Post-transcriptional Regulation of the Sodium/Iodide Symporter by Thyrotropin

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The Na+/I− symporter (NIS) is an intrinsic plasma membrane glycoprotein that mediates active I− transport in the thyroid gland (Dai, G., Levy, O., and Carrasco, N. (1996) Nature 379, 458–460), the first step in thyroid hormone biogenesis. Whereas relatively little is known about the mechanisms by which thyrotropin (TSH), the major hormonal regulator of thyroid function, regulates NIS activity, post-transcriptional events have been suggested to play a role (Kaminsky, S. M., Levy, O., Salvador, C., Dai, G., and Carrasco, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3789–3793). Here we show that TSH induces de novo NIS biosynthesis and modulates the long NIS half-life (∼5 days). In addition, we demonstrate that TSH is required for NIS targeting to or retention in the plasma membrane. We further show that NIS is a phosphoprotein and that TSH modulates its phosphorylation pattern. These results provide strong evidence of the major role played by post-transcriptional events in the regulation of NIS by TSH. Beyond their inherent interest, it is also of medical significance that these TSH-dependent regulatory mechanisms may be altered in the large proportion of thyroid cancers in which NIS is predominantly expressed in intracellular compartments, instead of being properly targeted to the plasma membrane.

The Na+/I− symporter (NIS) is an intrinsic plasma membrane protein that mediates the active transport of I− in the thyroid and other tissues such as salivary glands, gastric mucosa, and lactating mammary gland (1, 2). NIS is of central significance in thyroid pathophysiology as the route by which I− reaches the gland for thyroid hormone biosynthesis and as a means for diagnostic scintigraphic imaging and for radiiodide therapy in thyroid cancer (3). NIS couples the inward translocation of Na+ down its electrochemical gradient to the simultaneous inward translocation of I− against its electrochemical gradient (4−6) with a 2:1 Na+/I− stoichiometry (6). Cloning and sequencing of the rat NIS cDNA revealed a protein of 618 amino acids (7), which is highly homologous (87% identity) to the subsequently cloned human NIS (8). The current secondary structure model depicts NIS as a protein with 13 transmembrane segments, the amino terminus facing the extracellular side and the carboxyl terminus facing the cytosol, both of which we have demonstrated experimentally (9).

The iodine-containing thyroid hormones triiodothyronine and thyroxine play essential roles in promoting the development and maturation of the nervous system, skeletal muscle, and lungs and in regulating intermediary metabolism in virtually all tissues. Thyroid-stimulating hormone (TSH) is the primary hormonal regulator of thyroid function overall and has long been known to stimulate I− uptake activity in the thyroid (10). No thyroidal I− uptake is detected in humans whose serum TSH levels are suppressed (11). In addition, up-regulation of NIS thyroid expression and I− uptake activity by TSH has been demonstrated in rats in vivo (12), in the rat thyroid-derived FRTL-5 cell line (13), and in human thyroid primary cultures (14, 15). TSH up-regulates I− uptake activity by a cAMP-mediated increase in NIS transcription (13, 16–18). After TSH withdrawal a reduction of both intracellular cAMP levels and I− uptake activity is observed in FRTL-5 cells. This is a reversible process, as I− uptake activity can be restored either by TSH or agents that increase cAMP (13, 18). I− uptake activity surprisingly persists in membrane vesicles (MV) prepared from FRTL-5 cells that, when intact, have completely lost I− uptake activity due to prolonged TSH deprivation (19). This suggests that mechanisms other than transcriptional might also operate to regulate NIS activity in response to TSH.

Here we provide evidence for post-transcriptional regulation of NIS function by TSH. Our results show for the first time that NIS is a phosphoprotein and that the NIS phosphorylation pattern is regulated by TSH. Furthermore, our data indicate that in the absence of TSH, NIS is redistributed from the plasma membrane to intracellular compartments. This suggests that under TSH deprivation, the loss of I− transport activity in FRTL-5 cells is due to NIS intracellular distribution. Interestingly and contrary to expectations, NIS is overexpressed in some thyroid cancers, notwithstanding their decreased I− uptake activity (20, 21). Moreover, overexpressed NIS in these cells is predominantly retained intracellularly. The intracellular NIS redistribution pattern that we observed in FRTL-5 cells maintained in the absence of TSH resembles that reported in thyroid tumors, underscoring the importance of elucidating the mechanisms that govern the subcellular localization of NIS.
**Experimental Procedures**

**Cell Culture—**FRTL-5 rat thyroid cells, kindly provided by Dr. L. D. Kohn (National Institutes of Health, Bethesda, MD), were grown in Ham’s F-12 media (Life Technologies, Inc.) supplemented with 5% calf serum, 1 mM non-essential amino acids (Life Technologies, Inc.), 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and a six-hormone mixture (6H) containing insulin (1.5 μg/ml), hydrocortisone (1 μg/ml), dexamethasone (1 μg/ml), 1,3,5-triiodothyronine (1 μg/ml), l-tyrosine (10 μg/ml) and somatostatin (6.1 nm), and TSH (1 milliunits/ml) as reported previously (22). Cells were grown in a humidified atmosphere with 5% CO2 at 37 °C. To study the effect of TSH deprivation, FRTL-5 cells were kept in the same medium without TSH (5H). FRTL-5 cells are viable in this medium for at least 15 days (23). TSH was obtained from the National Hormone Pituitary Distribution Program.

**Preparation of Membrane Vesicles (MV)—**MV for I- transport were prepared as described previously (19). Briefly, FRTL-5 cells kept in TSH (+) or TSH (−) medium were washed, harvested, and resuspended in ice-cold 250 mM sucrose, 10 mM EGTA, 10 mM Hepes-KOH (pH 7.5), containing aprotinin (90 μg/ml) (Roche Molecular Biochemicals), leupeptin (4 μg/ml) (Roche Molecular Biochemicals), and phenylmethylsulfonyl fluoride (PMSF) (2.5 μM). Cells were disrupted with a motor-driven Teflon pestle homogenizer. The homogenate was centrifuged twice at 500 × g for 15 min at 4 °C, and the supernatant was centrifuged at 100,000 g for 20 min at 4 °C. The pellet was resuspended in ice-cold 250 mM sucrose, 1 mM MgCl2, 10 mM Hepes-KOH (pH 7.5), aliquoted, and stored in liquid nitrogen.

**Metabolic Labeling and Immunoprecipitation—**MV for I- transport assays in intact cells were performed with 90% confluent FRTL-5 cells in 12-well plates that were kept either in 6H or 5H medium (23). Briefly, after aspirating the culture medium, cells were washed two times with 0.5 ml of modified Hanks’ balanced salt solution (HBSS). Cells were incubated with HBSS buffer containing 20 μM Na125I (specific activity 50 Ci/mol) for 45 min at 37 °C in a humidified atmosphere with 5% CO2. Reactions were terminated by aspirating the radioactive solution and washing three times with cold HBSS. Intracellular 125I- was released by permeabilizing the cells with 500 μl of 95% cold ethanol and was quantitated in a gamma-counter. DNA in each well was determined by the diphenylamine method (19). I- uptake was expressed as picomoles of I- per μg of DNA in each well.

**FRTL-5 MV were assayed as described (19). MV were thawed at 37 °C and placed on ice. Aliquots containing 50 μg of protein (10 μl) were assayed for 125I- uptake by incubating at room temperature (RT) with an equal volume (10 μl) of a solution containing 20 μM Na125I (specific activity 50 Ci/mol) for 45 min at 37 °C in a humidified atmosphere with 5% CO2. Reactions were terminated by aspirating the radioactive solution and washing three times with cold HBSS. Intracellular 125I- was released by permeabilizing the cells with 500 μl of 95% cold ethanol and was quantitated in a gamma-counter. DNA in each well was determined by the diphenylamine method (19). I- uptake was expressed as picomoles of I- per μg of DNA in each well.

**Immunoblot Analysis—**SDS-9% polyacrylamide gel electrophoresis and electro blotting to nitrocellulose were performed as described previously (12). 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 (12) with 930 pM of affinity-purified anti-NIS (Ab) and 0.08 μg/ml of PMSF. After lysis, NIS was immunoprecipitated, subjected to electrophoresis, and electroblotted to nitrocellulose. NIS was visualized by autoradiography after 3 h at -70 °C. The NIS band was excised from the nitrocellulose and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (10 μg/ml) (Worthington) in 100 mM NH4HCO3 (pH 8.2), 1 mM CaCl2 for 24 h at 37 °C. Under these conditions, ~60% of the 32P was released from the nitrocellulose.

**Two-dimensional Tryptic Phosphopeptide Mapping of in Vivo Labeled NIS—**The phosphopeptide map was performed as described previously (25). Tryptic phosphopeptides were separated in two dimensions on cellulose thin layer plates by electrophoresis at pH 1.9 for 50 min at 4 °C for the anode and electroblotted to nitrocellulose at pH 3.0 for 30 min at the cathode. The plates were visualized by autoradiography after 3 days at -70 °C.
**RESULTS**

**TSH Differentially Regulates NIS Expression and $\Gamma^-$ Uptake Activity in FRTL-5 Cells**—We measured Na$^+$-dependent, perchlorate-inhibitable (i.e. NIS-mediated) $\Gamma^-$ uptake activity in intact FRTL-5 cells over the course of 10 days after TSH was removed from the culture medium and in MV prepared from these cells (Fig. 1A). MV are a pool of sealed vesicles from all subcellular compartments except the nuclear membrane. As reported previously (19), while $\Gamma^-$ transport activity decreased by 75% in intact cells 3 days after removal of TSH (Fig. 1A, empty bars), $\Gamma^-$ transport activity only decreased by 25% in MV (Fig. 1A, filled bars). By 5 days after TSH withdrawal, $\Gamma^-$ uptake was completely abolished in intact cells, whereas in MV it was still as high as 60% of the initial activity. To determine whether the reduction of $\Gamma^-$ uptake in intact cells was due to a decrease in NIS expression, we subjected MV from these cells to immunoblot analysis with anti-NIS Ab (Fig. 1B), and we monitored the ~85-kDa broad band corresponding to fully glycosylated NIS (12). Although NIS expression decreased by ~50% of its initial level after 3 days of TSH deprivation, NIS expression in MV remained detectable after 7–10 days (Fig. 1B). i.e. even after $\Gamma^-$ uptake in intact cells was completely abolished (Fig. 1A). That $\Gamma^-$ transport activity in MV from TSH-deprived cells persists during the entire time course is consistent with NIS expression in these cells.

**TSH Is Required for de Novo NIS Biosynthesis**—To test whether NIS is synthesized in the absence of TSH, cells that had been deprived of TSH for 5 days were metabolically labeled with 480 μCi/ml $[35S]$methionine/cysteine for 10 min. NIS immunoprecipitation and SDS-polyacrylamide gel electrophoresis analysis showed that de novo biosynthesis of NIS occurred only when cells were maintained in the presence of TSH (Fig. 2). NIS remained detectable for up to 10 days following TSH deprivation (Fig. 1B), i.e. in the absence of de novo NIS biosynthesis (Fig. 2), demonstrating that $\Gamma^-$ uptake observed in MV from TSH-deprived cells is mediated by NIS molecules synthesized prior to TSH removal.

**NIS Half-life Is Modulated by TSH**—The observation that NIS remains detectable after prolonged TSH deprivation in the absence of de novo NIS biosynthesis suggests that NIS has a long half-life. To determine the precise half-life of NIS and whether it is modulated by TSH, cells maintained in the presence of TSH were pulse-labeled with $[35S]$methionine/cysteine for 5 min and chased for different times in the presence (Fig. 3A) or absence of TSH (Fig. 3B). As indicated above, NIS migrates as an ~85-kDa broad band. The ~70-kDa band corresponds to a non-specific unrelated polypeptide that, unlike NIS, was also immunoprecipitated by preimmune serum (not shown). The half-life of NIS was determined to be ~5 days in the presence and ~3 days in the absence of TSH (Fig. 3C). This indicates that TSH modulates the long half-life of NIS, increasing it by 40%.

**TSH Regulates the Subcellular Distribution of NIS**—To assess the effect of TSH on NIS content at the plasma membrane, we performed cell surface biotinylation experiments in the presence of TSH and then over the course of 10 days after TSH was removed from the culture medium. To ensure that only polypeptides facing the extracellular milieu would be biotinylated, we utilized the NIS-specific and plasma membrane-impermeable biotinylating reagent Sulfo-NHS-SS-biotin. The entire biotinylated fraction was isolated with streptavidin-coated beads and was immunoblotted with anti-NIS Ab, whereas only 1:50 of the non-biotinylated fraction was loaded onto the gel (Fig. 4, A and B, respectively). Densitometric quantitation of the bands showed that NIS content at the plasma membrane decreased over time after TSH withdrawal in a fashion that correlated very closely with the corresponding decrease in NIS activity in intact cells (Fig. 4C). While 1 day of TSH deprivation causes a similar decrease in both intracellular and cell surface NIS, by 3 days after TSH withdrawal a more pronounced decrease in NIS content was detected at the plasma membrane than in intracellular compartments (Fig. 4C). This indicates that TSH regulates the subcellular distribution of NIS.

The possible regulatory role played by TSH in the subcellular distribution of NIS was further investigated by confocal immunofluorescence analysis of NIS subcellular localization in response to TSH withdrawal over a 10-day period (Fig. 5). As anti-NIS Ab recognizes a cytosol-facing epitope of NIS (i.e. the
activation and subcellular distribution of several transporters (27–32). NIS has several consensus sites for kinases, including those for cAMP-dependent protein kinase, protein kinase C, and CK-2. Furthermore, TSH actions in the thyroid are mainly mediated by cAMP, raising the possibility that phosphorylation might be involved in the regulation of NIS distribution. FRTL-5 cells were labeled with $^{32}$P, for 5 h and lysed. NIS was immunoprecipitated with anti-NIS Ab, and the immunoprecipitate was subjected to electrophoresis. The autoradiogram revealed that NIS was phosphorylated, independently of the presence of TSH in the culture medium (Fig. 6A). The results were plotted along with the corresponding I$^-$ transport activity values from intact cells (circles). All values were expressed as percentage relative to day 0. Values represent the means ± S.E. of at least three independent experiments.

**FIG. 4.** NIS at the cell surface decreases in close correlation with I$^-$ transport after TSH withdrawal. Cell surface biotinylation experiments were performed in FRTL-5 cells that were kept in the presence or absence of TSH. Cells were biotinylated with Sulfo-NHS-SS-biotin, a membrane-impermeable reagent, and lysed, and biotinylated proteins were separated from non-biotinylated proteins by precipitation with streptavidin. The membrane impermeability of Sulfo-NHS-SS-biotin was verified by demonstrating that the intracellular protein actin was not biotinylated (not shown). All biotinylated proteins were subjected to densitometric analysis (NIH program) for quantitation (TSH(+) and TSH(-) as calculated by the migration coefficient. Two phosphopeptides were resolved in the presence and three in the absence of TSH. Cells were kept in the presence or absence of TSH for 5 days and lysed. NIS was subjected to electrophoresis. The autoradiogram revealed that NIS was phosphorylated, independently of the presence of TSH in the culture medium (Fig. 6A). The results were plotted along with the corresponding I$^-$ transport activity values from intact cells (circles). All values were expressed as percentage relative to day 0. Values represent the means ± S.E. of at least three independent experiments.

**FIG. 3.** NIS has a long half-life, which is modulated by TSH. To determine the half-life of NIS, FRTL-5 cells were pulsed for 5 min with 480 μCi/ml of $[^{35}S]$methionine/cysteine in the presence of TSH. During the chase period an aliquot of cells was maintained in the presence of TSH (A) and a second aliquot kept in the absence of TSH (B). Chase periods are indicated in the horizontal axis. Samples were processed as described in Fig. 2. NIS bands were subjected to densitometric analysis (NIH program) for quantitation (TSH(+) and TSH(-); squares and dotted line). C, inset, scatter plot of NIS half-life from three independent experiments in the presence (+) and absence (-) of TSH. Student’s t test (unpaired) yielded $p < 0.0001$. Data fitting, S.D., and Student’s t test calculations were done with the Prism$^\text{TM}$ 2.0 software (GraphPad, San Diego, CA).

**TSH Modulates NIS Phosphorylation**—The mechanism by which TSH regulates the subcellular distribution of NIS is unknown. Phosphorylation has been shown to be implicated in...
DISCUSSION

The regulation of membrane transport proteins is a highly complex process that takes place at various levels (33–35). Here we show that this is the case for NIS regulation. NIS, being the transporter that mediates the first step (i.e. active I\(^{-}\)) uptake in thyroid hormone biosynthesis, provides a suitable regulatory target for TSH, which is the primary hormonal regulatory factor of thyroid function overall. It has long been clear that TSH stimulates thyroidal I\(^{-}\) uptake by up-regulating NIS transcription via cAMP (13, 17, 18). Our findings provide convincing experimental evidence that TSH also regulates NIS by post-transcriptional mechanisms.

With our high affinity anti-NIS Ab we demonstrated conclusively by immunoblot analysis that NIS is present in FRTL-5 cells as late as 10 days after TSH withdrawal (Fig. 1B) and that de novo NIS biosynthesis requires TSH (Fig. 2). Therefore, it is clear that any NIS molecules detected in TSH(-) FRTL-5 cells had to be synthesized prior to TSH withdrawal. This is consistent with NIS being a protein with an exceptionally long half-life, as suggested previously (17, 36). Indeed, by pulse-chase analysis we determined that NIS half-life is ~5 days in the presence and ~3 days in the absence of TSH (Fig. 3). Even though the NIS half-life in the absence of TSH is 40% shorter than in the presence of the hormone, it is still sufficiently long to account for the persistence of significant I\(^{-}\) uptake activity in MV from cells deprived of TSH (Fig. 1). It was the detection of this vesicular activity that first led to the suggestion that NIS might be regulated post-transcriptionally (19), a notion further supported by several subsequent reports (17, 36). In addition, it has recently been shown (14) that TSH markedly stimulates NIS mRNA and protein levels in both monolayer and follicle-forming human primary culture thyrocytes, whereas significant stimulation of I\(^{-}\) uptake is observed only in follicles, suggesting that NIS may be regulated by such post-transcriptional events as subcellular distribution.

Several transporters are modulated by post-transcriptional regulation of their trafficking to the plasma membrane and/or by internalization from the plasma membrane to intracellular compartments (37, 38). For example, the glucose transporter 4 (GLUT4) (35) is targeted to the plasma membrane in response to insulin, whereas the serotonin transporter is internalized in the presence of its antagonist cocaine (27). Therefore, it seems feasible that regulation of the subcellular distribution of NIS might also be a mechanism involved in modulating I\(^{-}\) uptake. We have shown a remarkably close correlation between NIS plasma membrane content and NIS activity (Fig. 4C), demonstrating that the progressive loss of NIS activity after TSH withdrawal is due to a decrease in the amount of NIS present at the cell surface. Furthermore, we observed that 3 days after TSH deprivation, intracellular NIS decreases at a slower rate than plasma membrane NIS (Fig. 4, A and B). These data support the notion that active NIS molecules, initially located in the plasma membrane while TSH is present, are redistributed to intracellular compartments in response to TSH withdrawal despite the lack of de novo NIS synthesis and the 40% reduction of the NIS half-life. This model explains the presence of NIS activity in MV from cells deprived of TSH that, when intact, exhibit no NIS activity. Clearly, TSH regulates I\(^{-}\) uptake in FRTL-5 cells by promoting NIS expression and by stabilizing mature NIS molecules in the plasma membrane, while de novo NIS synthesis and subcellular redistribution are post-transcriptional mechanisms that regulate I\(^{-}\) uptake in TSH(-) FRTL-5 cells.
uptake by modulating the subcellular distribution of NIS, without apparently influencing the intrinsic functional status of the NIS molecules, as proposed previously (19). In conclusion, TSH not only stimulates NIS transcription and biosynthesis, it is also required for targeting NIS to and/or retaining it at the plasma membrane. Future experiments might distinguish between these two possibilities.

The precise mechanism by which TSH regulates NIS distribution remains to be fully explored. NIS exhibits several consensus sites for the cAMP-dependent protein kinase, protein kinase C, and CK-2 kinases. We have observed that NIS is phosphorylated (Fig. 6A) and that the NIS phosphorylation pattern differs when cells are in the presence as compared to the absence of TSH (Fig. 6B). This demonstrates that TSH modulates NIS phosphorylation. Therefore, given that phosphorylation has been reported to play a role in regulating targeting of other transporters, such as the serotonin (27), phophorylation has been reported to play a role in regulating modulation of NIS phosphorylation. Therefore, given that phosphorylation is required for targeting NIS to and/or retaining it at the plasma membrane, and this decreases I uptake on scintigraphy (11). Conversely, the ability of thyroid radioiodide therapy in these cases.

The multifaceted TSH-NIS regulatory interaction shown here represents a key link in the negative feedback loop involving TSH and the thyroid hormones. First, the mentioned TSH actions on NIS lead, by different but mutually reinforcing mechanisms (i.e. transcriptional and post-transcriptional), to stimulation of I uptake resulting in higher thyroid hormone production and release. Then, a rise in thyroid hormone circulating levels ultimately inhibits TSH release in the pituitary gland, and this decreases I uptake in the thyroid.

The results presented here are highly relevant to thyroid cancer. It is of major diagnostic importance that most thyroid cancers exhibit decreased I uptake relative to the surrounding tissue on scintigraphy (11). Conversely, the ability of thyroid cancer cells to sufficiently transport I is the basis for radioiodide therapy to be effective against remnant thyroid malignant cancer cells to sufficiently transport I tissue on scintigraphy (11). Conversely, the ability of thyroid radioiodide therapy in these cases.

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REFERENCES

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