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Identification of a structural requirement for thyroid Na^+/I^- symporter (NIS) function from analysis of a mutation that causes human congenital hypothyroidism

Orlie Levy, Christopher S. Ginter, Antonio De la Vieja, Daniel Levy, Nancy Carrasco*

Albert Einstein College of Medicine, Department of Molecular Pharmacology, 1300 Morris Park Avenue, Bronx, NY 10461, USA

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Abstract Patients with congenital lack of I^- transport do not accumulate I^- in their thyroids, often resulting in severe hypothyroidism. A single amino acid substitution in the thyroid Na^+/I^- symporter (NIS), proline replacing threonine at position 354 (T354P), was recently identified as the cause of this condition in two independent patients [1,2]. Here we report that the lack of I^- transport activity in T354P NIS generated by site-directed mutagenesis, is not due to a structural change induced by proline, but rather to the absence of a hydroxyl group at the β-carbon of the amino acid residue at position 354. Hence, this hydroxyl group is essential for NIS function.

Key words: Na^+/I^- symporter; Congenital hypothyroidism; Iodide; Transport; Thyroid

1. Introduction

Iodide uptake into thyroid cells, the first step in the biosynthesis of the iodide-containing thyroid hormones T_3 and T_4, is mediated by the Na^+/I^- symporter (NIS). The cDNA encoding NIS was cloned in our laboratory [3,4], leading to numerous significant advances in NIS characterization (recently reviewed in [5,6]). Several cases of the rare genetic condition known as 'iodide trapping defect' or 'congenital lack of I^- transport' have been documented over the years [7,8]. Diagnosis is usually made in patients who exhibit coexistence of goiter with congenital hypothyroidism, low or absent thyroidal I^- uptake, and little or no I^- uptake by the salivary glands and gastric mucosa [7,8]. The availability of the rat and human NIS cDNAs [3,9] has made it possible for the first time to examine the molecular basis of this defect, and an identical mutation in NIS has recently been reported in two different patients. Fujiwara et al. [1] identified a homozygous missense mutation in a hypothyroid patient with an I^- transport defect. They found Pro instead of Thr at position 354 (T354P). Transfection of the mutant T354P NIS cDNA into human embryonic kidney (HEK)-293 cells did not elicit any I^- uptake activity. Hence, on this basis, the authors proposed that T354P probably disrupts, through a structural change, the ninth putative transmembrane helix of NIS, where the amino acid substitution is located.

More recently, Matsuda et al. [2] reported another case of an I^- transport defect with a loss of function mutation in the NIS gene. Strikingly, as noted above, this mutation is exactly the same as that in Fujiwara's report, i.e. T354P. The patient was euthyroid, apparently due to an extremely high I^- dietary intake, but he exhibited greatly increased NIS mRNA levels in his thyroid, and a slightly increased level of thyroid stimulating hormone (TSH) at the time of the open thyroid biopsy. This patient was born from a consanguineous marriage, and his daughter was heterozygous for the mutation but had no abnormal phenotype, suggesting the recessive nature of the disease. It is of considerable interest to compare these two cases. First, the comparison illustrates the variety of phenotypes that can be found for this condition, as one of the patients was hypothyroid and the other largely euthyroid. Second, whereas Fujiwara et al. [1] observed no I^- transport activity when they transfected the cDNA bearing the T354P mutation into HEK-293 cells, Matsuda et al. [2] reported a low but measurable and potentially significant level of activity (0.4% with respect to wild-type NIS). These authors proposed that the overexpression of NIS transcript in this patient's thyroid may result in a compensatory mechanism whereby the patient's intake of extremely large amounts of I^- produces a euthyroid phenotype. Moreover, both patients come from the same region in Japan, and thus the possibility that they share a common ancestor deserves to be explored.

NIS protein levels were not measured in either study, presumably due to the unavailability of anti-NIS antibodies (Abs) or enough thyroid tissue. Therefore, the mechanism by which these NIS mutations result in a loss of I^- uptake remained unknown. Hence, to characterize this transport defect at the molecular level, we sought to determine whether T354P NIS is a non-functional but stable polypeptide properly targeted to the plasma membrane, or a fully or partially functional protein that is retained in intracellular organelles as a result of the mutation, as is the case with some mutations in CFTR [10] and SGLT-1 [11] transporters.

2. Materials and methods

2.1. Site-directed mutagenesis

The following individual mutagenic oligonucleotides were generated to make the following substitutions at the threonine-354 position: T354P: CACCCTCAGCCCTGCATCCACC; T354G: CACCCTCAGCGGTGCATCCACC; T354A: CACCCTCAGCGCTGCATCCACC; T354Y: CACCCTCAGCTACGCATCCACC; T354S: CACCCTCAGCTCCGCATCCACC; T354C: CACCCTCAGCTGTGCATCCACC. 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 PCR extension with primers complementary to the 5′ end. Fragments with the mutant sequences were obtained by digesting the final PCR products with the appropriate unique restriction enzymes that would yield the smallest mutant fragments. These fragments were ligated into wild-type NIS cDNA and the mutant inserts were sequenced past their respective cloning sites.

*Corresponding author. Fax: (1) (718) 430 8922.
E-mail: carrasco@aecom.yu.edu

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2.2. Transfection of COS cells with NIS cDNA

COS cells were cultured and transfected as previously reported [3]. Briefly, COS cells were transfected by the DEAE-Dextran method with 3 μg/10-cm plate NIS cDNA in pSV.SPORT (Gibco-BRL) and two days post-transfection were assayed for iodide uptake as previously reported [3], and by immunoblot analysis.

2.3. Membrane preparations from NIS-expressing COS cells

Membranes from COS cells (10-cm plate) were isolated with protease inhibitors as described [12], except that after the centrifugation to remove nuclei the supernatant was incubated with 0.1 M Na_2C0_3, pH 11.2, for 45 min shaking at 4°C prior to high speed centrifugation at $100\,000 \times g$ to pellet membranes. Protein determination was performed as described [12].

2.4. Immunoblot analysis

SDS-9% PAGE and electroblotting to nitrocellulose were performed as previously described [12]. All samples were diluted 1:2 with sample buffer and heated at 37°C for 30 min prior to electrophoresis. Immunoblot analyses were also carried out as described [12], with affinity purified anti-NIS Ab at 3 μg/blot and a 1:1500 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham). Both incubations were performed for 1 h. Proteins were visualized by enhanced chemiluminescence (ECL) Western blot detection system (Amersham).

2.5. Immunofluorescence of COS cells expressing T354 NIS substitutions

COS cells were seeded onto polylysine coated cover slips one day after transfection (~50%). On the second day after transfection cells were fixed with 4% paraformaldehyde for 10 min, then washed 3 times with PBS. Cells were permeabilized with methanol for 5 min and washed 3 times with PBS. Cells were then blocked with 4% BSA/PBS for 1 h and subsequently incubated with affinity purified anti-NIS Ab (6 μg/μl) at 1:500 dilution in 4% BSA/PBS for 1 h. Cells were washed three times for 5 min with 0.2% Tween-20 in PBS and once with PBS only. Cover slips were then transferred onto 150 μl PBS containing goat anti-rabbit fluorescein conjugated secondary Ab (1:100 dilution) (Pierce) for 1 h in the dark and then washed as above. Cover slips were air-dried for 3 min and were then mounted onto slides containing 15 μl PBS in 50% glycerol with phenylenediamine. Cover slips were sealed with quick dry nail-polish and allowed to dry in the dark for 2 h at room temperature and then stored at 4°C in the dark. Cells were visualized in a BioRad MRC600 Laser Scanning confocal microscope equipped with a 60× oil immersion objective lens. Epifluorescent and fluorescent video images were collected, enhanced and analyzed using graphics software.

3. Results and discussion

3.1. Characterization of activity and expression of T354P in transfected COS cells

Having recently obtained a high affinity anti-NIS Ab against (rat) NIS [12], we decided to generate T354P (rat) NIS by site-directed mutagenesis and use the Ab to monitor T354P expression in COS cells. COS cells transfected with T354P NIS cDNA were assayed for I^- uptake activity as described [3], and found to display no I^- accumulation (Fig. 1A). Thus, these cells were indistinguishable from control non-transfected COS cells (Fig. 1A), which lack an endogenous I^- accumulating system [3,4]. In contrast, COS cells expressing wild-type (WT) NIS accumulated ~22 pmol I^-/μg DNA, which was inhibitable by perchlorate (ClO_4^-) (Fig. 1A, [3]). These data are consistent with the reported lack of I^- uptake in both HEK and COS cells expressing human T354P cDNA [1,2]. To assess whether the lack of I^- uptake activity in cells transfected with T354P cDNA was due to the absence of T354P expression, membranes from transfected COS cells were analyzed by immunoblot analysis using anti-NIS Ab. As we have previously shown [12], anti-NIS Ab recognizes 65-kDa and 90-kDa NIS polypeptides (differing from each other only in the degree of *N*-linked glycosylation) in COS cells expressing wild-type NIS. Remarkably, the levels of T354P observed upon immunoblot analysis of membranes from T354P-transfected COS cells were virtually identical to wild-type NIS (Fig. 1B, compare WT NIS to T354P). Therefore, T354P generated by site-directed mutagenesis is expressed in transfected COS cells, even though these cells exhibit no I^- transport activity. To determine whether T354P was properly targeted to the plasma membrane, we analyzed by indirect immunofluorescence with anti-NIS Ab both T354P and wild-type transfected cells. Comparable plasma membrane-associated staining was observed in both wild-type and T354P transfected cells (Fig. 1C, panel E). Staining in both wild-type and T354P transfected cells was absent when cells were not permeabilized (Fig. 1C, panels A and D), and also in permeabilized cells incubated with excess C-terminal peptide

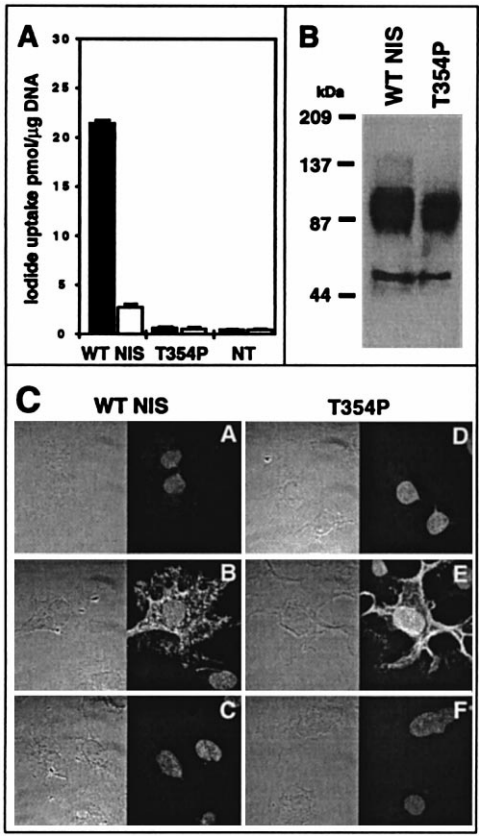

Fig. 1. Characterization of activity and expression of T354P NIS in transfected COS cells. A: Non-transfected COS cells (NT) or COS cells transfected either with WT or T354P NIS were assayed for iodide transport activity as reported [3] two days after transfection. Assays were performed in the presence of Na^+ (solid bars) and Na^+ plus perchlorate (clear bars). Shown are triplicate determinations of iodide uptake at the 45-min time point of a representative experiment ($n = 6$). B: Membranes were isolated from transfected COS cells, 20 μg were electrophoresed on SDS-9% PAGE and electrotransferred and immunoblotted with anti-NIS Ab as described previously [12]. C: Immunofluorescence of WT and T354P NIS transfected COS cells was performed as described in Section 2. All coverslips were incubated with anti-NIS and fluorescein conjugated goat anti-rabbit Abs. Top panels (A), non-permeabilized cells; middle panels (B), cells permeabilized with methanol; and bottom panels (C), cells permeabilized with methanol and incubated in the presence of C-terminal peptide (100 μg).

against which the Ab was raised (Fig. 1C, panels C and F), confirming that T354P is properly targeted to the plasma membrane.

3.2. Effect of other T354 substitutions on NIS expression and activity in COS cells

As indicated above, Fujiwara et al. [1] have suggested that the presence of Pro in place of Thr at position 354 induces a kink in the ninth putative transmembrane helix of NIS, thus conceivably explaining the lack of activity. To test this notion, we examined the possible role played by amino acids that stabilize or destabilize the α-helix. By site-directed mutagenesis we alternatively placed an Ala (an α-helix-stabilizer residue) or a Gly (an α-helix destabilizer residue) at position 354 instead of the original Thr. Interestingly, either substitution yielded a normally expressed (Fig. 2B) but non-functional NIS protein (Fig. 2A), indicating that the major factor causing the loss of function is the absence of Thr itself, rather than any structural effect on the α-helix that might result from the presence of either Pro or Gly at position 354.

This observation raised the possibility that, more specifically, the hydroxyl group of Thr is important for function, and that its loss renders the protein non-functional. To examine this hypothesis, we alternatively substituted T354 with two different hydroxyl-containing amino acids, Tyr and Ser. Like T354P, T354Y was non-functional and normally expressed (Fig. 2A,B), whereas T354S was completely functional and inhibitable by perchlorate, just like wild-type NIS (Fig. 2A,B). Indeed, the position of the hydroxyl group in Ser is far more similar to Thr than Tyr. In both Thr and Ser the hydroxyl group is at the β-carbon of the amino acid, whereas the hydroxyl group in Tyr is at the φ-carbon of the side chain phenyl group and consequently further away from the β-carbon of that amino acid. These data show specifically that the presence of a hydroxyl group in close proximity to the β-carbon of the residue at position 354 is essential for NIS function, a requirement satisfied either by the original Thr or by Ser. Moreover, a non-functional but normally expressed protein resulted when Cys was substituted for Thr at position 354 (T354C) (Fig. 2A,B), even though Cys is isosteric with Ser. The effect of the Cys substitution is due to the presence in this residue of a thiol (SH) instead of a hydroxyl group next to the β-carbon, underlining the significance of the hydroxyl group close to the β-carbon of the 354 residue for NIS function. Furthermore, comparable plasma membrane-associated fluorescence was observed in COS cells expressing all described substitutions at T354, demonstrating that all these T354 mutants are properly targeted to the plasma membrane (Fig. 2C, all B panels).

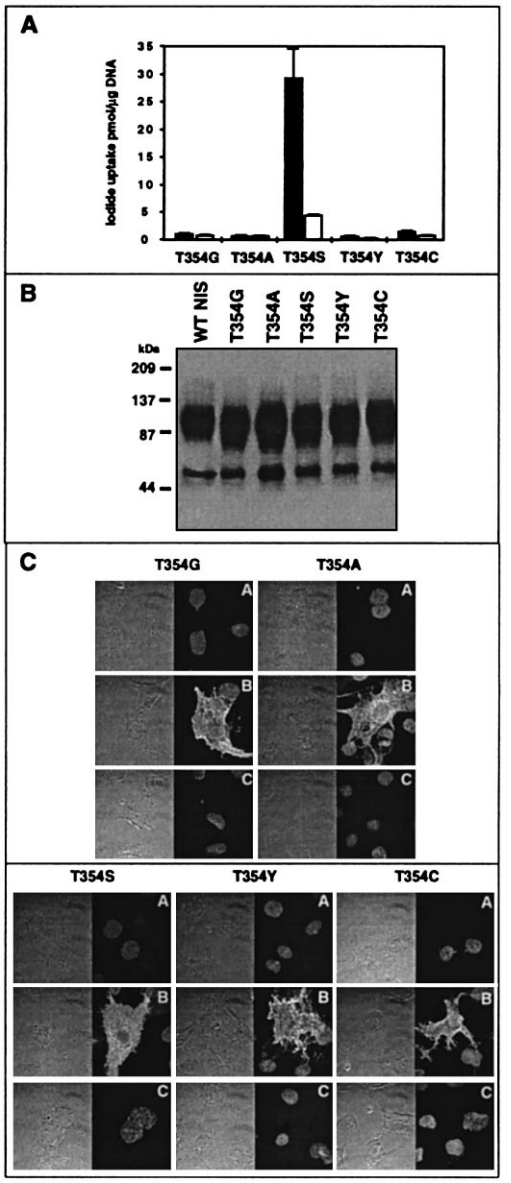

Fig. 2. Effect of other T354 substitutions on NIS expression and activity. A: COS cells were transfected with: lane 1, T354G; lane 2, T354A; lane 3, T354S; lane 4, T354Y; or lane 5, T354C NIS and assayed for iodide transport activity as reported [3] two days after transfection. Assays were performed in the presence of Na^+ (solid bars) and Na^+ plus perchlorate (clear bars). Shown are triplicate determinations of iodide uptake at the 45-min time point ($n=6$). B: Membranes were isolated from transfected COS cells, 20 μg were electrophoresed on SDS-9% PAGE, electrotransferred and immunoblotted with anti-NIS Ab as described previously [12]. C: Immunofluorescence of T354 substitution transfected COS cells was performed as described in Section 2 and as indicated in the legend to Fig. 1C.

3.3. Concluding remarks

In summary, we have determined with an anti-NIS antibody that T354P protein is expressed and properly targeted to the plasma membrane in COS cells transfected with this mutant. Therefore, we predict from these results that patients bearing this mutation properly synthesize and target mutant T354P. It will be of interest to assess levels of T354P protein and its localization in thyroid cells from patients bearing this mutation. We conclude further that the presence of Pro at position 354 does not render the transporter inactive as a result of a structural change in the α-helix, given that the T354A (an α-helix-stabilizer residue) substitution also yields an inactive NIS. Substitution of Thr^{354} with Ser results in fully active NIS, underlining the importance of the hydroxyl side chain at position 354 for NIS activity. More specifically, we have demonstrated that the presence of a hydroxyl group near the β-carbon of the residue at position 354 is essential for NIS function.

It is noteworthy that many serine and threonine residues are present every second or third residue in the putative transmembrane helix 9 of NIS (Fig. 3). The hydroxyl side chain at

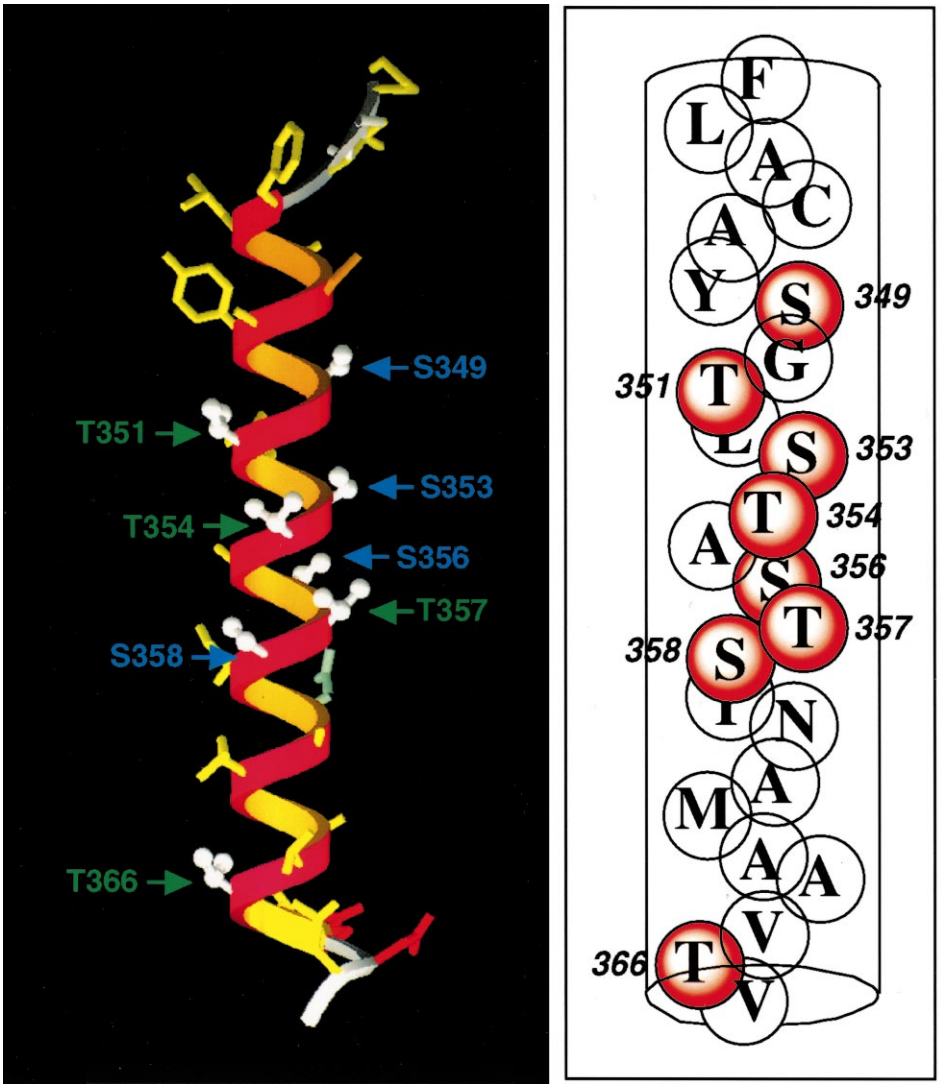

Fig. 3. Detail of putative NIS transmembrane domain nine. Left: α-helix backbone is depicted as a ribbon, Ser and Thr residues are represented in 'ball and stick' form (see arrows). Right: ball model of helix nine, only Ser and Thr residues are shaded. Top of the helices face the extracellular milieu whereas the bottom faces the cytosol.

position 354 could possibly play a role in the translocation of Na^+, and together with the cluster of serine and threonine residues present in helix 9, these hydroxyl groups could be involved in the Na^+ binding, translocation or coupling domains (Fig. 3). Supporting this notion is the fact that T354 is conserved in several Na^+-dependent cotransporters and has been postulated to be in a 'hot spot' for mutations [1]. Another NIS mutation has recently been reported [13], in which a stop codon was identified at position 272 (NIS 272X), resulting in a truncated NIS molecule. However, NIS 272X protein measurements were not performed due to the unavailability of anti-NIS Abs against the first half of the NIS molecule [13]. It is clear that the identification and characterization of other NIS mutations resulting in inactive transporters, combined with continued testing of the NIS secondary structure model, are likely to provide further insight into the precise mechanism of Na^+ and I^- translocation catalyzed by NIS.

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