Characterization of the Rat Thyroid Iodide Transporter Using Anti-peptide Antibodies

RELATIONSHIP BETWEEN ITS EXPRESSION AND ACTIVITY*

(Received for publication, May 9, 1997, and in revised form, June 2, 1997)

Agnès Paire, Françoise Bernier-Valentin, Samia Selmi-Ruby, and Bernard Rousse†

From INSERM, Unité 369, Faculté de Médecine Lyon-RTH Laennec, 69372 Lyon Cédex 08, France

Antipeptide antibodies directed against the C-terminal portion (amino acids 603–618) of the rat thyroid iodide transporter (rTIT) have been produced to characterize the molecular forms of rTIT in the rat thyroid and in the functional rat thyroid cell line, FRTL-5. rTIT is located on the basolateral membrane of rat thyroid follicular cells and randomly distributed on the plasma membrane of FRTL-5 cells that do not exhibit cell polarity. The major rTIT component corresponds to an 80–90-kDa glycosylated protein. After treatment of cell membrane fractions with N-glycosidase F or incubation of FRTL-5 cells with tunicamycin, rTIT has an apparent molecular mass of about 55 kDa. FRTL-5 cells cultured in the presence of TSH exhibit a high rTIT content and a high iodide uptake activity (IUA). Upon either removal of TSH or addition of cycloheximide, IUA declines more rapidly than rTIT. The half-life of rTIT was about 4 days. Re-exposure of 7-day TSH-deprived FRTL-5 cells to TSH causes a rapid synthesis of the glycosylated rTIT but a delayed re-induction of IUA. Tunicamycin totally prevents the TSH-dependent re-expression and activity of rTIT. Our data bring basic information on the location, structure, and turnover of rTIT and suggest that its activity is subjected to diverse control mechanisms including regulatory proteins.

Iodide trapping by epithelial thyroid cells is the first step of thyroid hormone synthesis within thyroid follicles. Thyroid iodide uptake brings into play a membrane transporter (1) defined as a Na+/iodide symportor (2). After the pioneering studies of Wolff (1), a definite step in the knowledge of the thyroid iodide transporter (TIT), has been made by Dai et al. (3) who cloned the cDNA of the rat TIT (rTIT) allowing the design of tools for further molecular analyses. Secondary structure prediction and hydrophatic profile analyses indicated that rTIT is a membrane protein with 12 membrane-spanning domains thus resembling the other Na+/dependent cotransporters. The availability of the rTIT primary sequence prompted us to produce antibodies to characterize the TIT protein of the rat and other animal species. We choose to generate polyclonal antibodies against a peptide corresponding to the last 16 amino acids (amino acids 603–618) of the rTIT. Antibodies that were raised exhibited a very high titer and a high specificity for a rat thyroid membrane glycoprotein fulfilling the criteria for being the rTIT. These antibodies have been used (a) to visualize the rTIT on rat thyroid tissue sections, (b) to characterize the molecular form(s) of rTIT in the rat thyroid and FRTL-5 cells, and (c) to determine the turnover of rTIT in FRTL-5 cells in relation with changes in their capacity to concentrate iodide.

EXPERIMENTAL PROCEDURES

Materials—Coon’s modified Ham’s F-12 medium was obtained from Seromed (Biochrom KG, Berlin). Bovine TSH (2 units/mg), insulin, cortisol, transferrin, glycy-l-histidyl-l-lysine acetate, tunicamycin, cycloheximide, and anti-rabbit IgG antibody conjugated to alkaline phosphatase were from Sigma. N-glycosidase F was purchased from Boehringer Mannheim and Immobilon P membrane from Millipore Corp. Na125I was obtained from ICN.

Preparation and Test of Antibodies—A peptide corresponding to the published sequence (3) of the C-terminal segment of the rTIT (amino acids 603–618) was synthesized by a solid-phase procedure using an automated peptide synthesizer (Nesystem Laboratories, Strasbourg, France). The peptide was conjugated to keyhole limpet hemocyanin using glutaraldehyde as a coupling reagent. Antisera were raised in rabbits by multipoint injections of 200 μg of the conjugate in complete Freund’s adjuvant. After 2, 4, 8, and 12 weeks, rabbits were given a booster injection. Blood was collected 10 days after the last injection. Two immune sera pAb 716 and pAb 792 were obtained. Sera were tested for their antibody titer against the peptide by enzyme-linked immunosorbant assay.

Multiwell plates from Corning Costar Corp. (Cambridge, MA) were coated with the unconjugated peptide (10 μg/ml) in carbonate buffer, pH 9.6, for 16 h at 4 °C. After saturation of residual binding sites with bovine serum albumin (5 mg/ml) in 10 mM Tris, 0.15 M NaCl, pH 7.4 (TN buffer), serial dilutions of antisera in TN buffer were added, and incubation was performed for 2 h at 37 °C. After washings in TN buffer containing 0.05% Tween 20 immune complexes were detected using an anti-rabbit IgG antibody conjugated to alkaline phosphatase. The enzyme activity was assayed using p-nitrophenyl phosphate as substrate. Absorbance measurements were made at 405 nm. Controls for autohydrolysis of the substrate and nonspecific binding of the conjugate were run in parallel.

Cell Culture—FRTL-5 cells, a continuous line of functional epithelial cells from Fisher rat thyroid (4), remain differentiated growing in 5% serum and require the presence of TSH for growth. Cells were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum, insulin (10 μg/ml), cortisol (10 μg/ml), transferrin (5 μg/ml), glycy-l-histidyl-l-lysine acetate (10 ng/ml), and TSH (1 munit/ml). They were subcultured in either 24-well plates or 60-mm culture dishes, and culture medium was changed every other day. Cells were allowed to reach confluence before any treatment was applied. FRT cells, a continuous line of non-functional but polarized thyroid cells, were grown in the same medium as FRTL-5 with two exceptions, normal calf serum was replaced by fetal calf serum and TSH was omitted.

Iodide Uptake Activity (IUA) Measurements—FRTL-5 cells, grown in 24-well plates, were washed twice with Earle’s balanced salt solution, pH 6.8, supplemented with 1 mM methimazole and 10 μM NaI and incubated in the same medium containing 0.5–1.0 μCi of carrier-free Na125I for 60 min at 37 °C. In each experimental condition, incubations were performed in the absence or in the presence of sodium perchlorate (0.1 mM) to measure the active iodide transport. At the end of the
Expression and Activity of the Thyroid Iodide Transporter

incubation period, the medium was aspirated, and cells were quickly washed twice with ice-cold Earle’s medium. The complete procedure was achieved within 40 s. Radiolabeled iodide taken up by the cells was released by the addition of 10% trichloracetic acid. Radioactivity of the trichloracetic acid-soluble fraction was measured in a γ counter from Packard Instruments. Incubations were made in duplicate. IUA was expressed in picomoles of iodide taken up per 10⁶ cells. Cells detached from culture wells by a 15-min incubation in PBS containing 0.25% trypsin and 0.02% EDTA were counted using an hemocytometer.

Immunofluorescence Labeling—Thyroid glands from Harlan Sprague Dawley rats (200–300 g body weight) rapidly removed after the animal sacrifice were fixed by immersion in the Bouin-Hollande solution. After paraffin embedding, 10-μm tissue sections were prepared and processed to allow immunofluorescence labeling. FRTL-5 cells were fixed in 4% paraformaldehyde for 30 min at 20 °C, then permeabilized using 1% Triton X-100 for 30 min at 20 °C and pre-incubated for 1 h at 20 °C in PBS/bovine serum albumin (1 mg/ml). Fixed and permeabilized FRTL-5 cells and rat thyroid tissue sections were incubated with the pAb 716 immune serum at a 1:10000 dilution overnight at 4 °C. After washings with 0.05% Triton X-100 in PBS, immune complexes were detected using a fluorescein-labeled secondary antibody. Images were prepared using the videomicroscope equipment previously described (5). Control incubations included the rabbit pre-immune serum, omission of the rabbit serum, and saturation of antibodies with the synthetic peptide.

SDS-PAGE and Western Blot—FRTL-5 cells, collected by scraping in PBS containing protease inhibitors soypritin, leupeptin, and aprotinin at a concentration of 1 μg/ml, were lysed by freezing-thawing. Cell homogenates were centrifuged at 100,000 × g for 60 min at 4 °C to obtain membrane fractions. Rat thyroid and adrenal homogenates were prepared in PBS supplemented with protease inhibitors in a glass-glass homogenizer. Particulate material was collected by centrifugation at 10,000 × g for 50 min at 4 °C. Protein was assayed by the Lowry method (16) after solubilization in 1% sodium deoxycholate. Protein samples (2–15 μg) from total cell extracts or membrane fractions were separated by SDS-PAGE on 7–8% acrylamide slab minigels and then transferred onto an Immobilon P membrane. Incubations with the immune serum and the secondary antibody conjugated to alkaline phosphatase were performed as described previously (5). Immunoblabeled membrane spots were quantified by videomage analysis (5).

RESULTS

Characterization of the rTIT Protein and Analysis of Its Cellular Distribution—The peptide (amino acids 603–618) selected by antigenicity prediction analyses from the rTIT primary sequence (3) and coupled to keyhole limpet hemocyanin has proved to be highly immunogenic. The anti-peptide antibody titer of the two immune sera, pAb 716 and pAb 792, was higher than 10⁴, the titer being defined as the highest dilution that gives a signal significantly higher than that of the controls in the enzyme-linked immunosorbent assay. This is illustrated for pAb 716 in Fig. 1. The pre-immune sera were devoid of activity. When tested on FRTL-5 cell extracts by Western blot, the two immune sera detected a main protein migrating as an 80–90-kDa component and some other bands of lower intensity with an apparent molecular mass ranging from 60 to 80 kDa. The 90–90-kDa protein was still detected on Western blot by using a 6 × 10⁻⁶ dilution of the pAb 716 immune serum (Fig. 1). The reactivity of anti-peptide antibodies toward the synthetic peptide analyzed by solid-phase immunoassay and the ability of the antibodies to label FRTL-5 cell protein(s) on Western blot were parallel on a large scale of dilution (up to 10⁻⁶) of the immune serum. The binding of anti-peptide antibodies to the immobilized peptide was inhibited by a prior incubation with the free peptide. The peptide concentration that produced a 50% inhibition was about 10⁻⁷ μ (data not shown). Anti-peptide antibodies (from pAb 716 immune serum) labeled the same bands in cell extracts from TSH-treated FRTL-5 cells and in rat thyroid particulate fractions (lanes 2 and 4 and lane 6 of Fig. 2A); an 80–90-kDa component was the most abundant in both cases. In contrast, FRTL-5 cells deprived of TSH for 7 days exhibited a lower amount of the 80–90-kDa protein and were devoid of the component of lower apparent molecular mass (60–65 kDa) (lanes 3 and 5 of Fig. 2A). The absence of protein labeling in cells (FRT cells) and tissue (rat adrenals) known to be devoid of iodide uptake activity gives evidence for the specificity of the reaction obtained with cells and tissue known to express TIT. Treatment of the TSH-treated FRTL-5 cell membrane fraction with N-glycosidase F led to a large shift in the distribution of the immunoreactive material; a main band with
an apparent molecular mass of about 55 kDa was formed. The deglycosylation reaction yielded the same product when performed on membranes or Nonidet P-40-solubilized membrane proteins (Fig. 2B). These results indicate that rTIT is a glycoprotein. That anti-peptide antibodies properly react with rTIT is further documented in Fig. 3. Anti-peptide antibodies intensely labeled the periphery of FRTL-5 cells cultured in the presence of TSH (Fig. 3A). The labeling was concentrated in the regions of cell-cell contacts but not limited to these parts of the plasma membrane. Looking apart from cell boundaries, there was a diffuse fluorescence becoming faint toward the middle of the cells. Numerous fluorescent dots also mainly located at the cell periphery were found over most cells. There was no labeling when the pre-immune serum was used instead of the immune serum (Fig. 3B) or when the immune serum was omitted. FRTL-5 cells deprived of TSH for 7 days were hardly labeled (Fig. 3C). Given the extreme low background, the low level of fluorescence of TSH-deprived cells probably corresponded to a specific labeling, the distribution of which did not appear different from that of TSH-treated cells. The immunofluorescence staining of rat thyroid sections was more easily definable. As shown at low (Fig. 3D) or high (Fig. 3E) magnification, the anti-peptide antibodies clearly labeled the basolateral plasma membrane domain which was remarkably delineated. On the opposite, the apical plasma membrane domain facing the follicle lumen was devoid of any fluorescence staining. The labeling intensity of thyocytes appeared rather homogenous within individual follicles and between follicles. As mentioned for FRTL-5 cells, the fluorescence background (nonspecific labeling) of tissue sections was very low, indicating that the anti-peptide antibodies react very selectively with the rTIT.

Relationship between rTIT Content and Iodide Uptake Activity of FRTL-5 Cells—FRTL-5 cells cultured in a TSH-free medium are known to progressively lose the IUA within 5–8 days (6) and to re-acquire rapidly this activity upon addition of TSH (7). The turnover of rTIT in FRTL-5 cells in relation to the loss and the re-establishment of IUA (associated with the withdrawal and re-addition of TSH, respectively) is reported in Figs. 4 and 5. Quantitative densitometric measurements on Western blot were carried out on the 80–90-kDa band which was considered as the mature, potentially active, rTIT protein. IUA and rTIT protein content of FRTL-5 cells maintained in the presence of TSH remained rather stable for up to 7 days. Removal of TSH from the culture medium led to a parallel 30% reduction of IUA and rTIT content within 1 day. From day 1 up to day 7 of TSH deprivation, there was a dissociation between the disappearance rate of rTIT and the decrease of IUA (Fig. 4B). At day 5, IUA was abolished whereas cells exhibited a rTIT content equal to about 30% of its initial value. We com-
Expression and Activity of the Thyroid Iodide Transporter

FRTL-5 were treated with forskolin instead of TSH (result not shown). As described previously (7), we found that the restoration of IUA was delayed. Twenty four h after TSH addition (day 8 from the beginning of the experiments), IUA reached only 15–20% of its initial (day 0) level whereas rTIT expression was maximum. Thereafter, IUA continued to increase and plateaued at a value about 50% lower than that obtained with FRTL-5 cells continuously cultured with TSH. Cycloheximide (3 μM) prevented the TSH-induced synthesis of rTIT. This indicates that at the concentration of 3 μM, cycloheximide very efficiently blocked protein synthesis in FRTL-5 cells. Tunicamycin, an inhibitor of the synthesis of N-linked oligosaccharides totally blocked the synthesis of the 80–90-kDa rTIT. Accordingly, tunicamycin prevented the TSH-dependent reinduction of IUA. It was verified that tunicamycin did not alter cell viability. An immunoreactive protein with an apparent molecular mass of 55 kDa (distinct from the 60–65-kDa protein normally found in both FRTEL-5 cells and rat thyroid) was transiently detected at days 8 and 9 (1 and 2 days after TSH addition). This 55-kDa protein could correspond to the non-glycosylated rTIT polypeptide; it has the same mobility in SDS-PAGE as the deglycosylated protein shown in Fig. 2B.

DISCUSSION

Antibodies directed against the synthetic peptide corresponding to the last 16 amino acids of the rTIT sequence (3) allowed the identification of rTIT protein(s) (a) on membrane fractions by Western blot and (b) on fixed cells by indirect immunofluorescence in both FRTEL-5 cells and rat thyroid gland. The major immunoreactive form of rTIT migrates as a broad band in SDS-PAGE; its apparent molecular mass of 80–90 kDa is substantially higher than that expected from the amino acid sequence, i.e. 65 kDa (3). This difference in size and the width of the electrophoretic band is probably attributable to the presence of N-linked oligosaccharide chains. Two potential N-linked glycosylation sites have been identified in the sixth and last extracellular loop of the predicted rTIT structure (3). On the opposite, the deglycosylated rTIT form obtained by N-glycosidase F treatment of FRTEL-5 cell membranes has an apparent molecular mass (about 55 kDa) lower than the predicted molecular mass of the polypeptide chain. Abnormal SDS binding, contributions of the intrinsic charge, and conformation of the protein as well as proteolytic cleavage are possible explanations for the abnormal electrophoretic mobility of the deglycosylated rTIT (8). The occurrence of a proteolytic cleavage during the deglycosylation reaction is unlikely. Indeed, an immunoreactive form of the rTIT of the same apparent size has been identified in tunicamycin-treated FRTEL-5 cells. The 55-kDa protein might thus correspond to the rTIT polypeptide chain. In addition to the 80–90-kDa component, both FRTEL-5 cells and rat thyroid gland contain a second immunoreactive component with an apparent molecular mass of 60–65 kDa that we interpret as a glycosylation intermediate. This is supported by the fact that this component (a) disappeared in TSH-deprived FRTEL-5 cells, (b) reappeared (without accumulation) after re-addition of TSH, and (c) was not formed in response to TSH when cells were treated with tunicamycin. The 80–90-kDa protein, being the most abundant immunoreactive species in both rat thyroid gland and FRTEL-5 cells, probably corresponds to the mature form of rTIT. This is strengthened by the fact that the anti-peptide antibodies that predominantly detected the 80–90-kDa protein, unequivocally labeled the basolateral membrane of thyrocytes where the functional form of TIT or sodium/iodide symporter should be located (9). It was recently reported that sera of patients with autoimmune thyroid diseases contain antibodies against TIT, some sera labeled a single band with an apparent molecular mass of about 80 kDa...
on FRTL-5 cell membranes (10), and some other sera inhibited the iodide uptake activity on CHO cells stably transfected with the rTIT cDNA but did not recognize rTIT on Western blot (11). These anti-TIT auto-antibodies probably interact with extra-cellular loops of rTIT. The 60–90-kDa protein detected by anti-peptide antibodies in the present study and the 80-kDa species labeled by human autoantibodies likely correspond to the same molecular entity.

Regulation of iodide transport by thyroid cells had been the subject of numerous studies (reviewed in Ref. 12) using different cell systems including FRTL-5 cells (2, 6, 7, 13). It has generally been accepted that TSH regulates IUA by acting on the synthesis of the TIT and/or the formation of an activating factor (2, 7, 14). Three conclusions can be drawn from the present study.

First, TSH is required for the maintenance of a steady-state level of rTIT in FRTL-5 cells, and the synthesis of rTIT is rapidly switched off or on upon suppression or re-addition of TSH. Indeed, the rate of disappearance of rTIT after TSH withdrawal was not different from the rTIT decay obtained by inhibiting protein synthesis. This is at variance with the model proposed by Kaminsky et al. (2) in which rTIT was supposed to constitutively reside in the plasma membrane. Addition of TSH to TSH-deprived FRTL-5 cells re-activated the expression of rTIT, and within 24 h, the rTIT cell content reached a steady-state level corresponding to the rTIT level of cells continuously cultured in the presence of TSH.

Second, the activity of rTIT appears to be subjected to regulatory processes. There was a marked dissociation in the time-dependent decrease of IUA and rTIT in cells treated with cycloheximide. The rapid initial loss of IUA after inhibition of protein synthesis in TSH-treated cells suggests the existence of a rTIT stimulatory protein with a turnover rate markedly higher than that of the rTIT. The involvement of such a stimulatory protein in iodide transport had been proposed long time ago by Knopp et al. (14) using dispersed thyroid cells and more recently further analyzed in FRTL-5 cells (2). However, the dissociation between IUA and rTIT content under cycloheximide treatment cannot be solely explained by the disappearance of an activating factor. Indeed, after the initial decrease, IUA remained rather stable for several days despite the progressive disappearance of rTIT. One has to postulate the implication of another regulatory event. The prolonged cycloheximide treatment could deplete cells in a long-lived protein endowed with an inhibitory action on rTIT. The dissociation between IUA and rTIT cell content after both TSH suppression and TSH re-addition also suggests the existence of an activating factor, the synthesis or the activity of which slowly decreases in TSH-deprived cells and slowly increases in cells re-exposed to TSH. Modulation of the activity of rTIT might possibly involve post-translational modifications of rTIT including phosphorylation or changes in the oligomerization state. Indeed, rTIT possesses a putative phosphorylation site for protein kinase A in its C-terminal part, and functional rTIT is possibly oligomeric.

Third, N-glycosylation of rTIT is essential for its synthesis and stability. In the presence of tunicamycin, the response of FRTL-5 cells (depleted in rTIT by a prolonged TSH deprivation) to TSH was limited to the transient formation of a low amount of a 55-kDa protein, presumably the rTIT polypeptide chain. As demonstrated for many other glycoproteins (15), the non-glycosylated form of rTIT likely accumulates in the endoplasmic reticulum where it is degraded. These data suggest that N-glycosylation is essential for correct folding and stabilization of the conformation of rTIT.

Acknowledgments—We are grateful to Prof. L. Nitsch (Naples, Italy) for providing us with the FRT and FRTL-5 cell lines. We thank Christine Audebet for her contribution to the management of the FRTL-5 cell line.

REFERENCES
1. Wolff, J. (1964) Physiol. Rev. 44, 45–90
2. Kaminsky, S. M., Levy, O., Salvador, C., Dai G., and Carrasco N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3789–3793
3. Dai, G., Levy, O., and Carrasco, N. (1996) Nature 379, 458–460
4. Ambesi-Impiumbato, F. S., Parkes, L. A. M., and Coon, H. G. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3455–3459
5. Rajas, F., Gire, V., and Rousset, B. (1996) J. Biol. Chem. 271, 29882–29880
6. Weiss, S. J., Philip, N. J., and Grollman, E. F. (1984) Endocrinology 114, 1090–1098
7. Weiss, S. J., Philip, N. J., Ambesi-Impiumbato, F. S., and Grollman, E. F. (1984) Endocrinology 114, 1099–1107
8. Weber, K., and Osborn, M. (1975) in The Proteins (Neurath, H., and Hill, R. L., eds) 3rd Ed., Vol. 1, pp. 179–225, Academic Press, New York
9. Chambard, M., Verrier, B., Gabrion, J., and Mauchamp, J. (1988) J. Cell Biol. 96, 1172–1177
10. Enso, T., Kogai, T., Nakazato, M., Saito, T., Kaneshige, M., and Onaya, T. (1996) Biochem. Biophys. Res. Commun. 224, 92–95
11. Endo, T., Kaneshige, M., Nakazato, M., Kogai, T., Saito, T., and Onaya, T. (1996) Biochem. Biophys. Res. Commun. 228, 199–202
12. Ekholm, R. (1990) Int. Rev. Cytol. 120, 241–288
13. Marcoes, C., Cohen, J. L., and Grollman, E. F. (1984) Endocrinology 115, 2123–2132
14. Knopp, J., Stole, V., and Teng, W. (1970) J. Biol. Chem. 245, 4403–4408
15. Fiedler, K., and Simons, K. (1995) Cell 81, 309–312
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275