 5A), and searched it in all protein sequences contained in the database using the ScanProsite software tool (http://ca.expasy.org/tools/scanprosite/) (22,23). Of a total of 312 retrieved sequences, all but 27 proteins belonged to the SLC5A family, of which NIS is a member. Given that SLC5A is a family of Na\(^+\)-dependent transporters, it is not surprising that a sequence linked to a likely role in Na\(^+\) binding and/or translocation is present in proteins of this family. Of note, LeuTAA TMS VIII contains 6 out of 10 residues of this sequence pattern, and 5 out of 6 of the critical amino acids.

According to our secondary structure model, amino acids that we found to be important in Na\(^+\)-dependent I transport are present on an extended surface that runs across one face of helix IX. During the transport cycle of NIS, Na\(^+\) may contact different residues due to movements of Na\(^+\) and/or rotation of helix IX. The negatively charged E368 and D369 at the end of TMS IX could constitute the Na\(^+\) gate, similarly to what happens in LeuTAA (20) and DAT (21).

In summary, we have shown that five OH-containing residues (T351, S353, T354, S356, and T357), N360 in NIS TMS IX, and D369 at the membrane-cytosol interface, are all critical for NIS function and probably for other transporters of the SLC5A family. These amino acids seem to play a critical role in Na\(^+\) binding and/or the lining of the Na\(^+\) translocation. Our results also suggest that Na\(^+\) binding/translocation does not occur solely through the N-terminal part of the protein, as has been suggested for SGLT1 as a general mechanism for the SLC5A family (24). We propose that the sequence pattern we have identified in TMS IX along with residues from other TMS may define a region involved in Na\(^+\) binding/translocation in members of the NIS family. The complete mechanism will be elucidated only when atomic-resolution structural data from different conformations of these transporters become available.
REFERENCES

1. Carrasco, N. (1993) Biochim Biophys Acta 1154(1), 65-82
2. De La Vieja, A., Dohan, O., Levy, O., and Carrasco, N. (2000) Physiol Rev 80(3), 1083-1105
3. Dohan, O., De la Vieja, A., Paroder, V., Riedel, C., Artani, M., Reed, M., Ginter, C. S., and Carrasco, N. (2003) Endocrin Rev 24(1), 48-77
4. Mazzaferrri, E. L. (1996) Carcinoma of follicular epithelium: radioiodide and other treatments and outcomes. In: Braverman, L. E., and Utiger, R. D. (eds). The Thyroid: a fundamental and clinical text, 7th Ed., Lippincott-Raven, Philadelphia
5. Wright, E. M., and Turk, E. (2004) Pflugers Arch 447(5), 510-518
6. Eskandari, S., Loo, D. D., Dai, G., Levy, O., Wright, E. M., and Carrasco, N. (1997) The Journal of biological chemistry 272(43), 27230-27238
7. Levy, O., De la Vieja, A., Ginter, C. S., Riedel, C., Dai, G., and Carrasco, N. (1998) The Journal of biological chemistry 273(35), 22657-22663
8. Turk, E., and Wright, E. M. (1997) The Journal of membrane biology 159(1), 1-20
9. Levy, O., Ginter, C. S., De la Vieja, A., Levy, D., and Carrasco, N. (1998) FEBS Lett 429(1), 36-40
10. De la Vieja, A., Ginter, C. S., and Carrasco, N. (2005) Mol Endocrinol 19(11), 2847-2858
11. Levy, O., Dai, G., Riedel, C., Ginter, C. S., Paul, E. M., Lebowitz, A. N., and Carrasco, N. (1997) Proc Natl Acad Sci USA 94(11), 5568-5573
12. De La Vieja, A., Ginter, C. S., and Carrasco, N. (2004) J Cell Sci 117(Pt 5), 677-687
13. de Wardener, H. E., He, F. J., and MacGregor, G. A. (2004) Kidney international 66(6), 2454-2466
14. Doege, H., Schurmann, A., Ohnimus, H., Monser, V., Holman, G. D., and Joost, H. G. (1998) Biochem J 329 ( Pt 2), 289-293
15. Pajor, A. M. (2001) The Journal of biological chemistry 276(32), 29961-29968
16. Pedersen, P. A., Nielsen, J. M., Rasmussen, J. H., and Jorgensen, P. L. (1998) Biochemistry 37(51), 17818-17827
17. Slotboom, D. J., Sobczak, I., Konings, W. N., and Lolkema, J. S. (1999) Proc Natl Acad Sci USA 96(25), 14282-14287
18. Zhang, Y., and Kanner, B. I. (1999) Proc Natl Acad Sci USA 96(4), 1710-1715
19. Kanner, B. I. (2006) The Journal of membrane biology 213(2), 89-100
20. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Nature 437(7056), 215-223
21. Loland, C. J., Granas, C., Javitch, J. A., and Gether, U. (2004) The Journal of biological chemistry 279(5), 3228-3238
22. Gattiker, A., Gasteiger, E., and Bairoch, A. (2002) Applied bioinformatics 1(2), 107-108
23. de Castro, E., Sigrist, C. J., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P. S., Gasteiger, E., Bairoch, A., and Hulo, N. (2006) Nucleic acids research 34(Web Server issue), W362-365
24. Loo, D. D., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E., and Wright, E. M. (1998) Proc Natl Acad Sci USA 95(13), 7789-7794
25. Dayhoff, M., Schwartz, R., and Orcutt, B. (1978) A model of evolutionary change in proteins. In: Dayhoff, M. (ed). Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington, DC

FOOTNOTES

*We are grateful to members of the Carrasco laboratory and Dr. Mario Amzel for discussions and critical reading of this manuscript. This project was supported by the National Institutes of Health DK-41544 and
The Ministry of Education and Culture (Ministerio de Educación y Cultura) of Spain PF 97 52094152. M.R. was supported by the National Institutes of Health 5T32 GM 07491 and a supplement of CA098390.

The abbreviations used are: NIS, sodium/iodide symporter; SGLT1, sodium/glucose co-transporter 1; SMVT, sodium/multivitamin transporter; SMCT, sodium/monocarboxylate transporter; LeuTAA, *Aquifex aeolicus* Na⁺-dependent leucine transporter; TMS, transmembrane segment.

**FIGURE LEGENDS**

**FIGURE 1. Schematic representation of NIS TMS IX.** NIS secondary structure model (A); helical net (B) and helical wheel (C) representations of TMS IX. Amino acids with OH groups at the β-carbon (Ser or Thr) are depicted over black background.

**FIGURE 2. Activity and expression of NIS mutants with TMS IX amino acids substitutions.** Nontransfected COS-7 cells (NT) or COS-7 cells transfected with either WT, Ala, Pro, Cys, Ser or Thr substituted NIS cDNA were analyzed. For steady-state Γ transport assays of NIS Ala, Pro and Cys substitutions (A) and; Ser and Thr substitutions (B), cells were incubated for 1 h in the presence of 20 μM Γ/140 mM Na⁺ (solid bars) or 20 μM Γ/140 mM Na⁺/80 μM perchlorate (open bars). Results are expressed in pmol Γ/μg DNA ± SEM. Values represent the average of at least 4 different experiments; in each experiment, activity was analyzed in triplicate. Immunoblot and the corresponding surface biotinylation analysis of Ala, Pro and Cys (C and D) and; Ser and Thr (E and F). Total protein of lysed cells (5 μg/lane) was isolated from NT cells or COS-7 cells transfected with NIS cDNA, electrophoresed, electrotransferred, and immunoblotted with 2 nM anti-NIS Ab. Cell-surface biotinylated proteins were labeled using 1 mg/ml Sulfo-NHS-SS-biotin. Immunoblot analysis of surface biotinylated polypeptides precipitated with streptavidin-agarose beads was performed with 2 nM anti-rNIS Ab. Nitrocellulose membranes were stripped and reprobed with anti-tubulin (total protein blots) or anti-Na⁺/K⁺-ATPase α-subunit Abs (biotinylated protein blots) (not shown) to determine the levels of NIS protein expression with respect to WT NIS. Immunofluorescence analysis of Ala, Pro and Cys (G) and; Ser and Thr (H) substitutions. Permeabilized cells were incubated with 2 nM anti-rNIS Ab, followed by fluorescein-conjugated anti-rabbit Ab.

**FIGURE 3. Iodide kinetic analysis of NIS TMS IX of (A) Ala, (B) Pro, Cys, Gly and Tyr; and (C) Ser/Thr substitutions.** Initial rates (4-min time points) of Γ uptake were determined at the indicated concentrations of Γ, as described in Experimental procedures. Calculated curves (smooth lines) were generated using the equation \( v= \frac{V_{\text{max}} \cdot [\text{I}]}{K_{\text{m}} + [\text{I}]} + 0.06 \cdot [\text{I}] + 0.60 \). The terms 0.06*[I]+0.60 correspond to background adjusted by least squares to the data obtained with NT cells. \( K_{\text{m}} \) and \( V_{\text{max}} \) values are indicated in the table. Values are the determination ± SEM of 3-16 experiments. Graphs correspond to a representative experiment. *The extremely low levels of transport activity by these NIS mutants precluded accurate determination of kinetic parameters.

**FIGURE 4. Iodide transport analysis with high Na⁺ concentration and Na⁺-dependent kinetic analysis.** Nontransfected COS-7 cells (NT) or COS-7 cells transfected with either WT, Ala, Pro, Cys, Gly and Tyr (A), or Ser and Thr (B) substituted NIS cDNA were analyzed. For steady-state Γ transport assays, cells were incubated for 1 h in the presence of 20 μM Γ/280 mM Na⁺ (gray bars) or 20 μM Γ/280 mM Na⁺/80 μM perchlorate (open bars). Results are expressed in pmol Γ/μg DNA ± SEM. Values represent the average of at least 4 different experiments; in each experiment, activity was analyzed in triplicate. Statistical significance was evaluated by t-test analysis (two-tailed); differences were considered significant at \( p < 0.05 \) (*), 0.05-0.001 (**) and <0.001 (***) (C) To assess Na⁺ dependence of Γ uptake, cells were incubated for 4 min with the indicated concentrations of Na⁺; isotonicity was maintained constant with choline chloride. Na⁺-dependence data were analyzed using the equation \( v= \frac{V_{\text{max}} \cdot [\text{Na}^+]^2}{K_{m}^2 + [\text{Na}^+]^2} + 0.001 \cdot [\text{Na}^+] + 0.87 \). The term 0.001*[Na⁺]+0.87 corresponds to the background adjusted by lineal regression analysis obtained with NT cells. \( V_{\text{max}}-\text{Na}^+ \) and \( K_{m}^-\text{Na}^+ \) values are indicated in the Table. Values are the determination ± SEM of 3-16 experiments. Graphs correspond to a representative experiment. *The extremely low levels of transport activity by these NIS mutants precluded accurate determination of kinetic parameters.
**Figure 5.** Multiple sequence alignment of TMS IX in some members of the SLC5A gene family (A). The sequence alignment was made using ClustalW with the PAM250 weight table (25). The amino acid numbering (345-367) corresponds to the rNIS sequence. The most conserved (black) and the closely related amino acids (gray) in NIS homologues are highlighted. Only Ser 353, Asn 360 and Glu 369 are conserved throughout. Thr 354, Ser 356 and Thr 357 are Ser or Thr in all homologues, except in SMIT transporters. The gene names and the gi accession numbers are written next to the transporter names. Two species of each transporter were selected for the analysis. A sequence pattern that contains all critical residues for transport in this paper is indicated at the bottom. **NIS TMS IX and Aquifex aeolicus LeuTaa TMS VIII sequence alignment (B).** Note that the OH-containing NIS residues 353 and 354 align with the OH-containing LeuTaa residues 354 and 355. Also, the NIS amide side-chain residue Asn 360 aligns with the LeuTaa amide side-chain residue Gln 361 and NIS E368 and D369 align with LeuTaa D369 and E370. LeuTaa contains 6 out of 10 residues in the sequence pattern that we defined as potentially involved in Na⁺ transport and 5 out of 6 of the critical residues for NIS transport within the pattern. **Multiple sequence alignment of TMX VIII in some members of SLC6 family (C).** Black triangles indicate important residues for NIS activity. Residues that coordinate Na⁺ in the LeuTaa crystal structure are indicated in grey. The star shows a residue that forms a salt bridge suggested to be an intracellular gate (20) that controls the access of Na⁺ and substrates in the SLC6 family (21).

**Figure 6.** Characterization of NIS TMS IX substitutions at positions 346, 360, 364, 368 and 369. Expression (A), cell surface biotinylation (B), I⁻ transport with 140 mM and 280 mM Na⁺ (C), I⁻ (D) and Na⁺-dependent kinetic analysis (E). Conditions were similar to those described in Figs. 2, 3 and 4, and in Experimental Procedures. For steady-state I⁻ transport assays (C) were incubated for 1 h in the presence of 20 μM I⁻/140 mM Na⁺ (solid bars) or 20 μM I⁻/280 mM Na⁺ (dashed bars). Results in the presence of perchlorate are shown in open bars. *The extremely low levels of transport activity by these NIS mutants precluded accurate determination of kinetic parameters. Statistical significance in (D) was evaluated by t-test analysis (two-tailed); differences were considered significant at p < 0.05 (*), 0.05-0.001 (**) and <0.001 (***)
Fig. 3 – De la Vieja et al.

**Table 1**

| Variant   | $K_{M-I}$ (µM) | $V_{max}$ (pmols/µg DNA)*4min |
|-----------|----------------|-------------------------------|
| NT        | -              | -                            |
| WT        | 23.7 ± 2.1     | 131.9 ± 28.3                 |
| S349A     | 25.4 ± 1.1     | 65.8 ± 2.6                   |
| T351A     | 60.7 ± 15.9    | 20.0 ± 6.4                   |
| S353A     | 49.9 ± 8.9     | 11.0 ± 5.0                   |
| T354A     | -              | -                            |
| S356A     | 72.8 ± 15.3    | 19.6 ± 4.6                   |
| T357A     | 93.0 ± 21.6    | 27.7 ± 14.7                  |
| S358A     | 18.9 ± 3.2     | 147.9 ± 50.4                 |
| T366A     | 19.2 ± 0.5     | 155.6 ± 11.6                 |

**Graph A**

**Graph B**

**Graph C**

**Graph D**
Fig. 4 – De la Vieja et al.

**C**

|        | $K_{M-Na^+}$ [mM] | $V_{max}$ [pmols/µg DNA]*4 min |
|--------|------------------|--------------------------------|
| NT     | -                | -                             |
| WT     | 63.2 ± 3.0       | 54.2 ± 8.6                    |
| S349A  | 65.7 ± 11.8      | 49.4 ± 12.1                   |
| S349C  | 130.4 ± 14.2     | 30.9 ± 5.8                    |
| T351A  | 116.2 ± 7.8      | 58. ± 1.3                     |
| T351S  | 99.3 ± 6.4       | 46.1 ± 10.5                   |
| S353A  | 141.2 ± 16.1     | 4.6 ± 0.8                     |
| S353T  | 96.6 ± 6.1       | 28.1 ± 9.7                    |
| T354A* | -                | -                             |
| T354S  | 90.3 ± 7.8       | 43.8 ± 11.4                   |
| T354C  | 114.1 ± 10.4     | 3.7 ± 0.7                     |
| T354P* | -                | -                             |
| T354G* | -                | -                             |
| T354Y* | -                | -                             |
| S356A  | 120.0 ± 9.5      | 6.7 ± 2.0                     |
| S356T  | 128.9 ± 24.0     | 48.8 ± 22.8                   |
| S356P* | -                | -                             |
| T357A  | 127.4 ± 26.7     | 6.9 ± 2.0                     |
| T357S  | 164.1 ± 10.5     | 49.6 ± 13.5                   |
| T357C  | 141.7 ± 15.0     | 4.3 ± 0.7                     |
| T357P* | -                | -                             |
| S358A  | 80.2 ± 3.7       | 78.9 ± 18.3                   |
| T366A  | 61.5 ± 9.9       | 77.8 ± 13.7                   |
Fig. 5 – De la Vieja et al.
Fig. 6 – De la Vieja et al.
Amino acid residues in transmembrane segment IX of the Na+/I- symporter (NIS) play a role in its Na+ dependence and are critical for transport activity

Antonio De la Vieja, Mia D. Reed, Christopher S. Ginter and Nancy Carrasco

J. Biol. Chem. published online July 2, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M700147200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts