The Na\(^{+}\)/I\(^{-}\) symporter (NIS), a 618-amino acid membrane glycoprotein that catalyzes the active accumulation of I\(^{-}\) into thyroid cells, was identified and characterized at the molecular level in our laboratory (Dai, G., Levy, O., and Carrasco, N. (1996) Nature 379, 458–460). Because mature NIS is highly glycosylated, it migrates in SDS-polyacrylamide gel electrophoresis as a broad polypeptide of higher molecular mass (90–110 kDa) than nonglycosylated NIS (50 kDa). Using site-directed mutagenesis, we substituted both separately and simultaneously the asparagine residues in all three putative N-linked glycosylation consensus sequences of NIS with glutamine and assessed the effects of the mutations on function and stability of NIS in COS cells. All mutants were active and displayed 50–90% of wild-type NIS activity, including the completely nonglycosylated triple mutant. This demonstrates that to a considerable extent, function and stability of NIS are preserved in the partial or even total absence of N-linked glycosylation. We also found that Asn\(^{225}\) is glycosylated, thus proving that the mentioned addition to demonstrating that the NH\(_{2}\) terminus faces extracellularly as well. A new secondary structure model consistent with these findings is proposed.

The uptake of I\(^{-}\) into thyroid cells, an essential step in the biosynthesis of thyroid hormones T\(_{3}\) and T\(_{4}\), is mediated by the Na\(^{+}\)/I\(^{-}\) symporter (NIS) (2).\(^{\dagger}\) NIS-catalyzed I\(^{-}\) accumulation is a Na\(^{+}\)-dependent active transport process that couples the energy released by the inward translocation of Na\(^{+}\) down its electrochemical gradient to driving the simultaneous inward translocation of I\(^{-}\) against its electrochemical gradient (2). The Na\(^{+}\) gradient acting as the driving force for I\(^{-}\) accumulation is maintained by the Na\(^{+}\)/K\(^{+}\) ATPase (2).

Using expression cloning in Xenopus laevis oocytes, we isolated the cDNA that encodes rat NIS, a highly hydrophobic 618-amino acid glycoprotein (1, 3). Advances in the characterization of NIS have recently been reviewed (4, 5). A thorough electrophysiological analysis of NIS activity was carried out in X. laevis oocytes injected with NIS cRNA, recording both pre-steady and steady state currents (6). The obtained electrophysiological and kinetic data are consistent with a mechanistic model in which Na\(^{+}\) binding is followed by I\(^{-}\) binding in an ordered fashion. Simultaneous recordings of ion fluxes and currents revealed a stoichiometry of 2 Na\(^{+}\)/I\(^{-}\} (6). A secondary structure model was proposed on the basis of hydrophathy analysis and secondary structure algorithms (1, 3). NIS was predicted to traverse the membrane 12 times and to have both the NH\(_{2}\) and COOH termini on the intracellular face of the membrane. A recently generated antibody against the COOH terminus of NIS recognizes a broad 85–110 kDa polypeptide, as visualized by immunoblot analysis of membrane proteins from FRTL-5 cells (a highly active line of rat thyroid-derived cells) (7). Indirect immunofluorescence analysis with anti-NIS Ab in FRTL-5 cells confirmed the cytosolic location of the NIS COOH terminus (7). Additionally, we have shown in rats that NIS protein expression is up-regulated by thyroid-stimulating hormone in vivo. NIS up-regulation was observed in rats with increased thyroid-stimulating hormone circulating levels resulting either from 6-propyl-2-thiouracil treatment or from an I\(^{-}\)-deficient diet (7). Conversely, NIS protein expression was decreased in hypophysectomized rats, which exhibit markedly lower thyroid-stimulating hormone levels. For the first time, mutations in NIS have recently been identified as causative of cases of congenital hypothyroidism (8–11).

We have demonstrated by in vitro peptidyl N-glycosidase F digestion that NIS is highly glycosylated in membranes from FRTL-5 cells, as well as in NIS-expressing X. laevis oocytes and COS cells. Complete N-glycosidase F digestion of mature 87-kDa NIS results in a deglycosylated 50-kDa NIS species (7). Paire et al. (12) have recently confirmed that mature NIS is a highly glycosylated protein in FRTL-5 cells. Two putative N-linked glycosylation sites (Asn\(^{245}\) and Asn\(^{497}\)) are present in the last predicted extracellular loop of NIS, and a third site (Asn\(^{225}\)), is located within the predicted third intracellular loop (1). In the present report we used site-directed mutagenesis to substitute, both separately and simultaneously, the asparagine residues in all three putative N-linked glycosylation consensus sequences of NIS with glutamine and assessed the effects of the mutations on function and stability of NIS in COS cells expressing mutant NIS, as compared with wild-type NIS. In addition to demonstrating that N-linked glycosylation is not essential for activity or stability of NIS, the results also show that Asn\(^{225}\) is glycosylated, thus proving that the mentioned hydrophilic loop that contains Asn\(^{225}\) is on the extracellular face of the membrane. A new secondary structure model consistent with this finding is proposed.
Site-directed Mutagenesis—The following individual mutagenic oligonucleotides were generated to substitute each asparagine residue in the NIS N-linked glycosylation consensus sequences to glutamine: N225Q, CTCGCTCGAACA/TCTCGCGG; N485Q, GGCTGACCA/GATCGTGC; and N497Q, GGAGGCC/ACCA/GCTCTCCAAC. The initial polymerase chain reaction chain extensions were performed using reverse primers complementary to the 3′ end. These fragments were gel purified and used for a second round polymerase chain reaction extension with complementary primers from the 5′ end. Fragments with the mutant sequences were obtained by digesting the final polymerase chain reaction 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. The double and triple mutants were obtained by combining individual mutants using the appropriate endogenous restriction sites.

The Flag epitope mutant was obtained by constructing an oligo: GGAATTCAATTATACCGACAGCGATGACAAATGGAGGGTGCAGAGCGCCCG. This oligo contains, at its 5′ end, a unique restriction site EcoRI followed by a start codon, the nucleotides corresponding to the eight-amin acid Flag sequence (DYKDDDK), and the first 20 bases of the 5′ end of the NIS coding region. A single polymerase chain reaction extension was performed with a reverse primer complementary to NIS. This fragment was digested with EcoRI/BglI and ligated into the same sites in pSV.Sport and wild-type NIS, respectively.

Growth of Cells and Transport Assays—COS cells were cultured and transfected in 10-cm dishes; 1 day after transfection cells were seeded onto six-well dishes where iodide transport activity was measured exactly as reported (1).

Immunoblot Analysis—SDS-9% polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose were performed as described previously (7). All samples were diluted 1:2 with loading buffer and heated at 37 °C for 30 min prior to electrophoresis. Immunoblot analyses were also carried out as described (7) with 2 μg of affinity-purified anti-NIS Ab (7) and a 1:5000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Pharmacia Biotech). Both incubations were performed for 1 h. Polypeptides were visualized by ECL Western blot detection system (Amersham Pharmacia Biotech). Generation of anti-NIS containing sera is described in Ref. 7.

Membrane Preparations from NIS-expressing COS Cells—Transfection of COS cells with NIS cDNA was performed as reported (1). Membranes from COS cells were prepared in the presence of protease inhibitors as described (13).

Peptidyl N-Glycosidase F Treatment—Membranes (40 μg) were resuspended in 10 μl of 0.5% Triton X-100 (pH 8.0) and 18.8 μl of water was added either with 3 μl of N-glycosidase F (600 milliunits, Boehringer Mannheim) or 1.2 μl of 50% glycerol. Membranes were then incubated overnight at 37 °C (18 h). 1.5 μl of 2% of lithium dodecyl sulfate was added whenever N-glycosidase F treatment was carried out in the presence of detergent. After overnight incubation, the reaction was diluted 1:2 with loading buffer (15 μl), and samples were incubated at 37 °C for 30 min prior to electrophoresis (7).

Immunofluorescence and Immunoprecipitation with Anti-NIS Ab—Immunofluorescence was performed as described (7). For in vivo labeling, COS cells were cultured and transfected in 10-cm dishes and seeded 24 h after transfection onto 6-cm dishes. COS cells were metabolically labeled with [35S]methionine/cysteine 48 h after transfection follow immunoprecipitation at the indicated time points with 1.50 dilution of anti-NIS serum (7).

RESULTS

Site-directed Mutagenesis of Putative NIS N-linked Glycosylation Sites—According to our previously proposed NIS secondary structure model there are two putative N-linked glycosylation sites located in the last extracellular loop at Asn485 and Asn497 (7). Aars648 and Asn497 were individually substituted with glutamine by site-directed mutagenesis, thus giving rise to single NIS mutants N485Q and N497Q. COS cells were transiently transfected with either wild-type, N485Q or N497Q NIS cDNA, and were studied 2 days after transfection to analyze NIS expression (Fig. 1A) and activity (see below, Fig. 2). On immunoblots of membranes from transfected COS cells, wild-type NIS appeared both as a partially glycosylated ~59-kDa and a mature glycosylated ~108-kDa polypeptide (Fig. 1A, lane 1). N485Q and N497Q each gave rise to partially glycosylated 55-kDa and mature ~92–95-kDa polypeptides (Fig. 1A, lanes 2 and 3, respectively), indicating that each mutant NIS polypeptide was subjected to partial N-linked glycosylation. Thereafter, both Asn485 and Asn497 were simultaneously substituted with glutamine by site-directed mutagenesis, generating the N485Q/N497Q double mutant. This also yielded a partially glycosylated ~52-kDa polypeptide and, unexpectedly, a mature ~72-kDa polypeptide (Fig. 1A, lane 4), demonstrating that at least one additional N-linked glycosylation site was still functional.

Although NIS contains another putative N-linked glycosylation site at position 225 (see Fig. 5A), this site lies within the predicted third intracellular loop. Because only N-linked consensus sequences located within extracellularly facing protein domains are exposed to N-linked glycosylation enzymes in the endoplasmic reticulum lumen during biosynthesis, N-linked glycosylation sites in intracellular domains are not glycosylated. Hence, to investigate whether Asn225 was glycosylated, notwithstanding its predicted intracellular location, Asn225 was similarly replaced with glutamine, and the resulting N225Q NIS mutant was assessed for expression and activity. Like single mutants N485Q and N497Q, single mutant N225Q yielded a partially glycosylated ~53-kDa and a mature ~82-
kDa NIS polypeptide (Fig. 1A, lane 5). The similarity in electrophoretic mobility between N225Q and the other two single mutants suggests that Asn\textsuperscript{225} is glycosylated. Moreover, these data show that N-linked glycosylation of NIS is not dramatically impeded when a single glycosylation site is removed by mutation, given that under these circumstances two N-linked glycosylation consensus sequences remain available. Thus, the triple mutant N225Q/N485Q/N497Q was generated and assessed as described above, appearing on immunoblots as a single ~50-kDa NIS polypeptide (Fig. 1A, lane 6) that comigrated with wild-type NIS expressed in \textit{Escherichia coli}, where N-linked glycosylation is absent (Fig. 1A, lane 7) (7). This result demonstrates that Asn\textsuperscript{225} is a functional N-linked glycosylation site and that the Asn\textsuperscript{225}-containing hydrophilic loop is therefore located on the extracellular side of the membrane (see secondary structure model of NIS below).

Peptidyl N-linked Deglycosylation of Wild-type and Mutant NIS Expressed in COS Cells—To confirm that the mature mutant NIS polypeptides observed in the immunoblots are indeed glycosylated, membranes from COS cells expressing either wild-type or mutant NIS were treated with N-glycosidase F, an enzyme that cleaves the bond between asparagine and N-acetyl-d-glucosamine, thus releasing N-linked oligosaccharides (Fig. 1B). Deglycosylation of NIS was carried out at a low concentration (0.1%) of lithium dodecyl sulfate to prevent aggregation of NIS, allowing the molecule to unfold completely and have all sites accessible to the enzyme (7). Deglycosylation of single and double mutants yielded ~50-kDa polypeptides (Fig. 1B, lanes 2, 4, 6, 8, 10, and 12), which comigrated with both the triple mutant and NIS expressed in \textit{E. coli} (Fig. 1B, lane 13). Therefore, the observed differences in apparent molecular masses between partially glycosylated mutant NIS polypeptides and the presumed mature forms were because of N-linked glycosylation. Although the predicted molecular mass of NIS is 65.2 kDa, the unglycosylated polypeptide clearly migrated faster (~50 kDa) than its predicted molecular mass, an electrophoretic behavior observed in many other hydrophobic polytopic membrane proteins (14–18).

Iodide Transport Activity of NIS Glycosylation Mutants—To examine the effect of the described mutations on NIS function, iodide transport assays were carried out in COS cells expressing wild-type and mutant NIS 2 days after transfection. Remarkably, all mutants exhibited perchlorate-sensitive NIS activity, at values ranging from 50 to 90% of the activity displayed by wild-type NIS (Fig. 2). Thus, all mutations had only a marginal modulatory effect on NIS activity. The single most significant aspect of these results is that all mutants were active, indicating that to a considerable extent function and targeting of NIS are preserved in the partial or even total absence of N-linked glycosylation. Triple mutant NIS, which is completely nonglycosylated, exhibited ~50% of wild-type NIS activity. This mutant is unquestionably of considerable value for further structure/function study of NIS (see “Discussion”).

Stability and Kinetic Analysis of NIS Triple Mutant—To ascertain whether the lack of N-linked glycosylation has an effect on the stability of NIS, COS cells transfected with triple mutant or wild-type NIS were labeled \textit{in vivo} with \[^{35}\text{S}\]methionine/cysteine for 10 min and subjected to chase periods of up to 56 h in the presence of unlabeled methionine and cysteine. Solubilized \[^{35}\text{S}\]methionine/cysteine-labeled proteins were immunoprecipitated with anti-NIS Ab and subjected to SDS-polyacrylamide gel electrophoresis. Wild-type NIS first appeared in the autoradiogram as a ~56-kDa precursor molecule that was then processed into the mature ~90–100-kDa polypeptide, remaining detectable for 39 h (Fig. 3A, lanes 1–3 and 5–8). When membranes from COS cells expressing wild-type NIS were treated with N-glycosidase F after a 30-min chase, NIS appeared as a ~52-kDa polypeptide in the corresponding autoradiogram (lane 4). This indicates that the ~56-kDa precursor is itself already partially glycosylated and that the ~52-kDa polypeptide species resulting from N-glycosidase F treatment corresponds to fully nonglycosylated NIS. Whereas triple mutant NIS differed markedly from wild-type NIS in that it appeared only as a ~52-kDa polypeptide that was never processed, triple mutant NIS nevertheless also remained detectable for 39 h, thus proving to be nearly as stable as wild-type NIS (Fig. 3A, lanes 9–12; the quantitation of lanes 5–12 is depicted in Fig. 3B). Therefore, it is clear that N-linked glycosylation is not an essential requirement for NIS stability.

Although the above data indicate that N-linked glycosylation is not essential for NIS function, it is of interest to determine whether the decrease in activity resulting from abolished N-linked glycosylation was because of a change in NIS affinity for iodide (\(K_m\)) or in the number of functional NIS molecules present in the membrane (apparent \(V_{max}\)). To examine this issue, a kinetic analysis of the triple mutant was carried out. The effect of varying concentrations of I\(^{-}\) (ranging from 2.5 to 160 \(\mu\)M) on NIS activity was studied in COS cells expressing either triple mutant or wild-type NIS (Fig. 3C). I\(^{-}\) uptake reached saturation at ~40 \(\mu\)M of I\(^{-}\) in COS cells expressing either wild-type or triple mutant NIS. The double reciprocal plot of the same data is presented in Fig. 3D. The calculated \(K_m\) for I\(^{-}\) was virtually identical in wild-type and triple mutant NIS (~41 ± 1.78 and 36 ± 1.32 \(\mu\)M, respectively) in four independent experiments. In contrast, the calculated \(V_{max}\) values were 210 ± 3.98 pmol I\(^{-}\)/\(\mu\)g DNA/4 min for wild-type NIS and 70 ± 2.72 pmol I\(^{-}\)/\(\mu\)g DNA/4 min for triple mutant NIS. Thus, the absence of N-linked glycosylation resulted in a ~3-fold decrease in the \(V_{max}\) without altering the \(K_m\) for I\(^{-}\). These results demonstrate that the reduction in I\(^{-}\) uptake catalyzed by the triple mutant is because of either a decreased number of active NIS molecules
in the plasma membrane or NIS molecules with a higher turn-over number than wild-type NIS rather than to a lower affinity of triple mutant NIS for I⁻.

Secondary Structure Model of NIS—In light of the data presented in Fig. 1, it is clear that Asn²²⁵ faces extracellularly and that our originally proposed secondary structure model of NIS should be revised. According to this original model, NIS was predicted to traverse the membrane 12 times and to have both the NH₂ and COOH termini on the intracellular face of the membrane (1). We have previously shown conclusively, using a site-directed polyclonal Ab against the COOH domain of NIS, that the COOH terminus of NIS faces intracellularly (7). However, our attempts to raise an anti-NH₂ terminus Ab have not been successful. Thus, to test directly whether the NH₂ terminus of NIS is located intracellularly or extracellularly, we used site-directed mutagenesis to engineer a NIS construct bearing a Flag epitope (sequence MDYKDDDDK) at the NH₂ terminus (N-Flag NIS), so that this domain could be detected with anti-Flag Ab.

I⁻ uptake in COS cells expressing N-Flag NIS was indistinguishable from wild-type NIS, indicating that the presence of the Flag sequence does not impair the activity of NIS (Fig. 4A). N-Flag NIS was clearly monitored with anti-Flag Ab by immunoblot analysis of membranes from COS cells expressing N-Flag NIS (Fig. 4B). Most tellingly, immunofluorescence analysis with anti-Flag Ab of COS cells expressing N-Flag NIS revealed immunofluorescence in both nonpermeabilized and permeabilized cells, demonstrating that the NH₂ terminus faces extracellularly (Fig. 4C, panel d). By contrast, immunofluorescence in these cells was present only in permeabilized cells when anti-NIS COOH terminus Ab was used (Fig. 4C, panels a and b), a finding consistent with the known intracellular location of the COOH terminus, as reported previously in FRTL-5 cells (7). Specificity of the immunoreactivity of the anti-Flag Ab was verified by the ability of the N-Flag peptide to competitively prevent immunofluorescence in these cells when added together with anti-Flag Ab (Fig. 4C, panels e and f).

In conclusion, a revision of the NIS secondary structure model should depict both the domain containing Asn²²⁵ and the NH₂ terminus facing extracellularly.

**DISCUSSION**

When the predicted amino acid sequence of NIS was compared with those of other cloned Na⁺/I⁻ cotransporters, the highest degree of homology (24.6% amino acid identity) was found to exist with the human Na⁺/glucose cotransporter, SGLT1 (1, 3). Both the sequence and hydropathy profile comparisons suggest that the membrane topology of NIS is similar to that of SGLT1. As we reported previously in connection with both NIS and SGLT1 (3), the amino acid sequence, hydropathy analysis, and secondary structure algorithms for both of these proteins are actually compatible with three different topological models. In the case of NIS, two of these models propose 12 helices, and one proposes 13 helices. Given the absence of a signal sequence in NIS, we had considered the 13-helix model less likely than the others because it predicts for the NH₂ and COOH termini to be on opposite sides of the membrane (i.e. on the extracellular and intracellular sides, respectively) (3).

In light of the evidence presented here on the glycosylation of Asn²²⁵ (Fig. 1) and the extracellular location of the NH₂ terminus (Fig. 4), we now propose that NIS most likely contains 13 transmembrane helices (Fig. 5B). We predict that the highly
hydrophobic region comprising amino acid residues 389–410 located between the previously proposed helices 9 and 10 forms a new transmembrane helix (Fig. 5A, dotted line). As a result of this change, helices 1–9 all have reversed polarity with respect to the original model, so that both the NH2 terminus and the hydrophilic loop containing Asn 225 are now placed on the extracellular side (Fig. 5B).

It is remarkable that current evidence supports precisely the 13-helix model that we initially regarded as less likely. It must be emphasized that the original model was favored on the basis of widely held expectations on the secondary structure of membrane transporters, such as the assumed link between the absence of a signal sequence and the placement of the NH2 terminus on the cytosolic side of the membrane. Not only does the present study prove that such a link is not always required, but it also leads to the conclusion that currently employed guidelines for assigning the polarity of transmembrane domains in this class of proteins are probably less reliable than is generally believed.

The 13-transmembrane domain model we propose here for NIS is consistent with recent findings reported on SGLT1 using N-linked glycosylation scanning mutagenesis (17). Although SGLT1 was also originally predicted to have 12 transmembrane domains, a new 14-transmembrane domain model has been proposed (17). In this model the first 13 domains of SGLT1 coincide with those we are presently predicting for NIS, with helix 14 corresponding to a highly hydrophobic carboxyl tail, which is not present in NIS. The 14-transmembrane domain SGLT1 model places the NH2 terminus on the extracellular face of the membrane. Our data are consistent with the topological information recently reviewed for the SGLT1 family of transporters (19).

Like NIS, such other transporters as SGLT1 and the GABA transporter have been found to be active in the absence of N-linked glycosylation. Whereas addition of tunicamycin (an inhibitor of the synthesis of N-linked oligosaccharides) to HeLa cells expressing GABA transporter abolishes GABA uptake (20), significant GABA uptake activity is detected in HeLa cells expressing nonglycosylated GABA transporter. Similarly, nonglycosylated SGLT1 is fully functional (17), even though the activity of SGLT1 is inhibited by tunicamycin (21). Our results are at variance with a conclusion reached in a report stating that N-linked glycosylation is essential for NIS folding and stability (12). Paire et al. (12) observed that tunicamycin prevented both the synthesis of mature NIS and the thyroid-stimulating hormone-dependent reinduction of NIS activity in FRTL-5 cells and hence reported that N-linked glycosylation is essential for NIS synthesis, folding, and stability (12). Because tunicamycin inhibits the synthesis of N-linked oligosaccharides and thus prevents N-linked glycosylation of all proteins in the
FIG. 5. Original and current NIS secondary structure models. A, original NIS secondary structure model with 12 putative transmembrane helices (1). Two putative N-linked glycosylation consensus sequences are indicated with bold Qs at positions 485 and 497. A third N-linked glycosylation sequence at position 225 is located in the third predicted intracellular loop. B, current NIS secondary structure model with 13 putative transmembrane helices. Both the hydrophilic loop containing Asn225 and the NH₂ terminus face extracellularly. All three N-linked glycosylation consensus sequences are indicated with bold Qs at positions 225, 485, and 497. Amino acid residues 389–410, originally located between helices 9 and 10 of the model shown in panel A (dotted line), form a new transmembrane domain (helix 10, panel B).
N-linked Glycosylation of the Thyroid Na$^{+}$/I$^{-}$ Symporter

A cell, it is clear that the observed effect of tunicamycin on NIS activity is not necessarily due specifically to the lack of N-linked glycosylation of NIS. The results presented here show rather that function and stability of NIS are considerably preserved in the partial or even total absence of N-linked glycosylation. Fully nonglycosylated triple mutant NIS is highly active, and both its affinity for iodide and its half-life are similar to those of wild-type NIS.

Even though the topology of eukaryotic Na$^{+}$-driven symporters has recently received considerable attention (17–19, 22, 23), relatively few proposed secondary models for these proteins have been unequivocally confirmed experimentally. N-linked glycosylation scanning mutagenesis has proved to be a valuable technique to analyze the topology of SGLT1, GABA (GAT-1), glycine (GLYT1), and glutamate/aspartate (GLAST-1) transporters, albeit with some limitations (Refs. 11, 17, 19, and 20, respectively). This technique is most effective when applied to the study of transporters that are functional even when devoid of carbohydrate moieties. As shown above, we have found that NIS is functional in a deglycosylated state, and therefore it is amenable to a topology analysis by N-linked glycosylation scanning mutagenesis to further probe our present secondary structure model. In the absence of tridimensional structure data, it is crucial to determine the topology of polytopic membrane proteins to design studies aimed at the elucidation of helix packing and understanding the roles played by specific protein domains and individual amino acid residues in the function of these proteins. The current secondary structure model will continue to be experimentally tested by a variety of biochemical, biophysical, and immunological methods with the ultimate goal of elucidating the molecular mechanism of I$^{-}$ transport into thyroid cells.

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