Molecular Characterization of V59E NIS, a Na⁺/I⁻ Symporter Mutant that Causes Congenital I⁻ Transport Defect

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I⁻ is actively transported into thyrocytes via the Na⁺/I⁻ symporter (NIS), a key glycoprotein located on the basolateral plasma membrane. The cDNA encoding rat NIS was identified in our laboratory, where an extensive structure/function characterization of NIS is being conducted. Several NIS mutants have been identified as causes of congenital I⁻ transport defect (ITD), including V59E NIS. ITD is characterized by low thyroid I⁻ uptake, low salivary/plasma I⁻ ratio, hypothyroidism, and goiter and may cause mental retardation if untreated. Studies of other ITD-causing NIS mutants have revealed valuable information regarding NIS structure/function. V59E NIS was reported to exhibit as much as 30% of the activity of wild-type NIS. However, this observation was at variance with the patients' phenotype of total lack of activity. We have thoroughly characterized V59E NIS and studied several amino acid substitutions at position 59. We demonstrated that, in contrast to the previous report, V59E NIS is inactive, although it is properly targeted to the plasma membrane. Glu and all other charged amino acids or Pro at position 59 also yielded nonfunctional NIS proteins. However, I⁻ uptake was rescued to different degrees by the other substitutions. Although the Kₘ values for Na⁺ and I⁻ were not altered in these active mutants, we found that the structural requirement for NIS function at position 59 is a neutral, helix-promoting amino acid. This result suggests that the region that contains V59 may be involved in intramembrane helix-helix interactions during the transport cycle without being in direct contact with the substrates. (Endocrinology 149: 3077–3084, 2008)
Materials and Methods

Site-directed mutagenesis

Site-directed mutagenesis was performed using the following oligos: V59A, GCA GCC GCT CCT SCG GGG CGT TCG; V95D, GCA GCC GCT CCT RAC GGG CGT TCG; V95E, GCA GCC GCT CCT SAG GGG CGT TCG; V95I, GCA GCC GCT CCT ATT GGG CGT TCG TGT; V95K, GCA GCC GCT CCT ARG GGG CGT TCG; V95L, GCA GCC GCT CCT GAG GGG CGT TCG; V95P, GCA GCC GCT CCT GAG GGG CGT TCG; V95Q, GCA GCC GCT CCT SAG GGG CGT TCG; V95R, GCA GCC GCT CCT ARG GGG CGT TCG TGT; V95T, GCA GCC GCT CCT AGG GGG CGT TCG TGT; and the T354P oligo was the same as in Levy et al. (22). Site-directed mutagenesis was carried out as previously described (27). The initial PCR extensions were performed using reverse primers complementary to the 3' end. These fragments were gel purified and used for a second round of PCR extension with primers complementary to the 5' end. Fragments with the mutant sequences were obtained by digestion of the final PCR products with the appropriate unique restriction enzyme to obtain the smallest mutant fragments. These fragments were then ligated into WT NIS cDNA (PS-VSport-rNIS), and the mutant inserts were sequenced past their respective cloning sites.

Cell culture and transient transfection of COS-7 cells

COS-7 cells were grown at 37 C with 5% CO2 and maintained in DMEM before transient transfection with 4 µg/plate rat NIS cDNA constructs using Lipofectamine Plus (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Transfected cells were split 24 h after transfection and analyzed by immunoblot, surface biotinylation, and immunofluorescence, assayed for I uptake, and measured by FACS to measure transfection efficiency 48 h after transfection.

Immunoblot analysis

Transfected cells were lysed by incubating them with complete lysis buffer [1% SDS, 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100] and then adding lysis buffer (complete lysis buffer without SDS) at a dilution of 1:10. After cell lysis, the protein concentration was quantified using the BCA protein measurement kit (Pierce, Rockford, IL). Samples were diluted with sample buffer and incubated at 37 C for 30 min followed by 95% SDS-PAGE, electroblotted to a nitrocellulose membrane, and immunoblotted with 2 nm of an affinity-purified anti-rNIS polyclonal antibody (directed against the last 16 amino acids of the rNIS C terminus) for 1 h and then incubated for 45 min with the antirabbit HRP secondary antibody (Jackson Laboratory, Bar Harbor, ME).

Cell surface biotinylation

Biotinylation of cell surface proteins was performed as previously described (19). Twenty-four hours after transfection, COS-7 cells were split into 12-well plates and maintained in DMEM for 24 h more. Transiently transfected cells were incubated with 1 mg/ml of the membrane-impermeable biotin reagent sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate-biotin (sulfo-NHS-SS-biotin) (Pierce), which covalently interacts with extracellular primary amines. The reaction was subsequently quenched with 100 mM glycine in PBS supplemented with 1 mM MgCl2 and 0.1 mM CaCl2. Cells were lysed and biotinylated proteins precipitated overnight with streptavidin-coated beads, heated with sample buffer containing 100 mM final dithiothreitol at 75 C for 5 min, subjected to SDS-PAGE, and immunoblotted with anti-rNIS. As a control, supernatants obtained after the overnight incubation were analyzed by immunoblot.

Steady-state I transport analysis

Transfected cells were assayed under steady-state conditions in triplicate. Forty-eight hours after transfection, cells were washed twice in Hanks' buffered salt solution (HBSS) [140 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.4 mM MgSO4, 0.5 mM MgCl2, 0.4 mM NaHPO4, 0.44 mM KH2PO4, and 5.55 mM glucose in 10 mM HEPES (pH 7.5)]. Cells were then incubated with 20 µM 125I (specific activity 100 mCi/mmol) and 140 mM Na (unless otherwise indicated) for 45 min to 1 h at 37 C in a humidified atmosphere with 5% CO2 and then washed twice more with HBSS (4 C). Accumulated I was released by incubating cells with 100% ethanol for at least 20 min at 4 C and quantitated in a γ-counter (Perkin-Elmer, Norwalk, CT). I uptake was standardized by determining the amount of DNA per cell by incubating cells with 5% trichloroacetic acid for 30 min at 4 C followed by an overnight incubation with diphenylamine solution.

I- and Na+-dependent kinetic analysis

For I-dependent kinetic analysis, cells were incubated with I concentrations ranging from 0.6–80 µM 125I and 140 mM NaCl for 2 min.
Initial rates of $\Gamma^-$ transport measured during kinetic analysis were analyzed by nonlinear regression using the following equation: $v([\Gamma^-]) = (V_{max} \times [\Gamma^-])/(K_m + [\Gamma^-]) + A \times [\Gamma^-] + B$. The terms $A$ and $B$ refer to background adjusted by least squares of the data obtained with nontransfected cells. For the Na$^+$-dependent kinetic analysis, cells were incubated with $20 \mu M \text{Na}^{2+}$ and a range of Na$^+$ concentrations between 0 and 140 mm for 2 min (isotonicity was maintained using choline-Cl). The equation used to analyze the initial rates is $v([\text{Na}^+]) = (V_{max} \times [\text{Na}^+]/(K_m^+ + [\text{Na}^+]^2) - A \times [\text{Na}^+] + B$. Data were analyzed by gnuplot (www.gnuplot.info).

**Results**

**V59E NIS is expressed but inactive**

To assess the transport properties of V59E NIS, we measured Na$^+$-dependent, perchlorate (ClO$_4^-$)-inhibitable (i.e. NIS-mediated) $\Gamma^-$ transport at 20 $\mu M$ $\Gamma^-$ and 140 mm Na$^+$ for 1 h in COS-7 cells expressing either V59E NIS or WT NIS. WT NIS-expressing cells accumulated 80–100 pmol $\Gamma^-$/μg DNA; $\Gamma^-$ uptake was inhibited by 40 $\mu M$ ClO$_4^-$ . Cells expressing V59E NIS showed no $\Gamma^-$ transport, just like nontransfected COS-7 cells (Fig. 2A). FACS analysis revealed that the transfection efficiency of WT and V59E NIS was similar (data not shown). Cell lysates from transfected COS-7 cells were subjected to SDS-PAGE and immunoblotted with a high-affinity anti-rNIS antibody. NIS expression levels were similar in cells transfected with WT or V59E NIS (Fig. 2B), each expressing the characteristic fully glycosylated mature (~110 kDa) and the partially glycosylated immature polypeptide (~55 kDa). In contrast, no polypeptides were detected in cell lysates from nontransfected COS-7 cells, which do not express endogenous NIS. The endogenously expressed α-subunit of the Na$^+$/K$^+$ ATPase was used as a loading control (Fig. 2B).

**V59E NIS is properly targeted to the plasma membrane**

Whereas the lack of $\Gamma^-$ transport mediated by the expressed V59E NIS protein (Fig. 2) suggested that it is inherently incapable of transporting $\Gamma^-$, it was also possible that V59E NIS was not targeted to the plasma membrane. Indeed, previous analysis of other ITD-causing NIS mutants revealed a lack of $\Gamma^-$ transport due to improper targeting of the mutant NIS proteins to the cell surface (20). Therefore, we assessed the presence of V59E NIS in the plasma membrane by cell surface biotinylation. Transfected COS-7 cells were treated with sulfo-NHS-SS-biotin, a membrane-impermeable biotin reagent that covalently interacts with extracellular primary amines. Immunoblot analysis of the biotinylated cell surface proteins in cells transfected with WT NIS showed a maturely glycosylated NIS polypeptide, represented by a broad band at about 110 kDa, indicating that, as expected, WT NIS is properly processed and targeted to the cell surface, where it mediates $\Gamma^-$ uptake. V59E NIS was also properly targeted to the plasma membrane. The endogenously expressed α-subunit of the Na$^+$/K$^+$ ATPase was used as a loading control. In contrast, the soluble protein tubulin was not biotinylated (data not shown). Therefore, the observed lack of $\Gamma^-$ transport is not due to improper protein maturation or faulty trafficking to the plasma membrane (Fig. 2C).

**The presence of charged amino acids or Pro at position 59 of NIS renders the protein inactive**

One possible explanation for the inactivity of the V59E NIS protein is the presence of the negatively charged side chain of Glu instead of the neutral side chain of Val at position 59. Thus, we substituted Val with other charged amino acids (Asp, Arg, and Lys). In addition, we also evaluated the effect of Pro, an α-helix breaker, at position 59. None of the NIS mutants with a charged residue or Pro at position 59 displayed $\Gamma^-$ transport (Fig. 3A), even though all (except the Pro mutant) were expressed at levels similar to those of WT NIS (Fig. 3B) and all were properly targeted to the plasma membrane, as determined by cell surface biotinylation (Fig. 3C). The endogenously expressed α-subunit of the Na$^+$/K$^+$ ATPase was used as a loading control. These results show that the presence of a charged amino acid or a Pro at position 59 renders NIS inactive.

**Neutral amino acids at position 59 rescue NIS activity**

The neutral amino acids Ala, Asn, Gln, Ile, Leu, Met, and Thr were individually substituted instead of Val at position 59. Each of the resulting NIS mutant proteins displayed $\Gamma^-$ transport at different levels, all significant but lower than those of WT NIS (Fig. 4A). Interestingly, mutants with amino acids at position 59 that had, like Val, branched side chains at the β-carbon (i.e. Ile and Thr) exhibited $\Gamma^-$ transport at levels comparable to those of WT NIS, independently of the
residue side-chain volume. Each of these NIS mutants was normally expressed after transfection into COS-7 cells (Fig. 4B), and each was also correctly targeted to the plasma membrane, as shown by cell surface biotinylation (Fig. 4C). The endogenously expressed α-subunit of the Na⁺/K⁺ ATPase was used as a loading control.

The decreased I⁻ transport in NIS mutants with neutral amino acid substitutions at position 59 is due to a change in \( V_{\text{max}} \). We performed kinetic analyses of those NIS mutants that displayed I⁻ transport (i.e. those containing Ala, Asn, Ile, Leu, Met, Gln, or Thr at position 59) to determine whether or not the change in I⁻ transport with respect to WT NIS was due to a decrease in the apparent affinity of NIS for Na⁺ or I⁻ (as reflected by a change in the respective \( K_m \) values) or to a lower NIS kinetic turnover number (as reflected by a change in the \( V_{\text{max}} \)). Cells were incubated with different concentrations of I⁻ (ranging from 0.6–80 \( \mu \)M) and 140 mM Na⁺ for 2 min to measure the initial rates of I⁻ transport (Fig. 5A). Na⁺-dependent kinetic experiments with the same NIS mutants using Na⁺ concentrations from 0–140 mM and 20 \( \mu \)M I⁻ were also performed to measure initial I⁻ transport rates as a function of the Na⁺ concentration (Fig. 5B). In both cases, each mutant protein exhibited \( K_m \) values similar to those of WT NIS but a lower \( V_{\text{max}} \) (Fig. 5C). Therefore, decreased transport in these mutants results from a lower NIS kinetic turnover.

The V59E/T354P NIS compound mutation is inactive

After demonstrating that V59E NIS is inactive, we decided to experimentally test the compound V59E/T354P NIS mutation because it is expressed in the three Japanese patients. To properly assess I⁻ uptake mediated by V59E/T354P NIS in relation to that mediated by WT NIS, we cotransfected 2 \( \mu \)g V59E and 2 \( \mu \)g T354P NIS cDNA into COS-7 cells and compared it with transport in cells transfected with 2 or 4 \( \mu \)g WT, V59E, or T354P NIS cDNA. In contrast to the results from Fujiwara et al. (10), who reported that V59E/T354P NIS exhibited approximately 20% of WT NIS I⁻ transport levels, we observed no I⁻ accumulation mediated by the compound mutant. Transfection ef-
Fig. 5. A, I⁻-dependent kinetic assays. cDNA constructs encoding WT NIS or NIS with amino acids at position 59 that were conducive to activity (V59A, I, L, M, N, Q, and T) were assayed for initial I⁻ transport rates (2 min) as a function of different I⁻ concentrations, ranging from 0.6125–80 μM, at a constant Na⁺ concentration of 140 mM Na⁺. Vmax and Km values were calculated with the equation v([I⁻]) = (Vmax×[I⁻]/Km + [I⁻]) + A×[I⁻] + B and the gnuplot program. B, Na⁺-dependent kinetic assays. Cells were assayed to determine the initial rates of I⁻ transport as a function of the external [Na⁺]. Cells were assayed for initial I⁻ transport rates (2 min) as a function of different Na⁺ concentrations, ranging from 0–140 mM, at a constant I⁻ concentration of 20 μM. Results were analyzed using the gnuplot program and applied to the equation v([Na⁺]) = (Vmax×[Na⁺]/[Km] + [Na⁺]) – A×[Na⁺] + B to determine Vmax and Km. C, Average Km (I⁻ and Na⁺) and Vmax (I⁻ and Na⁺) values as determined by kinetic analysis. The table lists the average Km and Vmax values from six I⁻-dependent kinetic assays (left) and six Na⁺-dependent kinetic assays (middle); in each experiment, activity was analyzed in triplicate. On the right, the position of the amino acid side chain of each amino acid is listed. The variation in Vmax and the lack of significant variation of Km values indicate that the decreased levels of I⁻ accumulation are not due to a change in the apparent affinity of the mutant NIS proteins for Na⁺ or I⁻. Interestingly, the amino acids that are branched at the β-carbon (such as Ile or Thr), i.e. equal to Val, accumulated the highest levels of I⁻. Graphs in A and B correspond to a representative experiment.

Discussion

The V59E NIS mutation was identified in three siblings in Japan (10, 24). The patients were heterozygous for both the T354P NIS mutation (inherited from their mother) and the V59E NIS mutation (inherited from their father). The three siblings had ITD, whereas their parents were healthy, compatible with the autosomal recessive character of ITD. Fujisawa et al. (10) cotransfected HEK293 cells with cDNA constructs encoding V59E and T354P NIS and reported 20% of WT NIS I⁻ transport in these cells, whereas HEK293 cells expressing V59E NIS transported almost 30% of WT NIS I⁻ transport. However, in the absence of I⁻ transport experiments in nontransfected cells and without a study of the effect of ClO₄⁻ in NIS-transfected cells, it is not possible to discriminate between NIS-mediated I⁻ uptake and nonspecific I⁻ binding and/or transport. In addition, the levels of expression of the different NIS proteins were not determined. Without this information, it is not feasible to accurately as-

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and determined that it is intrinsically inactive. Based on previous studies of some ITD-causing mutations, possible explanations for the inactivity caused by the replacement of Val with Glu include an inability to properly process and/or target the mutant protein to the plasma membrane (20) or a decreased ability to catalyze I\(^-\) transport owing to the difference in size and/or charge of the amino acid replacement (19, 21, 22); we investigated each of these possibilities.

Here we present a thorough analysis of the V59E NIS mutation and report that it renders NIS inactive because of the presence of the charged amino acid Glu, despite proper protein expression and plasma membrane targeting (Fig. 2). Furthermore, neutral amino acids branched at the \(\beta\)-carbon (Val, Ile, and Thr) and Ala were most conducive to NIS transport, compared with the other neutral amino acids that were substituted (Leu, Met, Gln, and Asn) (Fig. 4), whereas the presence of a charged amino acid prevented NIS activity, as did Pro (this result is most likely due to a bend in \(\alpha\)-helix formation) (Fig. 3). Lastly, we determined that the reduction in \(\Gamma\) transport observed in the mutant NIS molecules that are capable of transporting \(\Gamma\) (V59A, I, L, M, N, Q, and T) was due to a decrease in NIS kinetic turnover, as opposed to a change in the apparent affinity of the transporter for Na\(^+\) or \(\Gamma\) (Fig. 5).

According to our proposed secondary structure model (Fig. 1) (27), V59 is located in TMS II, close to the interface with the cytosol. The best explanation for our experimental data is that V59 is involved in intramembrane helix-helix contact. Given that all NIS mutants with substitutions at position 59 reached the cell surface, we conclude that charged residues at this position do not seem to affect correct folding and targeting; however, charged residues rendered NIS inactive, suggesting that accommodation of a charge of either polarity requires a large realignment of the two contacting helices so as to allow the charge to be compensated by a mobile charge at the membrane-cytosol interface. Replacements of V59 with neutral amino acids resulted in different levels of NIS activity, except for Pro, which rendered the protein inactive, most likely due to a disruption in \(\alpha\)-helix formation (28). On the other hand, the activity elicited by the other neutral residue substitutions cannot be explained on the basis of their helical propensity properties, which relate to the relative stability of helices having different amino acids at a particular position, given that Met has long been recognized as a strong helix former (30). In general terms, small (Ala) and \(\beta\)-branched (Val, Ile, and Thr) residues led to higher NIS activity, probably because they allow the WT orientation of the helices to be preserved, whereas larger residues (Leu, Met, Gln, and Asn), although still yielding active proteins, led to lower NIS activity, probably because a small rearrangement of the two helices involved. Elucidation of the three-dimensional structure of NIS would clarify the nature of the proposed helix-helix interactions.

The question remains as to how to explain the marked differences among the three siblings in phenotype and their improvement in thyroid function and clinical condition after treatment with KI. Fujiwara et al. (10) implicitly argue that when NIS function is diminished, excess \(\Gamma\) supply could compensate for the decreased transport capacity of NIS, and that differences in dietary \(\Gamma\) content could account for the
variability in phenotypes as observed in the three siblings (10). Their statement that NIS activity was not completely abolished is, as stated above, not compatible with the saliva/plasma I - concentrations of approximately 1, as found in all three patients. The clinical diagnosis of absence of NIS-mediated I - uptake, experimentally proven by the complete lack of I - transport by the same NIS mutants when transfected into COS-7 cells, leaves no other conclusion than that other mechanisms of I - entry into the thyroid must be present. The KI treatment that the siblings received (14 mg KI/d) is approximately 100 times the World Health Organization-recommended daily dietary I - supply (150 µg/d) (30). In addition, Toyoshima et al. (24) stated that the Japanese diet has a median daily I - content of 1.5 mg/d (24). Assuming a distribution volume of I - of about 2.5 liters in children, the plasma I - concentration in the siblings would have been approximately 164 µM, compared with 0.88 µM in adults in Western countries. It cannot be ruled out that at those high plasma I - concentrations, other channels or transporters such as Cl - channels or cystic fibrosis transmembrane conductance regulator (CFTR) may transport I - into the thyroid (31, 32). Even then, it is remarkable that this bypass apparently leads to sufficient thyroid hormone production and physical development in the absence of NIS.

Toyoshima et al. (24) reported that the mother of the three siblings consumed large quantities of laminaria, a dried kelp that contains 302 mg I /100 g. Interestingly, there was a strong correlation between the severity of the ITD symptoms in the three siblings and their feeding habits during infancy. The two oldest siblings exhibited symptoms of ITD that were significantly less severe than those of the youngest sibling. The oldest sibling was fed only the mother’s milk, which contained I - (because NIS is expressed in the lactating mammary gland) (6), and had the least severe ITD symptoms. The middle sibling was fed a combination of the mother’s milk and cow’s milk and exhibited symptoms less severe than those of the youngest sibling, who was fed only cow’s milk. Interestingly, it seems that the large quantities of laminaria consumed by the mother provided the two oldest children enough I - to produce T 3 and T 4 as did the 14-mg doses of KI that were taken later in life.

Our studies show that V59E NIS is inactive (as is T354P NIS). The ITD symptoms observed in the siblings were ameliorated after treatment with high doses of I - , first with the two older siblings whose cases of ITD were mild because of high I - concentrations in their diet as infants and later in life to the 14 mg KI treatment, suggesting that when plasma I - levels are extremely high, I - enters the thyroid by way of other channels or transporters.

In summary, we have thoroughly analyzed V59E NIS and V59E/T354P NIS and determined that the presence of a charged residue at position 59 in NIS completely impairs I - transport. We determined that the structural requirement at that position is a branched residue to allow proper helix-helix contact and the correct NIS kinetic turnover number. The patients’ clinical data published by Toyoshima et al. (24) indicate that the severity of the symptoms in each child was related to whether or not and for how long they were breastfed. This observation underscores the importance of providing adequate amounts of I - to infants, whether in maternal milk or formula, to ensure normal physical and mental development.

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