The iodide-transport-defect-causing mutation R124H: a δ-amino group at position 124 is critical for maturation and trafficking of the Na+/I− symporter

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Summary
Na+/I− symporter (NIS)-mediated active accumulation of I− in thyrocytes is a key step in the biosynthesis of the iodine-containing thyroid hormones T3 and T4. Several NIS mutants have been identified as a cause of congenital I− transport defect (ITD), and their investigation has yielded valuable mechanistic information on NIS. Here we report novel findings derived from the thorough characterization of the ITD-causing mutation R124H, located in the second intracellular loop (IL-2). R124H NIS is incompletely glycosylated and colocalizes with endoplasmic reticulum (ER)-resident protein markers. As a result, R124H NIS is not targeted to the plasma membrane and therefore does not mediate any I− transport in transfected COS-7 cells. Strikingly, however, the mutant is intrinsically active, as revealed by its ability to mediate I− transport in membrane vesicles. Of all the amino acid substitutions we carried out at position 124 (K, D, E, A, W, N and Q), only Gln restored targeting of NIS to the plasma membrane and NIS activity, suggesting a key structural role for the δ-amino group of R124 in the transporter’s maturation and cell surface targeting. Using our NIS homology model based on the structure of the Vibrio parahaemolyticus Na+/galactose symporter, we propose an interaction between the δ-amino group of either R or Q124 and the thiol group of C440, located in IL-6. We conclude that the interaction between IL-2 and IL-6 is critical for the local folding required for NIS maturation and plasma membrane trafficking.

Key words: Congenital iodide transport defect, Na+/I− symporter (NIS), Plasma membrane targeting, Impaired intracellular trafficking, Membrane vesicles

Introduction
The Na+/I− symporter (NIS) is a plasma membrane glycoprotein that mediates the active transport of I− into the thyroid gland and other tissues, such as salivary glands, gastric mucosa, small intestine and lactating breast (Altorjay et al., 2007; Nicola et al., 2009; Nicola et al., 2012; Tazebay et al., 2000; Wapnir et al., 2003). In the thyroid, I− accumulation is the first step in the biosynthesis of the iodine-containing hormones T3 and T4 (triiodothyronine and thyroxine, respectively).

Our group cloned NIS and has since characterized it extensively (Dai et al., 1996; Eskandari et al., 1997; Levy et al., 1997; Levy et al., 1998a; Riedel et al., 2001). Our experimentally tested secondary structure model for NIS shows a protein with 13 transmembrane segments (TMS) (Fig. 1A), an extracellularly facing N-terminus, and an intracellularly facing carboxy terminus (Levy et al., 1997; Levy et al., 1998a). Although NIS is Nglycosylated at three positions (Fig. 1A), glycosylation is not essential for stability, targeting or activity of NIS (Levy et al., 1998a). NIS activity results from coupling the inward translocation of Na+ down its electrochemical gradient to the inward transport of I− against its electrochemical gradient, with an electrogenic 2 Na+:1 I− stoichiometry (Eskandari et al., 1997). Significantly, NIS translocates different substrates with different stoichiometries, as NIS-mediated transport of perchlenate (ReO4−) or the environmental pollutant perchlorate (ClO4−) is electroneutral (1 Na+:1 ReO4−/ClO4−) (Dohán et al., 2007).

Iodide deficiency disorders (IDDs) remain a major health issue around the world as a direct result of insufficient dietary I− intake. Indeed, they are the most common preventable cause of mental retardation. Because I− is very scarce in the environment, normal thyroid physiology relies on adequate dietary I− intake and on proper NIS functioning (Dohán et al., 2003; Zimmermann, 2009). Fourteen cases of congenital hypothyroidism due to an I− transport defect (ITD) have been reported to date (Nicola et al., 2011; Spitzweg and Morris, 2010). ITD is an uncommon autosomal-recessive condition caused by naturally occurring mutations in the SLC5A5 gene, which encodes NIS.

Studies of different ITD-causing NIS mutations have uncovered key structure/function information on NIS (De la Vieja et al., 2004; De la Vieja et al., 2005; De la Vieja et al., 2007; Dohán et al., 2002; Levy et al., 1998b; Paroder-Belenitsky et al., 2011; Reed-Tsur et al., 2008). For instance, the analysis of the T354P mutation revealed that the hydroxyl group at the β-carbon of the residue at position 354 is essential for NIS function and that other...
-hydroxyl–containing residues in TMS IX are involved in Na\(^{+}\) binding/translocation (De la Vieja et al., 2007; Levy et al., 1998b). Similarly, the investigation of the G93R mutation led to the discovery that position 93 in TMS III is critical for substrate selectivity and stoichiometry. Furthermore, changes from an outwardly to an inwardly open conformation during the transport cycle use G93 as a pivot (Paroder-Belenitsky et al., 2011).

Szinnai et al. identified the ITD-causing mutant R124H NIS in a newborn carrying a homozygous transition at nucleotide 718 in exon 2 and reported that, when transiently transfected into COS-7 cells, the R124H NIS protein was inactive, even though it was properly targeted to the plasma membrane (Szinnai et al., 2006). However, we conducted a thorough molecular analysis of the R124H NIS mutant, in which we observed, by immunofluorescence, flow cytometry and cell surface biotinylation, that R124H NIS is retained intracellularly and therefore does not reach the cell surface. Strikingly, R124H NIS is intrinsically active, as determined by I\(^{-}\) transport assays in membrane vesicles. Of Lys, Ala, Trp, Asn, Asp, Glu or Gln, the only amino acid that restored NIS function when engineered at position 124 was Gln, suggesting that the presence of the \(\delta\)-amino group at position 124 is critical for proper NIS maturation and plasma membrane trafficking. Using our NIS homology model based on the structure of the \textit{Vibrio parahaemolyticus} Na\(^{+}\)/galactose symporter (Paroder-Belenitsky et al., 2011), we propose an interaction between the \(\delta\)-amino group of either R- or Q124 and the thiol group of C440. These data underscore the structural role played by residue R124 in bridging together IL-2 and 6, allowing a local folding required for NIS maturation and trafficking to the plasma membrane.

**Results**

**R124H NIS is intracellularly retained**

According to our experimentally tested NIS secondary structure model, Arg-124 is located in IL-2 (Fig. 1A). R124 is conserved
in NIS molecules from all species cloned to date and in 11 out of 12 members of solute carrier family 5 (SLC5A) (see Fig. 2A). COS-7 cells were transfected with wild-type (WT) or R124H NIS cDNA and assayed for I\(^-\) transport activity. At steady state, no perchlorate (ClO\(_4^--\))-inhibitable I\(^-\) accumulation was observed in R124H NIS-transfected cells, in contrast to control WT NIS-expressing cells (Fig. 1B) (Dai et al., 1996; De la Vieja et al., 2004; Dohán et al., 2002; Levy et al., 1997; Levy et al., 1998b). In indirect immunofluorescence experiments, R124H NIS-expressing cells showed intracellular staining, which did not co-localize with the plasma membrane marker Na\(^+\)/K\(^+\) ATPase (Fig. 1C), unlike WT NIS, which partially does (Fig. 1C) (De la Vieja et al., 2004; De la Vieja et al., 2005; Paroder-Belenitsky et al., 2011; Reed-Tsur et al., 2008). Additionally, immunofluorescence performed under non-permeabilized conditions to ascertain NIS expression only at the plasma membrane showed immunoreactivity in cells expressing WT NIS but not in R124H NIS-transfected cells (supplementary material Fig. S1). Consistently with our immunofluorescence data, flow cytometry (FACS) analysis showed that R124H NIS was not targeted to the plasma membrane (Fig. 1D). When FACS was performed under permeabilized conditions to assess total NIS protein expression, we observed positive staining in both WT- and R124H-expressing cells. In contrast, when the experiments were performed in non-permeabilized cells, using an anti-NIS VJ1 Ab directed against an extracellularly facing conformational epitope, positive staining was observed exclusively with WT NIS (Fig. 1D). Cell surface biotinylation showed the reported ~100 kDa mature NIS polypeptide at the plasma membrane (De la Vieja et al., 2005; Reed-Tsur et al., 2008), whereas no biotinylated NIS polypeptides were detected in R124H NIS-transfected cells (Fig. 1E), in agreement with the immunofluorescence and FACS data, indicating that R124H NIS did not reach the plasma membrane. Membrane fractions from cells expressing either WT or R124H NIS were subjected to immunoblot analysis. As reported (De la Vieja et al., 2005), the electrophoretic pattern of WT NIS comprises non-glycosylated (~55 kDa, band A), partially glycosylated (~60 kDa, band B), dimer of the partially glycosylated (~120 kDa, band BB), mature or fully glycosylated (~100 kDa, band C), and dimer of the mature (~200 kDa, band CC) polypeptides. The mature ~100 kDa NIS polypeptide was absent in R124H NIS-expressing cells, suggesting that R124H NIS was not fully processed (Fig. 1F).

**R124 is the only charged residue in IL-2 that is essential for NIS targeting to the cell surface**

Intracellularly facing charged residues have been shown to be important in trafficking of membrane proteins (Kasahara et al., 2004; Li et al., 2012; Wolff et al., 2010). IL-2 contains three basic (R111, R124, R127) and two acid residues (E119, E122). Besides R124, which is highly conserved, the other four of these charged residues are conserved to different extents among members of the SLC5 family (Fig. 2A). To examine the role of these residues, we engineered Ala substitutions at each position. In contrast to the results obtained with R124A, we observed that all of the other NIS mutants were properly targeted to the plasma membrane (Fig. 2B) and active, although to different extents than WT NIS (Fig. 2C). Therefore, R124 is the only charged residue in IL-2 that is critical for proper NIS maturation and plasma membrane expression.

**R124H NIS exhibits incomplete maturation**

We generated permanently transfected MDCK-II cells expressing either WT or R124H NIS. Cells expressing R124H NIS did not transport I\(^-\) (supplementary material Fig. S2A), in contrast to those expressing WT NIS. R124H NIS was expressed at levels comparable to those of WT NIS, even though fewer cells expressed R124H than WT NIS (supplementary material Fig. S2B, total). Clearly, R124H NIS did not reach the plasma membrane, as shown by FACS under non-permeabilized conditions (supplementary material Fig. 2B, PM). Therefore, the results in MDCK-II cells recapitulate those obtained in COS-7 cells (Fig. 1B,D). To investigate the impaired plasma membrane targeting of R124H NIS, we used endoglycosidase...
H (Endo H) treatment. As proteins that have matured beyond the medial Golgi are Endo H resistant, sensitivity to Endo H treatment indicates that a protein has not been processed past the medial-Golgi. Membrane fractions from MDCK-II cells expressing WT or R124H NIS were digested with Endo H and subjected to western blot analysis. The electrophoretic pattern of WT NIS comprises partially glycosylated (~60 kDa, band A) and mature or fully glycosylated (~100 kDa, band B) polypeptides (Fig. 3A) (Levy et al., 1997; Riedel et al., 2001). As observed in COS-7 cells (Fig. 1F), the mature NIS polypeptide was also absent in MDCK-II cells expressing R124H NIS. Endo H treatment caused the disappearance of partially glycosylated polypeptides from both WT and R124H NIS. However, as reported, the mature WT NIS polypeptide was Endo H resistant (Fig. 3A) (De la Vieja et al., 2005), whereas PNGase F treatment of WT NIS resulted in the disappearance of both the fully and partially glycosylated polypeptides, with a concomitant increase in the non-glycosylated polypeptide. Similarly, PNGase F digestion of R124H NIS caused the disappearance of the partially glycosylated band and an increase in the non-glycosylated polypeptide (supplementary material Fig. S3).

These findings show that R124H NIS is retained in intracellular compartments prior to medial-Golgi. To extend these results, we performed co-localization experiments of NIS expression with endoplasmic reticulum (ER) and Golgi resident markers. R124H NIS co-localized with the ER-residents proteins protein disulfide isomerase (PDI) (Fig. 3B) and calnexin (data not shown), but not with the cis-Golgi marker GM130 (Fig. 3C). These results, taken together, support the conclusion that R124H NIS is retained in the ER, that none of it matures beyond the medial-Golgi, and that the protein’s intracellular retention and its consequent failure to reach the cell surface account for the absence of NIS activity in R124H NIS-expressing cells.

**Intracellularly retained R124H NIS is intrinsically active**

In intact cells, I⁻ transport is only observed when NIS is properly targeted to the plasma membrane (Kaminsky et al., 1994). R124H NIS is not active in transfected COS-7 cells ultimately because it is not targeted to the cell surface. However, the lack of I⁻ transport activity in these cells does not necessarily mean that R124H NIS is intrinsically inactive. To examine whether or not R124H NIS is intrinsically functional, we prepared membrane vesicles (MVs) from non-transfected cells or cells expressing either WT or R124H NIS. As expected, MVs from WT NIS-expressing cells exhibited ClO₄⁻-inhibited I⁻ accumulation (De la Vieja et al., 2005). Strikingly, MVs from R124H NIS-expressing cells also displayed ClO₄⁻-sensitive I⁻ transport, albeit at a modest level (Fig. 4), indicating that, although trafficking of R124H NIS to the cell surface is impaired, the protein is functional.

**Gln at position 124 restores NIS activity**

To investigate whether R124H NIS was inactive because of the absence of the permanent positive charge provided by Arg, we engineered R124K NIS. Steady-state I⁻ transport assays performed in transiently transfected COS-7 cells showed that R124K was inactive (Fig. 5A). Like R124H NIS, R124K NIS was well expressed and intracellularly retained, as was evident from FACS analysis (Fig. 5B). Thus, the lack of the positive charge of Arg was not the cause of the impaired maturation of
R124H NIS. We further tested whether negatively charged residues (i.e., Asp and Glu) could restore NIS plasma membrane targeting. R124D and E did not display any significant $\Gamma^-$ uptake (Fig. 5A) and were not targeted to the plasma membrane (Fig. 5B). In addition, the substitution of a large (Trp) neutral residue at position 124 resulted in the absence of $\Gamma^-$ transport and no detectable plasma membrane expression, as assessed by FACS (Fig. 5A,B).

An important feature of the Arg side chain is its terminal $\delta$-guanidinium group. The nitrogen atoms of the guanidinium group are capable of forming hydrogen bonds or salt bridges, which might be important for the proper secondary and/or tertiary structure of NIS (Borders et al., 1994). Therefore, we replaced R124 with Asn or Gln. Like R124H, R124N NIS did not mature or reach the cell surface. In contrast, R124Q NIS, remarkably, did (Fig. 6A; supplementary material Fig. S4). Furthermore, in steady-state $\Gamma^-$ uptake assays, R124Q displayed $\text{ClO}_4^-$-sensitive $\Gamma^-$ transport, although to a lesser extent than WT NIS (Fig. 6B). In kinetic experiments, no significant variation in the $K_m$ for $\Gamma^-$ was observed in cells expressing R124Q with respect to WT NIS [$K_m$(R124Q): $7.2 \pm 2.3 \mu M$; $K_m$(WT): $8.9 \pm 1.6 \mu M$] (Fig. 6C), whereas the $V_{\text{max}}$ was lower in R124Q than in WT NIS [$V_{\text{max}}$(R124Q): $9.9 \pm 0.7 \mu M$; $V_{\text{max}}$(WT): $35.0 \pm 2.9 \mu M$] (Fig. 6C). These results suggest that the $\delta$-amino group of Arg and Gln may play a role in NIS folding. Because the replacement of Arg by Gln preserved NIS targeting to the cell surface, we conclude that a guanidinium group at position 124, rather than a positive charge, is required.

The interaction between R-124 and C-440 may be key for proper NIS folding

The only protein belonging to the SLC5 family whose crystal structure has been determined is the *Vibrio parahaemolyticus* Na+/galactose symporter (vSGLT) (Faham et al., 2008). Using the structure of this protein as a template, we have generated a homology model for NIS (Paroder-Belenitsky et al., 2011). To understand why a $\delta$-amino group is required at position 124 of NIS, we searched for potential interaction partners in our NIS homology model. We observed a possible interaction between the guanidinium group of R124 and the backbones of G434 and L437, and identified C440 as a residue that potentially interacts with the $\delta$-amino group of R- or Q124 NIS (Fig. 7A). To test this hypothesis, we engineered a C440A mutant in the background of WT or R124Q NIS and functionally tested these proteins in COS-7 cells. In steady-state $\Gamma^-$ uptake assays, C440A NIS displayed $\text{ClO}_4^-$-sensitive $\Gamma^-$ transport at the same level as WT NIS (Fig. 7B). However, in contrast to R124Q NIS, the double mutant R124Q/C440A did not exhibit any significant $\Gamma^-$ transport. Unlike C440A, which matured and reached the cell surface, the double mutant R124Q/C440A NIS did not (Fig. 7C). Additionally, MVs generated from transiently transfected COS-7 cells expressing R124Q/C440A did not display detectable $\Gamma^-$ uptake (not shown), indicating that either the protein was intrinsically inactive or its activity was so low as to be undetectable.
Having identified a mutant NIS protein that is intrinsically active but is retained intracellularly (R124H) and a mutant that is both inactive and retained intracellularly (R124Q/C440A), we sought to investigate whether other alterations in the IL-2/IL-6 interaction might result in molecules that are inactive but properly targeted to the plasma membrane. R124Q/C440S NIS turned out to be just such a molecule (Fig. 7B,C), suggesting that the hydrogen bond formed by the thiol group of C440 may be mimicked by S440 to achieve the local conformation required for targeting to the cell surface, but not mimicked closely enough to preserve all the conformations involved in the transport cycle.

**Discussion**

The detailed molecular analysis of several ITD-causing NIS mutants has yielded key mechanistic information on NIS structure/function, such as the following findings: a β-hydroxyl group at residue 354 is essential for NIS function (De la Vieja et al., 2007; Levy et al., 1998b); the presence of a charge or a large side-chain at position 395 interferes with NIS function (Dohan et al., 2002); any charged residue other than Glu at position 267 renders NIS inactive (De la Vieja et al., 2004), and a β-branched residue is required at position 59 for NIS to be active (Reed-Tsur et al., 2008). Furthermore, we have recently reported that position 93 is critical for substrate selectivity and stoichiometry (Paroder-Belenitsky et al., 2011). Five NIS mutants (V59E, G93R, T354P, G395R, and Q267E) have been shown to be fully glycosylated and properly targeted to the plasma membrane (De la Vieja et al., 2004; De la Vieja et al., 2007; Dohan et al., 2002; Levy et al., 1998b; Paroder-Belenitsky et al., 2011; Reed-Tsur et al., 2008). G543E, by contrast, was the first NIS mutant reported to mature only partially and be retained intracellularly rather than being targeted to the cell surface (De la Vieja et al., 2005).

Whereas Szinnai et al. (Szinnai et al., 2006) reported that R124H NIS, when transfected into COS-7 cells, reached the plasma membrane but was inactive, we here conclusively show, instead, that R124H NIS is not expressed at the plasma membrane. Only the immaturely glycosylated ~60 kDa R124H NIS and its ~120 kDa dimer were detected by immunoblot (Fig. 1F, Fig. 3A), species that, upon Endo H treatment, disappeared with a simultaneous increase in the nonglycosylated ~50 kDa species (Fig. 3A). The subcellular localization of R124H was thoroughly investigated by immunofluorescence and FACS analysis. We showed that R124H NIS was detectable as intracellular staining only in permeabilized cells but not in non-permeabilized cells (Fig. 1C,D; supplementary material Figs S1, S2). In addition, cell surface biotinylation experiments demonstrated that no R124H NIS molecules reached the plasma membrane (Fig. 1E). Furthermore, as R124H NIS is Endo H sensitive, this mutant is retained at some point before the medial-Golgi, the site where Endo H resistance is conferred. Co-localization studies conducted by confocal microscopy with intracellular organelle markers established that R124H NIS is primarily retained in the ER (Fig. 3B). Incomplete glycosylation of R124H NIS is unlikely to be the direct cause of the protein’s intracellular retention, given our previous demonstration that glycosylation is not essential for NIS plasma membrane localization or transport activity (Levy et al., 1998a). When all three glycosylated Asp residues were replaced with Gln, non-glycosylated NIS was still targeted to the cell surface and transported I" with a k_m for I" very similar to that of WT NIS (Levy et al., 1998a). Strikingly, when assayed in membrane vesicles, R124H NIS displayed ClO_4^- sensitive I" transport, albeit at lower levels than WT NIS, indicating that R124H NIS, while retained intracellularly in intact cells, remains intrinsically active.

During biosynthesis, integral plasma membrane proteins associate with molecular chaperones that facilitate their folding and keep them in the ER until they reach their proper conformation (Hartl et al., 2011). This quality control mechanism ensures that only properly folded proteins are delivered to their final destinations, thereby retaining and degrading misfolded polypeptides without preventing the export of properly folded proteins. However, several human disease–related mutant proteins with varying degrees of activity are retained in the ER, such as Duox2, CFTR, aquaporin-2, angiotensin I–converting enzyme, the V2 vasopressin receptor, the sulfonylurea receptor, the HERG K+ channel, the
melanocortin-4 receptor and the luteinizing hormone G protein-coupled receptor (Danilov et al., 2010; Grasberger et al., 2007; Li et al., 1993; Meimaridou et al., 2011; Morello et al., 2000; Newton et al., 2011; Partridge et al., 2001; Tamarappoo and Verkman, 1998; Zhou et al., 1999). Most ITD-causing NIS mutants are, like WT NIS, targeted to the plasma membrane. R124H NIS, on the other hand, is the first NIS mutant to be identified that, despite being active, does not leave the ER, causing congenital hypothyroidism.

R124 is located in IL-2 between TMS III and IV (Fig. 1A). Interestingly, R124 is highly conserved among members of solute carrier family 5 (SLC5A), unlike the many other charged residues in this loop, which are conserved to different extents (Fig. 2A). The only charged residue in this loop that proved crucial for targeting of NIS to the plasma membrane was R124 (Fig. 2B). However, a charged residue at position 124 is not a requirement for proper NIS maturation, as Lys, Glu, or Asp at this position did not restore plasma membrane NIS targeting (Fig. 5B). In addition, all of the amino acid substitutions at position 124 (K, D, E, A, W, N and Q), only Gln rescued NIS cell surface targeting and activity (Fig. 6), suggesting a critical role for the δ-amino group of R124. Gln has a δ-amino group that can be oriented to form a hydrogen bond equivalent to that formed by Arg. Indeed, analysis of Arg substitutions in the protein database indicates that Gln is often found in place of Arg, as frequently as Ile in place of Met, Trp in place of Val or Phe, and Thr or Ala in place of Ser. Only Lys in place of Arg is found more often (Henkoff and Henkoff, 1992).

Mutations in other members of the SLC5A family of transporters, such as the Na+/glucose symporters SLC5A1 (SGLT1) and SLC5A2 (SGLT2), also affect protein trafficking (Martin et al., 1997; Martin et al., 1996). However, the mechanisms that regulate the targeting of these transporters to the plasma membrane remain unknown (van den Heuvel et al., 2002; Wright et al., 2011). As previously mentioned, the R124 residue of NIS protein is well conserved among the members of the SLC5A family. Underscoring the importance of this Arg residue, autosomal recessive glucose-galactose malabsorption and renal glucosuria are caused by the inactivating mutations R135W in SGLT1 and R132H in SGLT2, respectively (see Fig. 2A) (Calado et al., 2006; Martin et al., 1996), pointing to a key role for this residue in SLC5A transporters. As in our own observations with R124H NIS, R135W in SGLT1 and R132H in SGLT2, respectively (De la Vieja et al., 2006; Martín et al., 1997), identifying that, despite being active, does not leave the ER, causing congenital hypothyroidism.

Materials and methods

Cell culture and transfections

COS-7 and MDCK-II cell lines obtained from the American Type Culture Collection were cultured in DMEM media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gemini BioProducts, Woodland, CA, USA), 10 mM glucose, 100 U/ml penicillin and 100 µg/ml streptomycin (Collgro, Herndon, VA, USA). Cells were transiently transfected with 4 µg of NIS cDNAs per 10 cm plate using Lipofectamine/Plus reagent (Life Technologies) following the manufacturer’s instructions. Cells were split 24 h after transfection and analyzed by FACs to determine the transfection efficiency (De la Vieja et al., 2005). Uptake activity, immunoblot, and surface biotinylation were assayed 48 h post-transfection.

MDCK-II polyclonal populations expressing WT or R124H NIS were selected and propagated in growth medium containing 1 g/L G418 (Mediatech, Herndon, VA, USA) as described (Paroder et al., 2006).

Site-directed mutagenesis

Generation of R124H, K, D, E, A, W, Q, and N mutants in human NIS cDNA cloned into pSVSport was as described (De la Vieja et al., 2004; De la Vieja et al., 2005; Dohan et al., 2002). Mutagenesis of R111A, R124A and H, R127A, E119A, I122A in human NIS cloned into pcDNA3.1; or R124Q, C440A, S and T in hemagglutinin-tagged human NIS cloned into pcDNA3.1 were introduced using QuickChange™ Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA, USA) as reported (Nicola et al., 2010). All constructs were sequenced to verify specific nucleotide substitutions.

Deglycosylation assays and immunoblotting

Protein deglycosylation was performed as previously described (De la Vieja et al., 2005; Levy et al., 1998a). Briefly, membrane fractions (5 µg) were incubated in glycoprotein denaturing buffer (New England Biolabs, Ipswich, MA, USA) containing 0.5% (w/v) sodium dodecyl sulfate and 40 mM dithiothreitol at 37°C (30 min). Deglycosylation reaction was performed with N-acetylglucosaminidase H (Endo H) (New England Biolabs) in 50 mM sodium citrate (pH 5.5) at 37°C for 2 h. As negative control, membrane vesicles were incubated in the same buffer without enzymee. SDS-PAGE, electrottransference to nitrocellulose membranes and immunoblotting was as described (Levy et al., 1997). Membranes were incubated with 4 µM of an affinity-purified polyclonal anti-human NIS Ab directed against the last 13 residues of the cytosolic NIS carboxyl terminus (Tazebay et al., 2000). Equal loading or purification controls were assessed by stripping and reprobing the same
Cell surface biotinylation
Biotinylation of cell surface proteins was performed as described (De la Vieja et al., 2004; De la Vieja et al., 2005). Briefly, transfected cells were washed with 1 mg/mL of the membrane-impermeable biotin reagent sulfo-NHS-SB-biotin (Pierce Chemical, Rockford, IL, USA), which covalently interacts with extracellular primary amines. Cells were lysed and biotinylated proteins were precipitated overnight with streptavidin-coated beads (Pierce Chemical). Beads were washed and adsorbed proteins were eluted with sample buffer containing 10 mM dithiothreitol at 75°C for 5 min, and analyzed by immunoblot.

Flow cytometry
Paraformaldehyde-fixed cells were incubated in PBS containing 0.2% BSA for non-permeabilized conditions or an additional 0.2% saponin for permeabilized conditions with anti-NIS VII mouse monoclonal Ab (dilution 1:50) directed against an undefined extracellular conformational epitope of NIS (Pohlenz et al., 2000). After washing, cells were incubated with 50 nM of Alexa-488-conjugated goat anti-mouse antibody (Life Technologies). The fluorescence of 10^6 cells per tube was assayed in FACSCalibur flow cytometer (BD Bio-sciences, San Jose, CA, USA).

Immunofluorescence and confocal microscopy
Cells were stained using indirect immunofluorescence procedures (Paroder et al., 2000). After washing, cells were incubated with 50 nM of Alexa-488-conjugated goat anti-mouse antibody (Life Technologies). The fluorescence of 10^6 cells per tube was assayed in FACSCalibur flow cytometer (BD Bio-sciences, San Jose, CA, USA).

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