Role of Megalin (gp330) in Transcytosis of Thyroglobulin by Thyroid Cells

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Michele Marino*, Gang Zheng†, Luca Chiovato§, Aldo Pinchera§, Dennis Brown‡, David Andrews‡, and Robert T. McCluskey‡

From the §Pathology Research Laboratory and the ¶Program in Membrane Biology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129 and the ¶Department of Endocrinology, University of Pisa, Pisa, Italy 56124

When thyroglobulin (Tg) is endocytosed by thyrocytes and transported to lysosomes, thyroid hormones (T4 and T3) are released. However, some internalized Tg is transcytosed intact into the bloodstream, thereby avoiding proteolytic cleavage. Here we show that megalin (gp330), a Tg receptor on thyroid cells, plays a role in Tg transcytosis. Following incubation with exogenous rat Tg at 37 °C, Fisher rat thyroid (FRTL-5) cells, a differentiated thyroid cell line, released T3 into the medium. However, when cells were incubated with Tg plus either of two megalin competitors, T3 release was increased, suggesting that Tg internalized by megalin bypassed the lysosomal pathway, possibly with release of undegraded Tg from cells. To assess this possibility, we performed experiments in which FRTL-5 cells were incubated with either unlabeled or 125I-labeled Tg at 37 °C to allow internalization, treated with heparin to remove cell surface-bound Tg, and further incubated at 37 °C to allow Tg release. Intact 330-kDa Tg was released into the medium, and the amount released was markedly reduced by megalin competitors. To investigate whether Tg release resulted from transcytosis, we studied FRTL-5 cells cultured as polarized layers with tight junctions on permeable filters in the upper chamber of dual chambered devices. Following the addition of Tg to the upper chamber and incubation at 37 °C, intact 330-kDa Tg was found in fluids collected from the lower chamber. The amount recovered was markedly reduced by megalin competitors, indicating that megalin mediates Tg transcytosis. We also studied Tg transcytosis in vivo, using a rat model of goiter induced by aminotriazole, in which increased release of thyrotropin induces massive colloid endocytosis. This was associated with increased megalin expression on thyrocytes and increased serum Tg levels, with reduced serum T3 levels, supporting the conclusion that megalin mediates Tg transcytosis. Tg transcytosis is a novel function of megalin, which usually transports ligands to lysosomes. Megalin-mediated transcytosis may regulate the extent of thyroid hormone release.

Hormone release by thyrocytes occurs after endocytosis of the precursor thyroglobulin (Tg)* (1, 2). Tg synthesized by thyrocytes is secreted into the follicle lumen, where it is stored as the major component of colloid (1, 2). Post-translational modifications lead to iodine-rich Tg forms that contain the thyroid hormones thyroxine (T4) and triiodothyronine (T3). Thyrocytes can phagocytose colloid under special circumstances. However, micropinocytosis (vesicular internalization) is the usual route of Tg uptake, which can result both from fluid phase pinocytosis and receptor-mediated endocytosis (1–10). Although receptors that mediate Tg endocytosis have not been fully characterized, we have recently obtained evidence that megalin (gp330) participates in this process (11, 12).

Megalin is a member of the low density lipoprotein (LDL) receptor family (13, 14) and has been shown to mediate endocytosis of multiple, unrelated ligands via coated pits, leading to delivery of ligands to lysosomes (15–21). In immunohistochemical studies, megalin has been found on the apical surface of a restricted group of absorptive epithelial cells, including thyrocytes (22, 23). Based on the assumption that physiological ligands are present in fluids to which megalin is exposed (24), we postulated that megalin on thyrocytes serves as a receptor for Tg. In support of this possibility, we demonstrated in previous studies (11, 12) that megalin is a high affinity Tg receptor and that it can mediate endocytosis of Tg by FRTL-5 cells, a differentiated Fisher rat thyroid cell line (25, 26). Here we show that megalin-mediated endocytosis of Tg by thyrocyte results in transcytosis rather than in proteolytic cleavage in the lysosomal pathway.

EXPERIMENTAL PROCEDURES

Materials—Tg was purified from frozen rat thyroids by ammonium sulfate precipitation and column fractionation, as described (27). Tg preparations were analyzed by both nonreducing and reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, using a rabbit anti-human Tg antibody cross-reactive with Tg from other species (Axle, Westbury, NY).

Radiolabeled Tg was prepared with 125I-Na (NEN Life Science Products) using IOIDO-BEADS (Pierce), according to the manufacturer’s instructions. The specific activity of the preparations ranged from 1500 to 7000 cpm/ng. Biotinylated Tg was prepared using a kit from Roche Molecular Biochemicals, according to the manufacturer’s instructions. The receptor-associated protein (RAP) was used as a glutathione

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† Recipient of an American Thyroid Association research grant for 1999. To whom correspondence should be addressed: Pathology Research Laboratory, Massachusetts General Hospital, Harvard Medical School, 149 13th St., Charlestown, MA 02129. Tel.: 617-726-5638; Fax: 617-726-5684; E-mail: m.marino@endoc.med.unipi.it.

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S-transferase (GST) fusion protein. DH5α bacteria harboring the pGEX-RAP expression construct were kindly provided by Dr. Joachim Herz (University of Texas Southern Medical Center, Dallas, TX). The production of RAP-GST and GST was performed as described (28).

Heparin (Sigma) was used because it effectively releases megalin-binding repeats, was previously described (30). A goat anti-GST antibody that reacts with megalin ectodomain epitopes in the second cluster of ligand binding repeats, were previously described (30). A goat anti-GST antibody was obtained from Amersham Pharmacia Biotech. Alkaline phosphatase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Bio-Rad. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Amersham Pharmacia Biotech. Alkaline phosphatase-conjugated streptavidin was from Vector (Burlingame, CA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were from Cappel (Durham, NC). FITC-conjugated donkey anti-goat IgG was from The Binding Site (San Diego, CA).

Cell Cultures—FRTL-5 cells (American Type Culture Collection, Manassas, VA) were cultured as described (25, 26), in Coon’s F-12 medium containing 5% fetal calf serum and a mixture of six hormones. An immortalized proximal tubule epithelial cell line (IRPT) was established as described (31). IRPT cells were cultured in Dulbeco’s modified Eagle’s medium containing 10% fetal bovine serum.

Radioiodine Labeling of FRTL-5 Cells—FRTL-5 cells were cultured in six-well plates until 80% confluence was reached. Cells were incubated at 37 °C for 30 min with methionine- and cysteine-free RPMI medium without serum, followed by incubation at 37 °C with [35S]methionine (100 μCi/well) in 2 ml of methionine and cysteine-free RPMI medium containing 5% fetal calf serum and the six-hormone mixture. After 1 h, the medium containing [35S]methionine was removed, cells were extensively washed with serum-free medium, and complete FRTL-5 tissue culture medium was readded. At different time points (0–48 h), the tissue culture medium was collected, and cell extracts were prepared. For this purpose, cells were detached with EDTA, washed with PBS, and lysed with 1% Triton X-100, 1% deoxycholate (Fishers) in Tris-buffered saline (pH 8.0), containing 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 5 mM e-amino-n-caproic acid, 5 mM benzamidine (all from Sigma), and 10 mM EDTA (Fishers).

To assess synthesis of Tg, following radioiodinelabeling, immunoprecipitation experiments were performed with cell extracts. Samples were incubated overnight with protein A-agarose beads (Amersham Pharmacia Biotech) coupled with the rabbit anti-Tg antibody or, as a control, with normal rabbit IgG. Beads were extensively washed and resuspended in nonreducing Laemmli buffer, followed by SDS-PAGE and autoradiography.

To assess secretion of Tg, following radioiodinelabeling, tissue culture media were added to plastic microtiter wells coated overnight with the anti-Tg antibody (1:500 in PBS) or, as a control, with normal rabbit IgG (20 μg/ml). After 3 h of incubation at 4 °C, wells were washed four times and cut out. Radioactivity was measured with a β-counter. Radioactivity in wells coated with normal rabbit IgG was subtracted, as background.

Thyroid Hormone Release Experiments—FRTL-5 cells were cultured in 24-well plates until 80–100% confluence was reached. The number of cells used in these experiments was 3.48 × 10⁷ cells/well. The mean amount of protein in cell lysates was 35.3 μg/well, as assessed using a commercial kit (Bio-Rad). Cells were incubated at 37 °C with unlabeled Tg, in Coon’s F-12 medium containing 5 mM CaCl₂, 0.5 mM MgCl₂, and 0.5% ovalbumin (Sigma). After 6 h, the medium was collected, and T3 was measured at the Massachusetts General Hospital Chemistry Laboratory by chemiluminescence. Values were normalized for the total amount of protein in the cell lysates. In certain experiments, Tg was added to the cells together with the megalin competitor RAP-GST (200 μg/ml) or the anti-megalin antibody 1H2 (200 μg/ml) or, as control, with GST (200 μg/ml) or normal mouse IgG (200 μg/ml).

Similar experiments were performed with [125I]-labeled Tg. For this purpose, we used FRTL-5 cells cultured in 24-well plates. [125I]-Tg was added to the cells in a volume of 500 μl at a concentration of 10 μg/ml. Cells were incubated at 37 °C for 6 h, followed by heparin treatment, and further incubation at 37 °C with PBS. The PBS was collected, and radioactivity was measured with a γ-counter.

Assessment of Polarity and of Tight Junctions in FRTL-5 and IRPT Cells Cultured on Permeable Filters—FRTL-5 and IRPT cells were cultured in high density large pore (3-μm) filters in cell culture inserts (Becton Dickinson, Mountain View, CA) placed in 24-well plates. These devices allow polarization of the cells and make it possible to trace transport of molecules across the cell layer, from the upper (insert) to the lower (cell culture well) chamber (32–37). Cells were used at complete confluence. The mean number of cells at confluence was 5.1 × 10⁴ cells/well, and the mean amount of protein in cell lysates was 4.96 μg/well.

Paracellular transport of [3H]mannitol was measured as described (38), by determining the expression of the tight junction-associated protein occludin (38), and by measuring the paracellular transport of [H]mannanititol. The mean number of cells at confluence was 5.1 × 10⁴ cells/well, and the mean amount of protein in cell lysates was 4.96 μg/well.

The expression of occludin by FRTL-5 and IRPT cells was assessed by Western blotting, using a mouse monoclonal antibody against human occludin, cross-reactive with rat occludin (38), purchased from Zymed Laboratories Inc. (South San Francisco, CA). Twelve-day confluent FRTL-5 cells or 9-day confluent IRPT cells were detached from the filters, washed, and lysed with 1% Triton X-100, 1% deoxycholate in Tris-buffered saline (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 5 mM e-amino-n-caproic acid, 5 mM benzamidine, and 10 mM EDTA. Cell extracts were subjected to nonreducing SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with the anti-occludin antibody (1 μg/ml), followed by horseradish peroxidase-conjugated goat anti-mouse IgG.

Transport of [H]mannanititol was measured in confluent FRTL-5 and IRPT cells as follows. Four μCi of [H]mannanititol (NEN Life Science Products) were added to the upper chamber, in a volume of 500 μl, in complete cell culture medium containing unlabeled mannitol (1 mM). The lower chamber was rinsed with 1 ml of medium without [H]mannanititol. Aliquots of the medium from the lower chamber were collected at various time points. Radioactivity was measured with a β-counter.

Results were compared with those obtained in filters without cells.

Transcytosis Experiments—Twelve-day confluent FRTL-5 cells or 9-day confluent IRPT cells on permeable filters were incubated at 37 °C with unlabeled Tg or lactoferrin (50 μg/ml in Coon’s F-12 medium, 5 mM CaCl₂, 0.5 mM MgCl₂, and 0.5% ovalbumin), to allow internalization. After 6 h, cells were washed with ice-cold PBS and incubated for 1 h at 4 °C with ice-cold heparin (100 units/ml) to remove cell-bound Tg (12). The medium containing heparin was removed, and cells were washed with warmed PBS, followed by incubation for 1 h at 37 °C with PBS to allow release of internalized Tg. Released Tg was detected in the PBS by enzyme-linked immunosorbent assay (ELISA) and by Western blotting to the Tg antibody. In inhibition experiments, we added with RAP-GST (200 μg/ml), 1H2 (200 μg/ml), GST (200 μg/ml), or normal mouse IgG (200 μg/ml).
incubations were performed at 4 °C, at which temperature transcytosis is inhibited (33). To assess the effect on Tg transport of the microtubule agent colchicine (39), cells were treated for 1 h at 37 °C with colchicine (Sigma) added both to the upper and the lower chamber at various concentrations (1–5 μM) in binding buffer. Before adding Tg, the buffer containing colchicine was removed, cells were washed, and transcytosis experiments were performed as described above.

**Binding and Uptake Experiments**—FRTL-5 cells, cultured in 96-well plates, were incubated at 37 °C for 24 h with unlabeled Tg (50 μg/ml in Coon's F-12 medium, 5 mM CaCl₂, 0.5 mM MgCl₂, 0.5% ovalbumin), for 6 h, as described previously (12). Cells were then incubated for 1 h at 4 °C with ice-cold heparin (100 units/ml), to release cell-bound Tg. The heparin was collected, and cells were lysed with H₂O on ice. Cell-bound Tg was detected in the heparin wash by ELISA, whereas internalized Tg was measured in cell lysates. In inhibition experiments, Tg was added to the cells together with RAP-GST (200 μg/ml), 1H2 (200 μg/ml), GST (200 μg/ml), or normal mouse IgG (200 μg/ml).

**ELISAs and Western Blotting**—For ELISAs, 96-well microtiter plates were coated with the samples to be tested for Tg and were incubated with the rabbit anti-Tg antibody (1:500), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000) and p-nitrophenyl phosphate. Absorbance was determined at 405 nm. The amount of Tg in samples to be tested was estimated using a serum reference absorbance produced by a Tg standard curve, obtained by coating microtiter wells with 1–1000 ng of purified Tg. Similar ELISA experiments were performed to measure lactoferrin, using a sheep anti-lactoferrin antibody (The Binding Site), followed by alkaline phosphatase-conjugated anti-sheep IgG (Bio-Rad).

For Western blotting, samples to be tested for Tg were subjected to SDS-PAGE under either nonreducing or reducing conditions and blotted onto nitrocellulose membranes, which were incubated with the rabbit anti-Tg antibody (1:500) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2500). Bands were detected using a chemiluminescent substrate kit (Kirkegaard & Perry). Similar experiments were performed for lactoferrin, using a sheep anti-lactoferrin antibody, followed by horseradish peroxidase-conjugated anti-sheep IgG (Bio-Rad).

**In Vivo Experiments**—The model of aminotriazole goiter in rats was used (40). Female Lewis rats weighing 100–120 g (Charles River Laboratories, Wilmington, MA) received aminotriazole (3-amino-1,2,4-triazole; Sigma), which was administered daily (20 mg/kg) followed by aminotriazole-conjugated streptavidin (1:3000) and p-nitrophenyl phosphate. Values of Tg in rat sera were calculated on a standard Lin-Log competitive radiolmmune assay-like curve, obtained by incubating anti-Tg-coated wells with biotin-labeled Tg plus unlabeled Tg (0.1–500 μg/ml), giving the arbitrary value of 1 unit/ml for the result obtained with 1 μg/ml of unlabeled Tg. Because this assay is not standardized, Tg values must be considered exclusively for internal comparison.

**Statistical Analysis**—Unpaired t test and regression analysis were performed using a personal computer software (Stat-View®, Abacus Concepts, Berkeley, CA).

**RESULTS**

**Analysis of Tg Preparations**—In the present study, we used purified rat Tg, which was prepared from frozen rat thyroids by ammonium sulfate precipitation and column fractionation, as described previously (27). The Tg preparations were tested by SDS-PAGE and Coomassie staining, performed both under nonreducing and reducing conditions (5% β-mercaptoethanol). Under nonreducing conditions, two bands were seen at about 660 and 330 kDa (Fig. 1, lane 1), and similar results were obtained by Western blotting. The 660-kDa band corresponded to covalently linked Tg dimers (41, 42). Size exclusion gel chromatography showed that almost all (~95%) of the 330-kDa band represented monomers derived from noncovalently associated Tg dimers that had been dissociated by SDS-PAGE, as previously reported (41, 42), with a small fraction (~5%) of free Tg monomers (not shown). As shown in Fig. 1 (lane 2), under reducing conditions, two bands, one slower (S) and one faster (F), were seen, as described previously (42, 43). Other Tg products with lower molecular masses were present in minimal amounts. Similar products of reduction have been previously described in human Tg (44–46). All of the Tg preparations used in the present study had an electrophoretic pattern similar to the one shown in Fig. 1.

**Analysis of FRTL-5 Cell Differentiation**—In the present study, we used FRTL-5 cells, a well-established, differentiated rat thyroid cell line (25, 26). In Tg-treated cultures (12), we showed that FRTL-5 cells express megalin in a TSH-dependent manner and that megalin on these cells can mediate binding and uptake of rat Tg. The degree of differentiation of FRTL-5 cells is known to vary between different batches of cells and under different culture conditions. For example, it has recently been reported (for a review, see Ref. 47) that certain batches of
FRTL-5 cells are tetraploid and poorly differentiated, as shown by their inability to synthesize and secrete Tg. Therefore, we assessed synthesis and secretion of Tg by the FRTL-5 cells we used, as a measure of their differentiation (47). For this purpose, we pulse-labeled FRTL-5 cells with \(^{35}\)S-methionine for 30 min. Synthesis of Tg was assessed in cell extracts by immunoprecipitation with a rabbit anti-Tg antibody, whereas secretion of Tg was tested by measuring binding of tissue culture supernatants to microtiter wells coated with an anti-Tg antibody. As shown in Fig. 2A, SDS-PAGE and autoradiography revealed the presence of 330-kDa Tg in the cell extracts immediately after labeling, with a peak at 30 min, with progressive reduction thereafter. At 8 h, radiolabeled Tg was no longer precipitated by the anti-Tg antibody. No Tg was precipitated by normal rabbit IgG, used as a control (not shown). As shown in Fig. 2B, FRTL-5 cells also secreted Tg into the medium. Thus, there was binding activity to the anti-Tg antibody in the tissue culture supernatants, seen first at 30 min after labeling, with a peak at 4 h, and with progressive reduction from 8 to 48 h. These results, as well as others described below, indicate that FRTL-5 cells used in this study were well differentiated.

**Megalin Mediates Thyroglobulin Transcytosis**

To investigate the fate of internalized Tg, we first demonstrated that FRTL-5 cells release T3 from exogenously added Tg. Following incubation of FRTL-5 cells with purified rat Tg at 37 °C, we measured T3 in the medium. Similar assays for T4 were not performed, because they are less sensitive and because, unlike T3, T4 can be released by cell surface proteases in addition to lysosomal degradation (48). Furthermore, T4 can be underestimated due to conversion into T3 by type 1 thyroid deiodinase. As shown in Fig. 3A, T3 was released by FRTL-5 cells incubated with Tg in concentrations exceeding 50 μg/ml, with increase at a higher concentration of Tg.

To investigate the role of megalin in T3 release, FRTL-5 cells were incubated with Tg plus either of two megalin competitors: the receptor-associated protein (RAP-GST fusion protein) or 1H2 (a monoclonal antibody against megalin). As noted above, T3 was not released at a Tg concentration of 50 μg/ml. However, when this concentration was co-incubated with RAP-GST or 1H2, release of T3 was detected (Fig. 3B). Furthermore, when cells were incubated with Tg at a concentration of 100 μg/ml, which by itself resulted in T3 release, co-incubation with RAP-GST or 1H2 led to a 5.5-fold increase of T3 release (Fig. 3B).

**Tg Endocytosed via Megalin Is Released Intact**—Based on our previous observation that megalin can mediate uptake of Tg (12) and because of the known ability of megalin to transport various ligands to lysosomes (15–21), the finding that megalin inhibitors enhanced Tg degradation in the lysosomal pathway (T3 release) was unexpected. We postulated that Tg endocytosed by megalin avoids the lysosomal pathway, possibly through a pathway leading to release of undegraded Tg. To investigate this possibility, FRTL-5 cells were incubated for 6 h at 37 °C with unlabeled Tg, to allow its internalization. Cells were then chilled and treated with heparin, which removes cell surface-bound Tg (12). Following further incubation at 37 °C, Tg was released into the medium, as detected by ELISA (Fig. 4A). Most of the released Tg represented internalized exoge-
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of purified Tg. Lane 2, Tg released, detected by Western blotting after 6% nonreducing SDS-PAGE. Lane 1, preparation of purified Tg. Lane 2, Tg released from FRTL-5 cells after incubation with the Tg preparation shown in lane 1. The arrows indicate bands corresponding to Tg. The figure is representative of one of five experiments. C, inhibitory effect of megalin competitors on the amount of Tg released. FRTL-5 cells were incubated with Tg, alone or in the presence of RAP-GST or 1H2, GST and normal mouse IgG (MIgG) were used as controls. Results are expressed as mean ± S.E. obtained in three experiments. D, release of endocytosed 125I-Tg by FRTL-5 cells. Cells were incubated at 37 °C with 125I-Tg (10 μg/ml), alone or in the presence of unlabeled Tg, RAP-GST, or 1H2, at a concentration of 50 μg/ml. GST and normal mouse IgG (MIgG) were used as controls. After 6 h, cells were treated with heparin and then incubated with warmed PBS to allow release of internalized Tg. Radioactivity was measured with a γ-counter. Values were normalized for the total amount of protein in the cell lysates. Values are expressed as mean ± S.E.

ous Tg, as shown by the finding that the amount released by cells incubated with buffer lacking Tg was minimal. Western blotting showed that the released Tg was intact, with an estimated mass of 330 kDa (Fig. 4B, lane 2), representing mainly, if not exclusively, Tg monomers derived from noncovalently associated Tg dimers dissociated by SDS-PAGE (41, 42). The amount of Tg released was ~12% of the amount of Tg given to the cells. Evidence that the Tg detected in the medium had been internalized by megalin was obtained in inhibition experiments. When FRTL-5 cells were incubated with Tg plus RAP-GST or 1H2, the amount of Tg released was markedly reduced (Fig. 4C). The mean inhibition produced by RAP-GST was ~71.3%, whereas the mean inhibition produced by 1H2 was ~64.0%.

To investigate to what extent the reduction in Tg release produced by megalin competitors resulted from reduced Tg binding and uptake by FRTL-5 cells, we performed Tg binding and uptake experiments, as described previously (12). In confirmation of our previous study (12), we found that FRTL-5 bound and internalized Tg and that these processes were markedly reduced by RAP-GST and 1H2 (not shown). By comparing the results obtained in Tg release experiments with those obtained in binding and uptake experiments, we estimated that ~80% of cell surface-bound Tg and ~70% of internalized Tg was released by FRTL-5 cells. These proportions were reduced by megalin competitors to ~55% and ~35% respectively. The results indicate that the effects of megalin competitors on Tg release by FRTL-5 cells did not result merely from a reduction of Tg binding and uptake and support the interpretation that a major portion of the Tg bound to and internalized by megalin had been released intact.

To further assess the results obtained in experiments with unlabeled Tg, we performed similar experiments using 125I-labeled Tg. As shown in Fig. 4D, 125I-Tg was released by FRTL-5 cells following its internalization. The amount of 125I-Tg released was ~10% of the amount given to the cells. When 125I-Tg was added to the cells in the presence of an excess of unlabeled Tg, RAP-GST, or 1H2, the amount of Tg released was markedly reduced, whereas no effect was produced by GST or normal mouse IgG, used as controls. Unlabeled Tg reduced the release of 125I-Tg by ~56.2%, whereas RAP-GST and 1H2 produced a mean inhibition of ~59.7 and ~81.1%, respectively.

Although the proportion of Tg released by FRTL-5 cells was similar in experiments with unlabeled Tg as compared with experiments using 125I-Tg, the amount of Tg released per μg of cell protein was greater in experiments with unlabeled Tg. This may be explained by the fact that in experiments with unlabeled Tg a lower number of cells was used. Thus, in experiments with unlabeled Tg, we used FRTL-5 cells cultured in 96-well plates, whereas in experiments with 125I-Tg we used FRTL-5 cells cultured in 24-well plates. Because the amount of Tg given to the cells in the two types of experiments was the same (5 μg/well), in experiments with unlabeled Tg there was a greater ratio between the amount of Tg given to the cells and the number of binding sites available. We have previously shown (12) in experiments with unlabeled Tg that under conditions similar to those used in the present study Tg binding sites are saturated. It is very likely that a greater amount of 125I-Tg would be needed to saturate FRTL-5 cell Tg binding sites when cells are cultured in 24-well plates.

Establishment of Polarized FRTL-5 and IRPT Cell Layers, Cultured on Filters in Dual Chambered Devices—Release of undegraded ligands following endocytosis can occur either through recycling to the cell surface at which endocytosis occurs or by transport across the cell to the opposite surface (transcytosis) (32–37). Evidence of transcytosis of intact 330-kDa Tg by cultured thyroid cells has been previously reported (49, 50). To study transcytosis, we established an in vitro model, based on the use of FRTL-5 cells cultured on permeable filters in dual chambered devices. In addition to FRTL-5 cells, we also used an immortalized rat renal proximal tubule cell line (IRPT cells) that expresses abundant megalin (31). In a previous study (12), we have shown that megalin on these cells can mediate binding and uptake of Tg.
Both FRTL-5 and IRPT cells were cultured on permeable filters until 100% confluence was reached. To assess polarization of the cell layers, we first evaluated the expression of megalin, by immunofluorescence staining on frozen sections cut perpendicular to the filters. As shown in Fig. 5, in FRTL-5 cells megalin was found at the upper surface (apical) of the cell layer but not at the lower surface (basolateral). Similar results were obtained with IRPT cells (not shown).

To further investigate polarization of FRTL-5 and IRPT, we performed electron microscopy on thin sections cut perpendicular to the filters. In both FRTL-5 and IRPT cells, we could discern an apical domain at the upper surface, which was clearly distinguished from the basolateral domain. As shown in Fig. 6, A and B, the two domains were separated by junctional complexes. Some clathrin-coated pits were seen at the apical membrane, which strongly indicates the existence of endocytic machinery at this surface. Furthermore, microvilli could be seen at the upper surface, since it is typical of apical membranes of polarized epithelial cells.

To determine whether FRTL-5 and IRPT cells cultured on permeable filters formed tight junctions, we performed several experiments. We first measured the TER of the cell layers for several days after reaching confluence. As shown in Fig. 7A, there was an increase of TER values in both FRTL-5 and IRPT cells, with a peak at day 12 of confluence in FRTL-5 cells (46.3 ± 6.4 ohms × cm²) and at day 9 of confluence in IRPT cells (115.3 ± 27.7 ohms × cm²).

We then assessed the expression of the tight junction-associated protein occludin (38). As shown in Fig. 7B, occludin was found by Western blotting in cell extracts from both FRTL-5 and IRPT cells at its expected molecular mass.

To obtain further evidence that FRTL-5 and IRPT cells formed tight layers when cultured on permeable filters, we performed experiments designed to measure transport through the cell layers of a radiolabeled molecule of very low mass (~1 kDa), namely [³H]mannitol. For this purpose, [³H]mannitol was added to the upper chamber of confluent FRTL-5 or IRPT cells. As shown in Fig. 8C, the amount of [³H]mannitol transported in 3 h from the upper to the lower chamber was minimal in both FRTL-5 (1.34% of the amount added to the upper chamber) and IRPT cells (3.68% of the amount added to the upper chamber), as compared with the amount transported through filters without cells (44.24%).

**Megalin on FRTL-5 Cells Mediates Apical to Basolateral Transcytosis of Intact Tg**—To study Tg transcytosis, we used 12-day confluent FRTL-5 cells, cultured on permeable filters in dual chambered devices. Cells were incubated at 37 °C with preparations of unlabeled Tg, containing both the 660- and 330-kDa forms, added to the upper chamber. Transferred Tg was measured in fluids collected from the lower chamber. As shown in Fig. 8A (bars 2 and 3), after 1–6 h of incubation, Tg was found by ELISA in the lower chamber. The amount of Tg found in the lower chamber of FRTL-5 cells incubated with buffer lacking Tg was minimal (Fig. 8A, bar 1). Western blotting showed that Tg in the lower chamber had a molecular mass of 330 kDa (Fig. 8B, lane 2). Evidence that Tg was transported through cells, rather than by leakage between cells, was provided by the finding that in experiments performed at 4 °C, at which temperature transcytosis is inhibited (33), Tg was not transported to the lower chamber (Fig. 8A, bar 1).
The selective transport of 330-kDa Tg provides further evidence that Tg had been transcytosed rather than trans-ported by paracellular leakage. Furthermore, when FRTL-5 cells were pretreated with the microtubule-disruptive agent colchicine (39), transport of Tg from the upper to the lower chamber was reduced, with a $\frac{40}{\%}$ inhibition at a colchicine concentration of 5 $\mu$M/liter (Fig. 8C).

We estimated that the amount of Tg added to FRTL-5 cells that was transcytosed at 37 °C was $\frac{3}{4}$% in 1 h and $\frac{6}{4}$% in 6 h. As shown in Fig. 8D, Tg transcytosis was markedly reduced when Tg was added together with RAP-GST or 1H2, suggesting that megalin largely mediates this process. The mean inhibition produced by RAP-GST was $\frac{82.2}{\%}$, whereas the mean inhibition produced by 1H2 was $\frac{64.3}{\%}$. The mean proportions of cell-bound and of internalized Tg transcytosed by FRTL-5 cells were 54.7 and 48.9% respectively, as measured in binding and uptake experiments. The inhibition of Tg transcytosis by megalin competitors did not result merely from a reduction of Tg binding and uptake.

**Megalin Mediates Thyroglobulin Transcytosis**

Megalin Mediates Thyroglobulin Transcytosis in Cultured Renal Proximal Tubule Cells (IRPT Cells)—To investigate whether megalin can mediate transcytosis of Tg by nonthyroid cells, we performed experiments with IRPT cells. We estimated that the amount of Tg added to IRPT cells that was transcytosed at 37 °C was $\frac{3}{4}$% in 1 h and $\frac{6}{4}$% in 6 h. As shown in Fig. 8D, Tg transcytosis was markedly reduced when Tg was added together with RAP-GST or 1H2, suggesting that megalin largely mediates this process. The mean inhibition produced by RAP-GST was $\frac{82.2}{\%}$, whereas the mean inhibition produced by 1H2 was $\frac{64.3}{\%}$. The mean proportions of cell-bound and of internalized Tg transcytosed by FRTL-5 cells were 54.7 and 48.9% respectively, as measured in binding and uptake experiments. The inhibition of Tg transcytosis by megalin competitors did not result merely from a reduction of Tg binding and uptake.
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To determine if increased megalin expression and function resulted from TSH stimulation rather than from a direct effect of aminotriazole on thyrocytes, some aminotriazole-treated rats also received T4 for 6 days, which partially reduced serum TSH levels (Table I). By immunofluorescence, thyroid cells in these rats showed less megalin expression than in rats treated with aminotriazole alone (Fig. 10C), which was confirmed by Western blotting in thyroid extracts (Fig. 10E, lane 3). Thus, the PD of the band corresponding to 330-kDa Tg was 43.7 ± 1.00 pixels/mm² in untreated rats and 20.0 ± 1.59 pixels/mm² in rats given aminotriazole for 6 days (p = 0.0001).

Based on the finding that megalin expression was increased on the apical surface of thyrocytes, we postulated that there would be increased ligand binding ability. In confirmation of our prediction, we found that RAP-GST bound to the apical surface of thyrocytes to a much greater extent in aminotriazole-treated rats than in untreated rats (Fig. 10, F and G). No binding of GST, used as a negative control, was seen (not shown).

Evidence of massive Tg endocytosis in aminotriazole-treated rats was provided not only by histological findings but also by immunofluorescence staining for Tg, which revealed a progressive reduction of colloid, with virtually complete depletion by 12 days (not shown). In addition, the amount of Tg in thyroid extracts assessed by Western blotting was reduced in aminotriazole-treated rats as compared with untreated rats (not shown). Thus, the PD of the band corresponding to 330-kDa Tg was 49.24 ± 1.00 pixels/mm² in untreated rats and 20.0 ± 1.59 pixels/mm² in rats given aminotriazole for 6 days (p = 0.0001).

To study megalin expression in the thyroid of aminotriazole treated rats, we performed immunofluorescence staining on thyroid frozen sections, using the monoclonal anti-megalin antibody 1H2. As shown in Fig. 10, A, B, and D, there was a striking increase in the intensity of megalin staining on the apical surface of thyrocytes at day 4 of treatment and later, as compared with untreated rats, whereas there was no change in the intensity of megalin staining in the parathyroid or in the kidney (not shown), indicating that megalin increase on thyrocytes was selective. To study megalin expression further, we performed Western blot analysis on thyroid extracts. As shown in Fig. 10E, megalin in thyroid extracts was increased in aminotriazole-treated rats (lane 2) as compared with untreated rats (lane 1), whereas no difference was observed in kidney extracts (not shown). To quantify megalin expression in the thyroid, the PD of the band corresponding to megalin was measured. As assessed in groups of six rats, the mean PD of the megalin band was significantly (p = 0.0046) higher in rats treated with aminotriazole for 6 days (53.0 ± 12.45 pixels/mm²) than in untreated rats (19.7 ± 3.35 pixels/mm²), whereas the PD of the megalin band in Western blotting performed with kidney extracts did not differ between the two groups of rats.

Megalin-mediated Transcytosis of Tg in Aminotriazole-treated Rats—To investigate megalin-mediated transcytosis of Tg in vivo, we studied the well established model of aminotriazole goiter in rats (40). Aminotriazole inhibits iodination of newly synthesized Tg, resulting in increased TSH release from the pituitary. After several days, progressive changes occur in the thyroid, due to the stimulatory effects of TSH, characterized by enlargement and proliferation of thyroid cells, with massive endocytosis of Tg from the colloid. By 10–12 days, the colloid is almost completely depleted. We performed studies on rats given aminotriazole for 2–12 days and found histological alterations in the thyroid similar to those described in the study of Strum and Karnovsky (40).

To study megalin expression in the thyroid of aminotriazole treated rats, we performed immunofluorescence staining on thyroid frozen sections, using the monoclonal anti-megalin antibody 1H2. As shown in Fig. 10, A, B, and D, there was a striking increase in the intensity of megalin staining on the
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Fig. 10. Studies on aminotriazole-treated rats. A–D, increased thyroid megalin expression in aminotriazole rats, detected by immunofluorescence staining. Frozen thyroid sections were incubated with the mouse monoclonal anti-megalin antibody 1H2 followed by FITC-labeled anti-mouse IgG. A, normal rat. Megalin staining is seen mainly on the apical surface of thyrocytes. B, aminotriazole-treated rat (6 days). Apical staining is more intense and broader. The amount of colloid is slightly reduced. C, rat treated with aminotriazole and T4 (6 days). Staining for megalin is less than in the aminotriazole-rat not given T4 (B). D, aminotriazole treated rat (12 days). There is more intense megalin staining. Colloid is largely depleted. Magnification is × 100. The figure is representative of one of three experiments. E, increased megalin expression in thyroid extract from an aminotriazole rat, detected by nonreducing 5–16% SDS-PAGE and Western blotting, performed using 1H2. Lane 1, normal rat. Lane 2, aminotriazole-treated rat (6 days). Lane 3, rat treated with aminotriazole and T4 (6 days). The arrow indicates bands corresponding to megalin. Similar results were obtained in other experiments performed with five rats in each group. F–G, increased binding of exogenous RAP to thyroid sections from aminotriazole-treated rats. Fixed sections were incubated with RAP-GST, followed by goat anti-GST antibody and FITC-labeled anti-goat IgG. Control sections incubated with GST alone showed no staining (not shown). F, normal rat. There is little or no binding of RAP-GST. G, aminotriazole-treated rat (6 days). There is a striking increase of binding. Magnification is × 100. The figure is representative of one of three experiments. H, increased Tg in sera from aminotriazole rats, detected by 6% SDS-PAGE and Western blotting. Fifty μl of serum were added to each lane. Lanes 1 and 2, normal untreated rats. Lanes 3 and 4, aminotriazole-treated rats (6 days). Lanes 5 and 6, rats treated with aminotriazole plus T4 (6 days). The arrows indicate bands corresponding to Tg.

Table I

| Assay              | Untreated (n = 6) | Aminotriazole, 6 days (n = 6) | Aminotriazole, 12 days (n = 6) | Aminotriazole + T4, 6 days (n = 6) |
|--------------------|-------------------|-------------------------------|-------------------------------|-----------------------------------|
| TSH (ng/ml)        | 5.75 ± 2.6        | 12.1 ± 10.3                   | 40.25 ± 28.7                 | 8.31 ± 2.20                      |
| Tt3 (ng/dl)        | 88.1 ± 17.2       | 62 ± 13.5                     | 14.2 ± 11.5                  | 78.7 ± 13.2                      |
| Tg (units/ml)      | 272 ± 225         | 643 ± 268                     | 1037 ± 42                    | 476 ± 261                        |
| n = 6              |                   |                               |                               |                                   |

*p = 0.0166 versus untreated by Student’s t test.

**p = 0.018 versus untreated by Student’s t test.

***p = 0.0001 versus untreated by Student’s t test.

****p = 0.030 versus untreated by Student’s t test.

*****p = 0.0021 versus untreated by Student’s t test.

Reduction of serum T3 levels, as seen at 6 days and to an even greater extent at 12 days (Table I). This result was expected based on the assumption that the massive Tg endocytosis resulted mainly in transcytosis rather than in proteolytic cleavage in the lysosomal pathway with hormone release. To examine further the conclusion that the effects of aminotriazole on serum Tg and T3 were due to increased transcytosis of Tg via megalin, we analyzed the relation between levels of thyroid megalin expression, assessed by Western blotting, and serum levels of Tg and T3, in groups of six rats (untreated, treated with aminotriazole alone, or treated with aminotriazole plus T4 for 6 days). We found that serum Tg levels showed a significant positive correlation with the PD of the thyroid megalin band by Western blotting (p = 0.034 and r = 0.611), whereas T3 levels were negatively correlated (p = 0.0007 and r = 0.837).

**Discussion**

In the present study, we provide evidence that megalin-mediated endocytosis of Tg by thyroid cells largely results in its transcytosis, thereby avoiding the lysosomal pathway, where proteolytic cleavage of Tg results in hormone release. We propose that this novel function of megalin plays a role in the regulation of thyroid hormone release.

In a previous study (12), we demonstrated that FRTL-5 cells, an established, differentiated rat thyroid cell line (25, 26), can bind and internalize exogenous Tg via megalin. Here we studied intracellular proteolytic processing of Tg by measuring the amount of T3 released by FRTL-5 cells following incubation with exogenous unlabeled Tg. This assay can be considered specific for the intracellular cleavage of Tg in the lysosomal pathway. Thus, unlike T4, T3 is not appreciably released by cell surface proteases (1, 48). Furthermore, although some internalized Tg may undergo partial cleavage in prelysosomes with release of T4, this process does not result in an appreciable release of T3, which instead occurs almost entirely in lysosomes (1, 48).

Because megalin has been shown to transport various ligands to lysosomes (15–21), we initially expected to find that megalin-mediated uptake of Tg would result in release of T3 and that, therefore, interference with megalin would result in reduced T3 release. However, we found that T3 release from
exogenous Tg by FRTL-5 cells was increased up to 5-fold by either of two megalin competitors, the receptor-associated protein RAP or the monoclonal anti-megalin antibody 1H2. Moreover, the increase in T3 release occurred despite the fact that RAP and 1H2 reduce Tg uptake in FRTL-5 cells (12). These observations provide evidence for the conclusion that Tg internalized by megalin avoids the lysosomal pathway and indicate that other means of Tg endocytosis, such as fluid phase uptake or uptake by low affinity receptors (1–10), lead to its proteolytic cleavage in the lysosomal pathway. In this regard, we previously showed that megalin only partially mediates Tg uptake by FRTL-5 cells (12), supporting the notion that Tg endocytosis also occurs via other mechanisms (1–10).

Based on the observation that megalin mediates Tg uptake (12) but not T3 release, we postulated that Tg endocytosed by megalin is transported through a pathway that leads to the release of undegraded Tg from the cells. In support of this, we found that FRTL-5 cells released a considerable proportion of the internalized Tg (~70%) intact into the medium and that the amount was markedly reduced (by ~65–70%) by megalin competitors.

Release of ligands following endocytosis occurs either by recycling to the cell surface where endocytosis takes place or by transepithelial transport (transcytosis) of the ligand to the opposite cell surface (32–37). Herzog and associates (49, 50) have shown that Tg is transcytosed intact across thyrocytes from the apical to the basolateral surface in cultured pig thyroid follicles. Here we provide evidence that Tg transcytosis is mediated by megalin, using polarized FRTL-5 cells cultured in dual chambered devices, with the apical surface facing the upper chamber. When Tg was added to the upper chamber, at which surface megalin was exclusively expressed, fluids collected from the lower chamber were shown to contain intact Tg, indicating that a certain amount had transversed the layers. The amount of Tg transported to the lower chamber was markedly reduced by megalin competitors (by ~65–80%), indicating that much of the Tg transcytosis was mediated by megalin. The inhibitory effects of megalin competitors on Tg transcytosis only partially resulted from a reduction of Tg binding and uptake via megalin by FRTL-5 cells. Thus, transcytosis of the fraction of Tg bound to or internalized by FRTL-5 cells was appreciably reduced by megalin competitors. This indicates that intracellular routing of Tg was affected by RAP and 1H2. However, the mechanisms by which inhibition of megalin at the intracellular level reduces Tg transcytosis are unknown but may be related to alterations in Tg-megalin complexes that interfere with signals that target to transcytosis. Clearly, further studies are needed to investigate how targeting of Tg to transcytosis occurs.

The validity of the conclusion that Tg internalized by megalin is not transported to the lysosomal pathway but is rather transcytosed across FRTL-5 cells depends on the specificity of the megalin competitors we used. Both RAP and 1H2 have been extensively used in studies on megalin function and are well established megalin inhibitors (15–21). 1H2 is entirely specific for megalin (30), and, although RAP binds to certain other members of the LDL receptor family (notably the low density lipoprotein receptor-related protein and the very low density lipoprotein receptor (15, 22)), these receptors are not expressed by thyrocytes, including FRTL-5 cells (12, 22). Therefore, in dealing with FRTL-5 cells, the effects of RAP can be considered specific for megalin. Furthermore, there is no evidence that these competitors may affect cellular processes in ways other than by inhibiting megalin function. The possibility that by blocking megalin the competitors may indirectly affect other processes in the cell cannot be entirely excluded, but it is not supported by available evidence. Indeed, the primary megalin function is to bind and internalize ligands present in the extracellular fluid (15–21), although their intracellular fate varies depending on the ligand and cell type.

At which stage of the endosomal pathway Tg internalized by megalin is diverted from the lysosomal pathway is unknown. Studies aimed at tracing the intracellular route of Tg, either by electron microscopy or by other techniques, may offer a direct and detailed view of the intracellular fate of Tg. However, such studies may be inconclusive. Thus, it may be difficult to trace Tg through the lysosomal pathway using antibodies, because of the progressive loss of its immunoreactivity, which begins in early endosomes (1, 48). Furthermore, an alternative method such as using Tg coupled to gold particles or to other substrates may affect its intracellular trafficking.

Our interpretation that in FRTL-5 cells transport of Tg from the upper to the lower chamber was by transcytosis rather than by paracellular leakage is supported by several lines of evidence. (i) Tg transport was inhibited by low temperature and by the microtubule-disruptive agent colchicine, as is characteristic of transcytosis (32–37, 39). (ii) Tg transport was selective for the 330-kDa form of Tg. (iii) A smaller megalin ligand, namely lactoferrin (15), which was internalized by FRTL-5 cells, did not reach the lower chamber. (iv) Tg transport was inhibited by megalin competitors, which indicates specificity of the process, as occurs with receptor-mediated transcytosis but not with paracellular leakage. (v) FRTL-5 cells formed a tight junctional barrier, as shown by the presence of intercellular junctional complexes by electron microscopy, by the expression of occludin, and by development of increased TER with time in culture. (vi) There was only minimal paracellular transport of [3H]mannitol, a molecule of very low mass (~1 kDa), as compared with Tg (transport at 1 h as follows: mannitol, 0.28% of the amount added; Tg, ~3%).

Our conclusion that megalin-mediated transcytosis of Tg through FRTL-5 cells was from the apical to the basolateral surface depends on the use of polarized cells, with the apical surface facing the upper chamber. The evidence supporting such polarity includes the demonstration by immunofluorescence microscopy of megalin exclusively at the upper surface of the cell layer. In all polarized cells studied that express megalin, this receptor has been seen only at the apical surface (22, 23). Furthermore, electron microscopic examination of FRTL-5 cells cultured on the permeable filters showed evidence of polarity, with microvilli and clathrin-coated pits at the apical membrane, a finding that indicates the existence of endocytic machinery at this surface. In addition, some other studies (51–53) describe features of polarity in FRTL-5 cells, including the demonstration that they possess microvilli only on the surface facing the medium (51, 52) and secrete extracellular matrix only at the opposite surface (53). Nevertheless, other studies (54, 55) have concluded that FRTL-5 cells are not polarized and are incapable of forming tight junctions, based on the finding of endogenously synthesized Tg both in the upper and lower chambers of cells cultured on permeable filters and on the relatively low TER, as compared with the known TER of other cells, such as FRT cells (54). However, based on our present findings, the presence of Tg in the lower chamber may be explained by transcytosis of endogenously synthesized Tg secreted into the upper chamber. Furthermore, a low TER excludes neither polarity nor the presence of tight junctions, because TER can be affected by other factors, such as cell density (56). For example, in the renal proximal tubule epithelium in situ, an extremely low TER is present, and yet this epithelium is highly polarized and has a functionally important tight junctional barrier, as well as vectorial transepithelial...
transport processes (57). Furthermore, although the TER of FRTL-5 cells was low, "nonzero" TER values suggest the existence of a tight junctional barrier (56), a conclusion supported by electron microscopic findings, by the expression of occludin by FRTL-5 cells, and by the low transport of [3H]mannitol. It should be noted that the degree of differentiation of FRTL-5 cells may vary from one batch to another. Thus, it has been reported recently (47) that some batches of FRTL-5 cells lack the ability to synthesize and secrete Tg, signs of differentiated thyroid function. In the present study, we have used well differentiated FRTL-5 cells, as shown by their ability to synthesize and secrete Tg. It is possible that the disparate results reported concerning the polarity of FRTL-5 cells are due to different degrees of differentiation of the cells used.

Our results obtained with IRPT cells, a rat renal proximal tubule cell line that expresses abundant megalin (31), show that megalin can mediate Tg transcytosis in cultured cells other than thyroid cells and support the conclusions reached in experiments with FRTL-5 cells. Like FRTL-5 cells, the IRPT cells as cultured here on permeable filters were polarized and formed a tight junctional barrier. We found that Tg was transcytosed by IRPT cells and that this process was markedly reduced by megalin competitors, whereas lactoferrin, another megalin ligand, was internalized but not transcytosed by IRPT cells.

Our experiments using the model of aminotriazole goiter in rats (40) support our findings with FRTL-5 cells and indicate that megalin mediates transcytosis of Tg in vivo. This experimental model is characterized by massive endocytosis of Tg from the thyroid follicle lumen, with severe, progressive depletion of colloid, beginning within several days of treatment, as a consequence of enhanced TSH secretion from the pituitary. Here we showed that the thyroid alterations included a striking increase of megalin expression on the apical surface of thyrocytes, associated with enhanced ligand binding ability, as demonstrated by increased binding of exogenous RAP to the apical surface of thyrocytes. Evidence that the effects of aminotriazole were due to TSH stimulation was provided by the finding that co-administration of T4, which reduced serum TSH levels, partially prevented the increase in megalin expression. The evidence that thyroid megalin expression in vivo is TSH-dependent extends our previous in vitro observations with FRTL-5 cells (12). Assuming that much of the Tg in aminotriazole-treated rats is endocytosed from the colloid by megalin, we postulated that there would be increased serum levels of intact Tg, as a consequence of transcytosis. Indeed, serum levels of intact 330-kDa Tg were increased and were significantly correlated in individual rats with the levels of megalin expression by thyroid cells. Furthermore, serum T3 levels were reduced and inversely correlated with the levels of megalin expression in the thyroid. An initial reduction of serum thyroid hormones is known to result from the effect of aminotriazole on iodination of newly synthesized Tg, which is the first form of Tg to be endocytosed (40). This leads to increased TSH secretion from the pituitary, which is thought to compensate for the suppressive effect of aminotriazole on thyroid hormone release by increasing endocytosis of previously stored Tg, most of which is fully iodinated and hormonogenic (40). However, low levels of T3 were still present after 6 and 12 days of aminotriazole treatment, indicating that despite massive colloid endocytosis there was no proportionate release of thyroid hormones. The findings of increased serum Tg levels, combined with low levels of T3 and with increased megalin function on thyrocytes, support our hypothesis that Tg internalized via megalin is not subjected to proteolytic cleavage in the lysosomal pathway but rather transcytosed from the colloid into the bloodstream. Nevertheless, the evidence of megalin-mediated transcytosis of Tg in vivo is indirect and requires further documentation.

Earlier studies (15–21) have shown that megalin can mediate endocytosis of various ligands, with transport to lysosomes and degradation, in several types of absorptive epithelial cells, to which its expression is largely restricted (22–23). However, Zlokovic and associates (58, 59) provided evidence that megalin is expressed in low levels on brain endothelial cells, where it can mediate transcytosis of apolipoprotein J across the blood-brain barrier. Several other ligands internalized by receptors on brain endothelial cells, including ligands of the LDL receptor and of the LDL receptor-related protein, have been shown to be transcytosed intact across the blood-brain barrier (58). In other cells, notably epithelial cells and fibroblasts, the same ligands are transported to lysosomes and degraded following receptor-mediated endocytosis (15–21, 60). These results indicate that brain endothelial cells have special mechanisms that favor transcytosis. Nevertheless, in the present study we show that megalin can mediate transcytosis of Tg in two types of epithelial cells, thyrocytes and immortalized renal proximal tubule cells, in which other ligands internalized by megalin are degraded in lysosomes, including lactoferrin, as shown here. The results indicate that the ligand Tg somehow accounts for transcytosis in these cells. Because Tg is not normally present in the glomerular filtrate, the finding that megalin on immortalized renal proximal tubule cells mediates Tg transcytosis has no in vivo physiological significance by itself. Nevertheless, the finding suggests the possibility that some ligands reabsorbed by megalin from the glomerular filtrate are returned to the circulation by transcytosis. Further studies are needed to investigate this hypothesis.

The reasons why receptor-mediated endocytosis sometimes results in transcytosis of ligands are not entirely understood and clearly vary in different cells and with different receptors and ligands (36). One factor is the pH dependence of the ligand-receptor binding (61). Thus, many ligands dissociate from their receptors in prelysosomal endocytic vesicles, which have a pH of 5.2–5.0, following which ligands enter lysosomes (61). Ligands that do not dissociate at these low pH levels may remain combined with the receptor and bypass the lysosomal pathway to undergo transcytosis (33). Another possible mechanism for diverting Tg from its lysosomal pathway may be that megalin interferes with the interaction of Tg with molecules that target endocytosed proteins to lysosomes. In this regard, Tg has been shown to bear a signal that is recognized by the mannose 6-phosphate receptor (62), which is known to target lysosomal enzymes to lysosomes, either from the biosynthetic or endocytic pathway (63). Although endocytosis of Tg by thyroid cells does not appear to be mediated by mannose 6-phosphate receptors (7), it is possible that these receptors favor transport to lysosomes of Tg molecules that have been endocytosed by other means. Thus, it is conceivable that Tg complexed with megalin is not recognized by the mannose 6-phosphate receptors, which might thereby reduce Tg targeting to the lysosomal pathway. Further studies are needed to investigate the mechanisms by which transcytosis of Tg via megalin occurs.

Transcytosis of Tg endocytosed from the colloid is thought to be one of the mechanisms that account for the presence of intact Tg in the circulation (42, 49, 50), where the levels have been shown to be increased under conditions with heightened TSH stimulation (64, 65). Although circulating Tg can be degraded by macrophages with extrathyroidal release of thyroid hormone (66, 67), the contribution of this mechanism to the total amount of thyroid hormone in the circulation is likely to be negligible, as compared with the contribution of intrathyroidal degradation of Tg. Thus, diversion of Tg from its degra-
pathway in the thyroid as a consequence of megalin-mediated transcytosis may effectively reduce the extent of T4 and T3 release and their levels in the circulation. For this hypothesis to be true, transcytosed Tg should be hormonogenic. Indeed, we were able to demonstrate that FRTL-5 cells can release T3 from transcytosed Tg (collected from the lower chamber and purified) (not shown).

Although the evidence that megalin mediates Tg transcytosis rather than its transport to the lysosomal pathway was unexpected, certain considerations indicate the possible benefit of such a function. High affinity receptors serve to mediate endocytosis of ligands that are present in low concentrations in extracellular fluids and thereby to compete with fluid phase endocytosis. However, Tg in the colloid is very highly concentrated, suggesting that uptake by fluid phase or low affinity receptors should be sufficient mechanisms for hormone release (1, 2, 68). Thus, Tg in the colloid reaches concentrations up to 750 mg/ml, much of which is in a covalently cross-linked multimerized insoluble form, with an average concentration in humans of 590 mg/ml (68). Newly synthesized Tg, which is soluble and also very highly concentrated (1, 2, 68), is thought to be the first available for endocytosis (the “last come first served” hypothesis) (68, 69). We propose that megalin competes with fluid phase uptake and low affinity receptors, especially under circumstances that lead to massive Tg endocytosis, such as intense TSH stimulation, thereby preventing excessive hormone release.

A possible example in human disease where megalin-mediated transcytosis of Tg may be beneficial is Graves disease, where, by competing with mechanisms that lead to Tg proteolytic cleavage in the lysosomal pathway, it may help reduce excessive thyroid hormone release. In this disease, TSH receptor-stimulating autoantibodies mimic the effects of TSH on thyroid cells (70), resulting in markedly increased Tg endocytosis with colloid depletion (71), associated with enhanced levels of circulating intact Tg (72). On the other hand, it can be argued that Tg transcytosis may be harmful under certain conditions, in particular iodine deficiency, where the process could lead to a loss of a critical Tg reserve available for hormone release. However, TSH is not appreciably increased in conditions, in particular iodine deficiency, where the process of such a function. High affinity receptors serve to mediate endocytosis of ligands that are present in low concentrations in extracellular fluids and thereby to compete with fluid phase endocytosis.

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