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invited review

Role of thyroglobulin endocytic pathways in the control of thyroid hormone release

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Marinò, Michele, and Robert T. McCluskey. Role of thyroglobulin endocytic pathways in the control of thyroid hormone release. *Am J Physiol Cell Physiol* 279: C1295–C1306, 2000.—Thyroglobulin (Tg), the thyroid hormone precursor, is synthesized by thyrocytes and secreted into the colloid. Hormone release requires uptake of Tg by thyrocytes and degradation in lysosomes. This process must be precisely regulated. Tg uptake occurs mainly by micropinocytosis, which can result from both fluid-phase pinocytosis and receptor-mediated endocytosis. Because Tg is highly concentrated in the colloid, fluid-phase pinocytosis or low-affinity receptors should provide sufficient Tg uptake for hormone release; high-affinity receptors may serve to target Tg away from lysosomes, through recycling into the colloid or by transcytosis into the bloodstream. Several apical receptors have been suggested to play roles in Tg uptake and intracellular trafficking. A thyroid asialoglycoprotein receptor may internalize and recycle immature forms of Tg back to the colloid, a function also attributed to an as yet unidentified *N*-acetylglucosamine receptor. Megalin mediates Tg uptake by thyrocytes, especially under intense thyroid-stimulating hormone stimulation, resulting in transcytosis of Tg from the colloid to the bloodstream, a function that prevents excessive hormone release.

receptor; endocytosis

THYROGLOBULIN (Tg), the precursor of thyroid hormones, is synthesized by thyrocytes and secreted into the lumen of thyroid follicles, where it is stored as the major component of colloid. At the cell-colloid interface, post-transitional modifications of Tg occur, which are characterized by coupling of tyrosyl residues with iodide, leading to the formation of thyroid hormone residues within the Tg molecule. Hormone release generally requires uptake of Tg from the colloid by thyrocytes and proteolytic cleavage along the lysosomal pathway. However, thyroxine (T4), but not triodothyronine (T3), can also be released to some extent by extracellular proteolysis of Tg within the colloid, at the apical surface of thyrocytes (8, 28). Furthermore, some Tg molecules that reach the circulation can be degraded peripherally after uptake by macrophages, especially Kupffer cells (7, 9). However, the contribution of this mechanism to the total pool of circulating thyroid hormones is probably minimal.

It is obvious that the process of internalization and degradation of Tg by thyrocytes must be strictly regulated to provide appropriate amounts of thyroid hormones and to avoid excessive hormone release. A major problem stems from the extremely high concentration of Tg within the colloid, which can reach up to 800 mg/ml. Even though much of the Tg is insoluble and, therefore, not readily available for uptake, the concentration of soluble Tg available to thyrocytes is probably also relatively high. If effective mechanisms of Tg internalization (whether receptor-mediated or fluid-phase uptake) were to deliver hormone-rich forms of Tg to lysosomes unchecked, excessive hormone release would ensue. Indeed, the problem of avoiding excessive hormone release may be of greater importance than the often discussed issue of how to avoid the wasteful process of internalizing and degrading immature forms of Tg that are poor in hormone residues. Figure 1 illustrates the principal intracellular pathways that Tg

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follows after micropinocytosis. In the present review, we consider mechanisms that are known or have been postulated to promote and control Tg endocytosis by thyroid cells and hormone release, under physiological and pathological conditions, with particular emphasis on the role of Tg receptors. To introduce the subject, we review the general features of structure, synthesis, and secretion of Tg.

STRUCTURE, SYNTHESIS, AND SECRETION OF TG

In its major form, Tg is a 660-kDa glycoprotein with a sedimentation coefficient of 19S, composed of two identical 330-kDa subunits (monomers) (28). Complete sequences of human, bovine, and mouse Tg cDNA have been obtained (12, 57, 100), as well as a partial sequence of rat Tg cDNA (23, 34). The ~8.5-kb human Tg gene is located on the long arm of chromosome 8 and encodes 2,767 amino acid residues, representing the Tg monomer (28, 57, 100). Transcription of the Tg gene requires a complex interplay of at least three transcription factors (TTF1, TTF2, and Pax 8), which are also involved in transcription of thyroperoxidase (TPO) and of the thyroid sodium/iodide symporter (NIS) genes (22, 73). The thyroid-stimulating hormone (TSH) upregulates Tg synthesis by acting directly on the Tg promoter and indirectly through the thyroid transcription factors (22, 28).

The thyroid gland is composed of histological/functional units named follicles that consist of a single layer of polarized epithelial cells (thyrocytes) that surround a spherical lumen that contains colloid, a viscous substance composed mainly of Tg (10, 27, 28). Tg is produced exclusively by thyrocytes and follows the usual biosynthetic pathway. It is synthesized and initially processed in the endoplasmic reticulum with the formation of dimers and with addition of *N*-linked glycoside residues, and then is further processed in the Golgi apparatus, especially by modification of carbohydrate residues (43, 44, 46, 47).

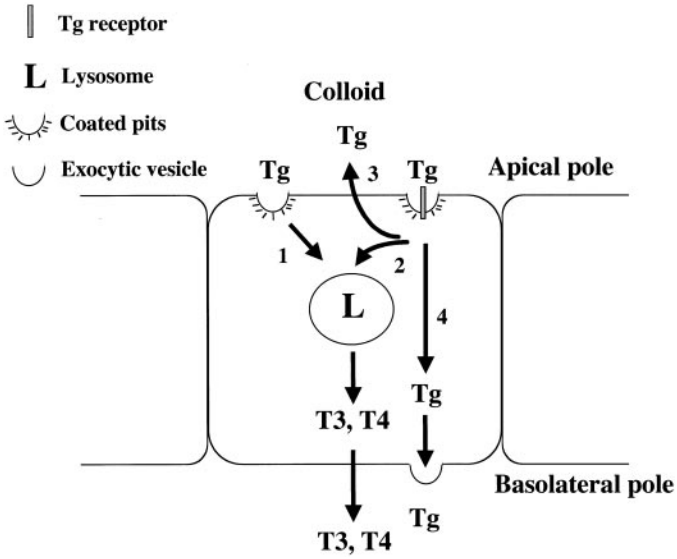

Fig. 1. Schematic representation of the principal intracellular pathways of thyroglobulin (Tg) after endocytosis by thyroid epithelial cells. *1*: Tg is internalized by fluid-phase nonspecific micropinocytosis and transported to lysosomes, where it is degraded with release of thyroid hormones [triodothyronine (T3) and thyroxine (T4)]. *2*: Tg is internalized by an unidentified low-affinity receptor and possibly transported to lysosomes. *3*: Tg is internalized by a receptor (possibly the asialoglycoprotein receptor) and recycled back into the colloid. *4*: Tg is internalized by megalin (gp330) and transported by transcytosis at the basolateral surface of thyroid cells, where it is released by exocytosis into the bloodstream. This process reduces the extent of thyroid hormone release because Tg escapes the lysosomal pathway.

Tg molecules contain numerous *N*-linked complex carbohydrate groups (for review, see Ref. 28). In addition, there are two *O*-linked groups, including chondroitin sulfate units and phosphate (28). During its transit through the Golgi apparatus, some high-mannose type *N*-glycans are converted into *N*-acetyllactosamine chains, some of which have accessible *N*-acetylglucosamine moieties (28, 68). Tg is transported via vesicles from the trans-Golgi network to the apical surface of thyrocytes, where it is released into the lumen of thyroid follicles and stored as the major protein component of colloid (>95%). There is evidence that secretion of Tg is a regulated process rather than a constitutive process (1, 45). This may be useful in achieving balance between release and uptake of Tg, which, under normal conditions, must be equal.

Formation of thyroid hormone residues within the Tg molecule occurs at the cell-colloid interface by coupling of tyrosyl residues of Tg with iodide, a process that is carried out by TPO. Five hormonogenic sites have been identified in human Tg (28), and it has been estimated that, under physiological conditions, a Tg molecule contains on average 2.28 molecules of T4 and 0.29 molecules of T3 (95). However, the degree of hormone content varies among different Tg molecules (28, 35), and Tg molecules that are poor in iodide and hormone content are usually referred to as immature. Tg molecules within the colloid also differ with respect to the extent and type of glycosylation, and there is a correlation between the iodine content of Tg and the nature of its complex carbohydrate residues (28, 91).

Heterogeneity of Tg within the colloid is not restricted to its iodine and carbohydrate content. Thus, although the predominant form of Tg is the 19S, 660-kDa dimer, free 330-kDa monomers can be found in minimal amounts (27, 28). Reduction or degradation of 660-kDa and 330-kDa Tg molecules can lead to the formation of smaller polypeptides, some of which are present in trace amounts in the colloid. Furthermore, some Tg dimers form tetramers with a coefficient of sedimentation of 27S, which are also found in minimal amounts in the colloid (28).

Because newly secreted Tg molecules are soluble and adjacent to the apical surface of thyrocytes, they are the first available for uptake by thyrocytes ["last come first served" hypothesis, proposed and supported by elegant experiments by Schneider (87)]. Newly secreted molecules that escape uptake proceed into the colloid, where they undergo further modifications, characterized in part by the formation of highly con-

centrated (up to 750 mg/ml), insoluble, covalently cross-linked, multimerized forms (3). Thus most of the Tg in the colloid is in storage, to be solubilized and taken up by thyrocytes only under special circumstances, such as iodine deprivation or intense TSH stimulation.

MECHANISMS OF Tg RETRIEVAL BY THYROID CELLS

In rodents, thyrocytes can internalize Tg from the colloid by phagocytosis (macropinocytosis) as the result of acute TSH stimulation, as described by Wetzel et al. (103) in 1965 and later by van den Hove et al. (99). After TSH injection in hypohysectomized rats, pseudopods form at the apical membrane of thyrocytes and engulf luminal material, forming so-called "colloid droplets" that fuse with lysosomes, where Tg is degraded (99, 103). However, the importance of this pathway is unclear, because pseudopods and colloid droplets have not been observed in species other than rats (27, 83).

In most species, including humans, uptake of Tg by thyrocytes occurs exclusively by micropinocytosis, which can be either nonspecific (fluid phase) or receptor mediated (Fig. 1). Both forms of micropinocytosis (also called endocytosis or vesicular internalization) involve the formation of small vesicles at the plasma membrane, mainly in coated pits, which invaginate to form intracellular vesicles that fuse with endosomes (79, 97). Nonspecific endocytosis is a constitutive process by which substances are taken up in proportion to their concentration in the adjacent extracellular fluid (79, 97). In contrast, receptor-mediated endocytosis involves specific binding of certain substances (ligands) to cell surface receptors, often with high affinity, with the result that even minor components of the extracellular fluid can be internalized in large amounts (79, 97). It is unlikely that high-affinity receptors are needed to mediate internalization of large amounts of Tg, in view of its high concentration in the colloid. As discussed later, a high-affinity Tg receptor (megalin, gp330) is expressed on the apical surface of thyrocytes, but its function is controlled in ways that prevent excessive hormone release. There is also evidence of low-affinity receptors on thyrocytes, but their role in Tg uptake is not firmly established.

There is evidence that micropinocytosis of Tg can occur both by fluid-phase uptake and receptor-mediated endocytosis, although the relative importance of these two pathways is uncertain and may vary under different conditions. Micropinocytosis of Tg was first described by Seljelid et al. (88) in 1970, who identified small endocytic vesicles that contained Tg in rat thyroid cells in vivo. Later, Bernier-Valentin et al. (4) described clathrin-coated microvesicles that contained Tg in vivo and in cultured pig thyroid follicles. Although the presence of Tg in clathrin-coated pits is sometimes cited as evidence of receptor-mediated endocytosis, substances taken up nonspecifically can also be demonstrated in these structures, especially if they are highly concentrated in extracellular fluids (79, 97). Indeed, Kostrouch et al. (50) found that gold-labeled Tg and albumin were internalized to a similar extent by micropinocytosis via coated pits in porcine thyroid cells.

One way to distinguish between receptor-mediated endocytosis and nonspecific uptake is to determine whether the process is saturable (54). Thus receptors, especially those of high affinity, become completely occupied even at relatively low concentrations of ligands and are rendered incapable of further uptake (54). In contrast, fluid-phase uptake, which is virtually nonsaturable, continues to increase in proportion to the concentrations of ligands in the extracellular fluid. In studies performed with a differentiated rat thyroid cell line (FRTL-5 cells), we found that, although Tg uptake was in part receptor mediated (by megalin) (62), the entire process was not saturable (Marinò M, Zheng G, and McCluskey RT, unpublished observations), suggesting that nonspecific Tg uptake prevails when thyroid cells are exposed to high concentrations of Tg, as may occur in vivo. Similar conclusions had been previously reached by van den Hove et al. (99) using thyroid hemilobes in culture.

Another way to investigate receptor-mediated endocytosis vs. nonspecific uptake is to determine whether certain forms of Tg (differing from others with respect to their hormone content or type of glycosylation) are preferentially taken up from the colloid. Although there is some evidence of selective uptake of different forms of Tg, as discussed in detail later, interpretation of such studies is hampered by insufficient knowledge of the concentration of different forms of Tg in the colloid adjacent to thyrocytes, confounded by the knowledge that certain forms of internalized Tg are recycled to the apical surface of thyroid cells, which could lead to the erroneous conclusion that there is reduced uptake. There are no satisfactory methods to investigate the problem of selective uptake of Tg in vivo. On the basis of the assumption that the concentration of soluble Tg is even only moderately high, and that the constitutive process of vesicular internalization is as active as in other cells, fluid-phase uptake of Tg should be considerable. On the other hand, it is conceivable that in the absence of intense TSH stimulation, constitutive micropinocytosis by thyrocytes is normally low, thereby preventing excessive hormone release and colloid depletion.

INTRACELLULAR FATES OF INTERNALIZED Tg MOLECULES

After endocytosis, vesicles that contain Tg quickly lose their clathrin coats and fuse with early endosomes (4). However, internalized Tg can then follow different intracellular pathways. Kostrouch et al. (50) tracked the intracellular route of microvesicles that contained Tg, with the use of immunogold-labeled Tg molecules and antibodies against organelle markers, and found that some Tg is transported through early endosomes to lysosomes. During this route, there is progressive loss of Tg immunoreactivity, which indicates Tg deg-

radation (27, 83). However, Kostrouch et al. (49) also provided evidence that some Tg molecules internalized by thyroid cells can be recycled back into the follicle lumen, probably mainly through the Golgi apparatus, as indicated by experiments of Miquelis and associates (68). Herzog and associates (36, 81) have demonstrated another pathway of endocytosed Tg that avoids the lysosomes, namely, vesicular transport from the apical to the basolateral surface (transcytosis) (70). This pathway is considered to be one of the mechanisms that account for the presence of Tg in the circulation (21, 25, 26, 36, 81). As discussed later, the cell surface receptor megalin has been shown to mediate Tg transcytosis (61).

TG RECEPTORS ON THYROID CELLS

Over the last 20 years, several attempts have been made to identify and characterize Tg receptors on thyroid cells. As reviewed by Hatipoglu and Schneider (35), specific receptors might be found either on the apical surface, where they could mediate endocytosis, or on intracellular membranes, where they might influence intracellular trafficking. Several candidate receptors have been proposed (Table 1), and recently we provided evidence that megalin can mediate Tg endocytosis. Next, we review the various putative or established receptors and their roles in Tg endocytosis and intracellular trafficking.

The Thyroid Asialoglycoprotein Receptor

In 1979, Consiglio et al. (18) demonstrated binding of ^{125}I-labeled bovine Tg to thyroid membrane preparations as well as to membrane preparations from nonthyroid tissues (liver, brain, retroorbital tissue). Specificity of binding was shown by the inhibitory effect of unlabeled Tg. The binding of Tg to thyroid membranes was optimal at low pH (~4.5), and it was selective for poorly iodinated forms of Tg. Our analysis of their data obtained in inhibition experiments with unlabeled Tg (18) indicates that the binding affinity of Tg to the thyroid membrane preparation used was rather low [dissociation constant (K_d) ~800 nM].

Because binding of Tg to thyroid membranes was markedly increased by enzymatic removal of sialic acid units from Tg molecules, it was suggested that the responsible receptor might be similar to the asialoglycoprotein receptor of the liver (18). The hepatic receptor is expressed on the basolateral (sinusoidal) surface of hepatocytes, where it serves to bind and internalize serum glycoproteins from which sialic acid residues have been removed, therefore having exposed galactose or *N*-acetylgalactosamine residues (2, 24). Additional evidence of a thyroid asialoglycoprotein receptor was obtained in studies based on binding of partially deglycosylated Tg preparations to thyroid membranes or to thyroid cells in culture (19, 80, 82, 89). However, in these studies, it was not possible to identify the carbohydrate residues of Tg responsible for binding or to exclude a role of unrelated receptors or other mechanisms of binding (19).

Direct demonstration of the thyroid asialoglycoprotein receptor was made only after cDNA encoding the rat hepatic receptor became available (24). The deduced structure of the rat hepatic asialoglycoprotein receptor showed it to be an integral transmembrane glycoprotein composed of three subunits, named rat hepatic lectin-1 (RHL-1), rat hepatic lectin-2 (RHL-2), and rat hepatic lectin-3 (RHL-3) (24). The three subunits are encoded by two separate genes, one for RHL-1 and one for RHL-2 and RHL-3 (24). Although it was initially thought that the asialoglycoprotein receptor was expressed exclusively in the liver, more recently, mRNAs for RHL-1 and RHL-2/3 have been found in several rat tissues (33). In 1995, Pacifico et al. (75) demonstrated that asialoglycoprotein receptor mRNA is present in the thyroid of adult rats and showed that the protein is expressed on the apical surface of polarized FRT cells, a poorly differentiated rat thyroid cell line (75). Furthermore, the same group (76) showed that the levels of expression of the RHL-1 subunit of the asialoglycoprotein receptor in a rat thyroid cell line

Table 1. *Tg endocytic receptors and their role in Tg endocytosis and intracellular trafficking*

Characterized Receptors	Tg Motifs Recognized	Role	References
Asialoglycoprotein receptor	Exposed galactose or *N*-acetylgalactosamine residues	Recycling of immature Tg molecules?	18, 19, 69, 75, 76, 80, 82
Megalin (gp330)	Multiple amino acid determinants	Endocytosis and transcytosis	60–62, 108
Postulated or Poorly Characterized Receptors	**Tg Motifs Recognized**	**Postulated Role**	**References**
N-acetylglucosamine receptor	Exposed *N*-acetylglucosamine residues	Recycling of immature Tg	5, 6, 19, 65, 67, 68, 72, 96
Protein disulfide isomerase	Exposed *N*-acetylglucosamine residues	Recycling of immature Tg	65
Mannose-6-phosphate receptor	Mannose-6-phosphate groups	Tg targeting to lysosomes	37, 53, 86
Low-affinity receptors	?	?	31, 32, 53
Heparan sulfate proteogycans	Positively charged protein determinants	Facilitate binding to megalin in rodents	58, 60

Tg, thyroglobulin.

(PCC13) are dependent on the presence of TSH in the cell culture medium, suggesting a thyroid-specific function of the receptor. In addition, they showed in ligand blot binding assays that asialo-Tg binds to a recombinant protein that corresponds to the carbohydrate recognition domain of the RHL-1 subunit (rCRD-RHL-1) of the asialoglycoprotein receptor (76). In another study (69), the same group provided evidence for saturable surface binding of rat Tg to cultured PCC13 cells, which was inhibited by rCRD-RHL-1 and by an antiserum raised against rCRD-RHL-1. Our evaluation of the data from Montuori and associates (69) suggests that Tg bound with high affinity to PCC13 cells (K_d ~1.25 nM) and with low affinity to the asialoglycoprotein receptor on the cell surface of PCC13 cells [inhibition constant (K_i) ~500 nM]. Furthermore, they showed that rCRD-RHL-1 specifically binds to rat desialated Tg. However, Tg binding to the recombinant RHL-1 subunit of the asialoglycoprotein receptor does not reproduce the conditions under which binding occurs to the intact receptor on cells. Thus it is likely that high-affinity ligand binding requires interaction with three adjacent units (RHL-1, RHL-2, and RHL-3), as has been shown for hepatic binding of asialoglycoproteins (84). Furthermore, only ligands with three adjacent exposed galactose residues bind avidly to the hepatic receptor (84), and the interrelationship of galactose residues on asialo-Tg is unknown. Unexpectedly, deglycosylation of Tg did not affect its binding ability to rCRD-RHL-1, suggesting that protein determinants of Tg are important for binding (69). Similarly, there is evidence that the hepatic asialoglycoprotein receptor can recognize amino acid determinants in addition to carbohydrates, as shown by its ability to bind deglycosylated lactoferrin (2). However, further studies are needed to define the nature and importance of the protein determinants. In an attempt to identify Tg binding sites for the asialoglycoprotein receptor, Montuori et al. (69) found that binding to rCRD-RHL-1 was restricted to a 68-kDa Tg polypeptide, derived from native Tg by thermolysin digestion. The 68-kDa Tg polypeptide was found to correspond to the NH_2-terminal region of Tg, which includes an important site of T4 formation (12, 23, 34, 57, 100).

Although the primary structures of the hepatic and thyroid asialoglycoprotein receptors are probably the same, they may be different in the organization of the subunits on the cell surface or in glycosylation, which could influence ligand binding. Furthermore, the hepatic receptor normally functions optimally at neutral pH, whereas the thyroid asialoglycoprotein receptor shows optimal binding at low pH.

The function of the asialoglycoprotein receptor in the thyroid has not been firmly established. Nevertheless, it seems likely that this receptor serves to internalize preferentially immature Tg molecules and divert them from the lysosomal pathway, as initially postulated by Consiglio and associates (18, 19). Thus the receptor is known to be expressed on the apical surface of cultured thyrocytes (75), and if it is similarly located on thyrocytes in vivo, it would be in a position to participate in Tg uptake from the colloid, even though its affinity for Tg may be low, especially at neutral pH. Endocytosis by the receptor would probably favor immature Tg molecules, which have a lower level of sialation (28). Because Tg binds to the asialoglycoprotein receptor not only at neutral pH (69) but also at low pH, it is likely that any Tg internalized by the asialoglycoprotein receptor would avoid the lysosomal pathway. Thus many ligands dissociate from their receptors in prelysosomal endocytic vesicles, which have a pH of ~5, after which ligands enter lysosomes (20). Ligands that do not dissociate at these low pH levels may remain combined with the receptor and bypass the lysosomal pathway, to undergo either recycling to the cell surface from which they have been internalized or transcytosis to the opposite surface (20, 100).

Recently, evidence of an unexpected function of the thyroid asialoglycoprotein receptor has been reported. In studies designed to investigate autocrine functions of Tg, Kohn and associates (93, 94, 98) have obtained evidence that the interaction of asialo-Tg with the asialoglycoprotein receptor results in suppression of several thyroid-specific genes, including those encoding TTF1, TTF2, Pax 8, or NIS. The mechanism by which this endocytic receptor may function in signal transduction remains to be established.

The Postulated N-Acetylglucosamine Receptor

In 1981, Consiglio et al. (19) reported that *N*-acetylglucosamine residues of Tg are important for its binding to thyroid membrane preparations. In particular, they showed that digestion of asialo-Tg with galactosidase, which exposes *N*-acetylglucosamine residues, resulted in increased binding of Tg to thyroid membranes, compared with native Tg or with asialo-Tg. The role of *N*-acetylglucosamine determinants was later extensively studied by Miquelis and co-workers (67). In 1987, using radiolabeled asialoagalacto-bovine serum albumin (BSA) as a probe, they found apparently high-affinity (saturation point 13 nM), calcium-dependent binding to thyroid membrane preparations, which was inhibited by unlabeled native Tg and, to an even greater extent, by asialo-Tg and asialoagalacto-Tg. Because the studies employed thyroid membrane preparations rather than purified receptors, the possibility that more than one receptor accounted for binding cannot be excluded. In particular, as discussed later, binding of asialo-Tg, which has exposed galactose but not exposed *N*-acetylglucosamine groups, suggests a role of the asialoglycoprotein receptor.

In 1993, Miquelis et al. (68) proposed that the postulated *N*-acetylglucosamine receptor serves to recycle immature forms of Tg back to the colloid. They used radiolabeled asialoagalacto-BSA as a probe for receptor-mediated endocytosis and radiolabeled mannose-BSA as a marker of fluid-phase endocytosis. They found that internalization of the two compounds by thyroid hemilobes in primary cultures resulted in two

different intracellular fates. Thus mannose-BSA was mainly degraded by thyroid cells, whereas asialoagalacto-BSA was released undegraded in substantial amounts after endocytosis. Furthermore, radiolabeled ovomucoid, a glycoprotein with exposed *N*-acetylglucosamine residues, was also used as a probe for receptor-mediated endocytosis, and it was found to accumulate in the Golgi after endocytosis. The authors postulated that the receptor bears a signal that prevents transport of proteins with exposed *N*-acetylglucosamine units to the lysosomal pathway and instead targets them to the Golgi apparatus for reprocessing and return to the colloid.

In 1996, Mziaut et al. (72) showed that binding of Tg to FRTL-5 cells was not appreciably inhibited by coincubation of the cells with Tg plus asialo-agalacto-BSA, suggesting that not only carbohydrate determinants but also protein determinants of Tg are involved in its binding to thyroid cells and that protein and carbohydrate determinants cooperate in binding. Furthermore, in another study, the same group determined that a peptide sequence in the NH_2-terminal portion (Ser-798–Met-1172) is involved in Tg binding to thyroid cell membranes (66). As noted earlier, the asialoglycoprotein receptor also recognizes protein determinants of Tg (69), which may, therefore, account for some of the binding of deglycosylated Tg seen by Mziaut and associates (72).

To localize the postulated *N*-acetylglucosamine receptor, Miquelis and associates (67) applied radiolabeled asialoagalacto-BSA to rabbit and porcine thyroid sections and found binding mainly to the apical surface of thyrocytes and in subapical regions. In an attempt to characterize the receptor, the same group (67) identified and purified a ~45-kDa molecule, which was found to bind to asialoagalacto-BSA. In a later study (96), with the use of antibodies developed against the ~45-kDa molecule, they showed by immunofluorescence its apical and subapical localization in thyroid cells. However, cloning of this molecule, which was first thought to be the elusive *N*-acetylglucosamine receptor (5), was, on further analysis, conclusively shown to be an unrelated protein, hnRNP M (6).

Although an *N*-acetylglucosamine receptor has not been isolated, Miquelis and associates (68) have provided compelling evidence for a mechanism that functions at acidic pH to bind proteins with exposed *N*-acetylglucosamine residues and recycle them after their internalization by thyroid epithelial cells. However, the evidence that Tg recycling is mediated by a yet unidentified *N*-acetylglucosamine receptor is indirect in part because, as mentioned earlier, it derives from studies in which asialoagalacto-BSA, and not Tg, was used as a ligand. It is worth considering the possibility that the functions attributed to the postulated *N*-acetylglucosamine receptor are in fact carried out by another receptor, in particular the asialoglycoprotein receptor. Thus studies performed by separate groups have provided evidence that Tg binding to the asialoglycoprotein receptor (18, 19, 69) and to a putative *N*-acetylglucosamine receptor (66) occurs at low pH and involves the NH_2-terminal portion of the Tg molecule. However, as discussed next, there are also some reasons to suspect that a molecular chaperone, protein disulfide isomerase (PDI), rather than a membrane receptor, may account for the functions believed to result from a *N*-acetylglucosamine receptor.

Protein Disulfide Isomerase

Recently, Mezgrhani et al. (65) studied the role of PDI in Tg binding to FRTL-5 cells. PDI is principally a resident endoplasmic reticulum protein, which is thought to function as a chaperone in the Tg synthetic pathway. Mezgrhani et al. (65) showed that PDI is also secreted by FRTL-5 cells and combines with the cell membrane. The binding of Tg to FRTL-5 cells at low pH was inhibited by antibodies against PDI and was selective for immature forms of Tg (65). On the basis of the finding that PDI binds Tg at low pH levels (ranging from 5.0 to 6.0), the authors postulated that PDI-Tg interactions may occur in vivo in any of the acidic compartments of the exocytic and endocytic pathways (65). Interaction of PDI with Tg internalized from the colloid might, therefore, occur in endosomes or prelysosomes.

The same authors noted that PDI has several characteristics of the postulated *N*-acetylglucosamine receptor discussed earlier, including similar estimated molecular masses and the ability to bind to ovomucoid, an *N*-acetylglucosamine-bearing glycoprotein that competes with Tg binding sites on the thyroid cell membrane (65). They suggested that PDI may be responsible for the recycling of immature Tg molecules from the endosomal compartment back to the colloid rather than an unidentified *N*-acetylglucosamine receptor. However, further studies are clearly needed to investigate this hypothesis.

The Mannose 6-Phosphate Receptor

It is well established that after lysosomal enzymes are synthesized in the endoplasmic reticulum, they acquire a mannose 6-phosphate group in the Golgi apparatus, which targets them to lysosomes rather than the secretory pathway (41, 101). The targeting occurs via a Golgi membrane receptor that binds protein bearing exposed mannose 6-phosphate residues (41, 101). In 1987, Herzog and associates (37) provided evidence that porcine Tg bears exposed mannose 6-phosphate groups, despite the fact that it is not transported to lysosomes from the biosynthetic pathway but, rather, secreted into the colloid. Two years later, Scheel and Herzog (86) performed an immunohistochemical study of the mannose 6-phosphate receptor in cultured thyroid cells and showed that its pattern of distribution differs from that of other cell types. Thus only trace amounts of the receptor were found in the Golgi apparatus, which may explain why Tg is not targeted to lysosomes from the synthetic pathway. Presumably, lysosomal enzymes bind to the receptor in the Golgi apparatus with greater affinity. Predominant localization of the mannose 6-phosphate receptor was

found in elements of the endocytic pathway, such as coated pits and endosomes, leading to the hypothesis that the mannose 6-phosphate receptor may somehow be involved in Tg endocytosis. However, in 1992, Lemansky and Herzog (53) reported that Tg endocytosis by cultured pig thyroid follicles with reverse polarity does not involve exposed mannose 6-phosphate groups, but the study did provide evidence for low-affinity Tg binding sites, as discussed later. Although it is conceivable that the mannose 6-phosphate receptor in endosomes may help target Tg internalized by other means to the lysosomal pathway, Lemansky and Herzog (53) found no evidence that the extent of Tg degradation by cultured pig thyroid follicles was related to mannose 6-phosphate groups.

Low-Affinity Binding Sites

In studies mentioned earlier, Lemansky and Herzog (53) found that radiolabeled Tg bound with low affinity to porcine thyrocytes, in a saturable manner and that uptake of radiolabeled Tg by thyroid cells was inhibited by unlabeled Tg. However, the receptor(s) has not been identified. Nevertheless, the findings support a role of low-affinity receptors in Tg uptake, which could be important under special circumstances. Thus the receptor(s) may not be able to compete with fluid-phase uptake under physiological conditions but may do so under conditions that upregulate its expression. Further studies are needed to investigate this possibility.

Recently, Giraud et al. (31) obtained evidence of selective, moderately high-affinity binding of radiolabeled Tg to thyrocytes in cultured "inside out" porcine follicles as well as to cultured Chinese hamster ovary cells and Madin-Darby canine kidney cells. More recently, the authors have provided preliminary evidence that Tg interaction with these binding sites occurs through a sequence in the COOH-terminal region of human Tg (32). The receptors responsible for binding have not been identified, and it is not known whether they play a role in Tg endocytosis.

Megalin (gp330)

Megalin was first identified as the major pathogenic autoantigen in Heymann nephritis, a rat model of membranous glomerulonephritis (42, 43). However, there is no evidence that a homologous antigen is involved in human membranous glomerulonephritis (15, 59). In 1989, partial cDNAs encoding megalin were isolated that showed homology with the low-density lipoprotein (LDL) receptor, indicating that megalin is a member of the LDL receptor family (78), which includes the LDL receptor, the very low-density lipoprotein receptor, and the LDL receptor-related protein (39, 104). Saito et al. (85) subsequently obtained complete rat megalin cDNA, which encodes a protein composed of 4,660 amino acid residues. The complete primary structure of human megalin has also been obtained, and it is shown to be highly homologous to rat megalin and similar in size (38). The structure of megalin is characterized by a large extracellular domain, with four cysteine-rich ligand binding repeats, a single transmembrane domain, and a relatively short cytoplasmic tail (38, 85). The cytoplasmic tail bears a sequence that leads to endocytosis after ligand binding on the cell surface. In immunohistochemical studies, megalin has been found principally on the apical surface of a restricted group of absorptive epithelial cells, including renal proximal tubule cells, epididymal cells, type II pneumocytes, and thyroid epithelial cells (55, 107). Studies carried out in vitro have shown that megalin can bind multiple unrelated ligands and that it can mediate endocytosis of ligands by cultured cells on which it is expressed (39, 51, 104, 106).

On the basis of the assumption that physiological ligands of megalin may be identified by consideration of the composition of fluids to which it is exposed in various organs (106), we postulated that megalin on thyrocytes serves as a receptor for Tg. In support of this, we found that Tg binds to megalin in solid-phase assays, with characteristics of high-affinity receptor-ligand interactions (108). Thus rat Tg bound to purified rat megalin in a concentration-dependent, calcium-dependent, saturable manner, with a mean estimated K_d of 9.2 ± 0.6 nM (108). In another study (60), we found that megalin binding sites of rat Tg are located in the COOH-terminal portion of the molecule.

We then demonstrated megalin expression by FRTL-5 cells and found that it was dependent on the presence of TSH in the cell culture medium, suggesting a thyroid-specific function of megalin (62). The TSH dependence of megalin expression was also demonstrated later in vivo in rats (61). To investigate megalin interactions with Tg in cultured cells, we used FRTL-5 cells and IRPT cells, an immortalized rat renal proximal tubule cell line that expresses megalin (62). Using several techniques, we found that Tg binds to megalin on the cell surface of both FRTL-5 and IRPT cells. The binding of Tg to megalin on FRTL-5 cells was saturable and of high affinity (K_d 11.2 ± 3.0 nM) and was markedly reduced by megalin competitors, namely, the receptor-associated protein and a monoclonal anti-megalin antibody. Furthermore, we found that megalin contributes to Tg uptake. Thus after incubation of FRTL-5 and IRPT cells with rat Tg at 37°C, we found that the amount internalized was reduced by ~50% by megalin competitors.

Because megalin has been shown to mediate uptake and transport of ligands to lysosomes in several cell types (39, 51, 104, 107), we originally expected that megalin-mediated endocytosis of Tg would lead to its degradation, with release of thyroid hormones. Instead, we found that Tg internalized by megalin on thyroid cells was not degraded but transported intact from the apical to the basolateral membrane of thyroid cells by transcytosis (61), a pathway originally described by Herzog and associates (36, 81). We first showed that release of T3 from Tg exogenously internalized by FRTL-5 cells, used as a measure of Tg degradation, was increased by megalin competitors,

suggesting that Tg internalized by megalin bypassed the lysosomal pathway and that megalin competes with other mechanisms of Tg endocytosis that lead to lysosomal degradation (61). To investigate transcytosis, we used FRTL-5 and IRPT cells cultured on permeable filters in dual-chambered devices, which permit the study of passage of substances added to the upper chamber across the cell layer to the lower chamber. Under the culture conditions used, FRTL-5 and IRPT cells showed features of polarity, notably megalin expression, exclusively at the upper surface of the cell layer, where microvilli and coated pits are seen by electron microscopy (61). Furthermore, FRTL-5 and IRPT cells form tight layers, as shown by the presence of junctional complexes demonstrated by electron microscopy, by the expression of the tight junction-associated protein occludin, and by the relatively low passage from the upper to the lower chamber of a molecule of very low mass, [^{3}H]mannitol (61).

After addition of preparations that contained both 660-kDa and 330-kDa Tg to the upper chamber and incubation at 37°C, intact 330-kDa Tg was found in fluids collected from the lower chamber (61), and the amount recovered was markedly reduced by megalin competitors. We also studied Tg transcytosis in vivo, with the use of a rat model of goiter induced by aminotriazole, in which increased release of TSH induced massive colloid endocytosis (92). We found that rats treated with aminotriazole for several days showed strikingly increased megalin expression on thyrocytes and increased serum Tg levels, with reduced serum T3 levels (61), which supports the conclusion that megalin mediates Tg transcytosis, diverting Tg from the lysosomal pathway.

Megalin-mediated transcytosis of Tg may serve to reduce the extent of thyroid hormone release mainly in special circumstances. Under physiological conditions (normal TSH stimulation), megalin expression on thyrocytes is relatively low (61, 62), and the knowledge that serum Tg levels in normal subjects are low or undetectable (90) is consistent with the interpretation that transcytosis is normally low. However, under conditions of intense TSH stimulation, such as in aminotriazole-treated rats, there is a striking increase of megalin expression on thyrocytes, associated with heightened megalin-mediated transcytosis of Tg, as evidenced by elevated levels of circulating Tg and reduced levels of serum T3. This indicates that internalized Tg has bypassed the lysosomal pathway (61). Further studies are needed to investigate this hypothesis in patients with increased TSH or TSH-like stimulation, such as those with Graves' disease.

Even though transcytosis of Tg by megalin prevents hormone release within thyrocytes, some hormone release may occur after uptake of the transcytosed circulating Tg by macrophages, especially Kupffer cells, as mentioned earlier (8, 28). This process may contribute to the total pool of thyroid hormones in the circulation, although only to a minimal extent.

Heparan Sulfate Proteoglycans

Several megalin ligands are heparin-binding proteins, and heparin inhibits their interaction with the receptor (39, 51, 104). These findings also apply to rat Tg (60, 62, 108). Thus we have found that *1*) rat Tg is a moderately high-affinity heparin-binding protein (K_d ~47 nM) (60, 62); *2*) heparin inhibits rat Tg binding to megalin in solid-phase assays and releases rat Tg from immobilized megalin (108); and *3*) heparin releases virtually all of the rat Tg bound to the surface of cultured thyroid cells, from both megalin and other binding sites (62).

Studies that deal with proteins that bind to heparin and to cell surface receptors, including members of the LDL receptor family, have provided evidence that the heparin- and receptor-binding sites are functionally and spatially related (16). For some of these proteins, efficient binding and uptake by cell surface receptors require binding to cell surface heparin-like molecules, namely, heparan sulfate proteoglycans (HSPGs) (13, 14, 16, 17, 40, 52, 56, 71, 102, 105). HSPGs contain core proteins, to which heparan sulfate (HS) glycosaminoglycan chains are attached, and are present on the surface of most vertebrate cells as well as in the extracellular matrix (77). The binding of HSPGs to proteins is due to the interaction of negatively charged regions of HSPGs with positively charged regions of proteins. We identified a region in the partially known sequence of rat Tg [Arg-689–Lys-703, corresponding to Arg-2489–Lys-2503 in the complete, homologous sequence of mouse Tg (12)] that is rich in positively charged amino acid residues and contains a Cardin and Weintraub (11) heparin-binding consensus sequence (Ser-693–Pro-699: SRRLKRP). We found that this region, located in the COOH-terminal portion of the molecule, is a Tg heparin-binding site and provided evidence that it is also functionally involved in megalin binding, even though this heparin-binding sequence of Tg itself does not bind to megalin (60). Thus an antibody against the Tg heparin-binding sequence almost completely inhibited Tg binding to megalin in solid-phase assays. On the basis of these findings and on the knowledge that HSPGs are expressed on thyroid cells (29, 30), we investigated rat Tg interactions with HSPGs (58). We showed, in solid-phase assays, that unlabeled rat Tg binds to a HS preparation in a dose-dependent, saturable manner with moderately high affinity (K_d ~19 nM, constant of inhibition ~25 nM). We then studied the role of HSPGs in rat Tg binding to FRTL-5 cells and found that Tg binding was reduced by HS and HSPGs preparations and by enzymatic removal of HSPGs from the cell surface. Furthermore, the antibody against the COOH-terminal rat Tg heparin-binding site involved in megalin binding virtually abolished Tg binding to HS and to FRTL-5 cells, suggesting that combined interactions of rat Tg with HSPGs and with megalin are involved in rat Tg binding to thyroid cells.

The role of HSPGs in Tg binding to thyroid cells may be restricted to rodents, because the heparin binding sequence of rat Tg we identified (Ser-693–Pro-699:

SRRLKRP) (60) is conserved in mice (Ser-2492–Pro-2498: SRRLKRP) (12) but not in humans (Ala-2493–Ser-2499: ARALKRS) (57). Furthermore, we found that although human Tg binds to megalin, it does not appreciably bind to heparin (unpublished observations), which is not surprising in view of our previous finding that substitution by glycine of any of the positively charged amino acid residues in the rat Tg heparin-binding sequence results in a dramatic reduction of its heparin-binding ability (60). The heparin- and HSPG-binding ability of rat Tg may represent the consequence of an evolutionary adaptation of the thyroid to the necessity of a high metabolic rate in rodents. The binding to HSPGs may render Tg uptake and hormone release more efficient, resulting in a higher metabolic rate than in large mammals.

Tg RECEPTORS AND THYROID DISEASE

The study of mechanisms of Tg uptake by thyroid cells is of potential relevance to the understanding of the pathogenesis of certain thyroid diseases. Mutations of the Tg gene that result in amino acid substitutions are associated with some sporadic or familial, congenital forms of goiter (63, 64). In these diseases, Tg accumulation in the thyroid is thought to result from a defect in intracellular trafficking of Tg, due to misfolding of the molecule in the endoplasmic reticulum (48, 63). However, it is also plausible that modifications of the Tg structure may result in impaired Tg binding and internalization by cell surface receptors, thereby promoting excessive retention of Tg in the colloid. In addition, it is conceivable that somatic mutations of Tg receptors may result in impaired Tg endocytosis. However, no supporting evidence is currently available, and further studies are needed to investigate this possibility.

The only evidence that Tg receptors are somehow involved in thyroid diseases is provided by the presence of autoantibodies against megalin in some patients with autoimmune thyroiditis. In a recent study (59), we found that ~50% of patients with autoimmune thyroiditis and ~10% of patients with Graves' disease have circulating autoantibodies against megalin. Similar immunological abnormalities were seen in a minority of patients with nontoxic goiter and differentiated thyroid cancer, associated with serological evidence of thyroid autoimmunity. Autoantibodies against megalin were detected by binding of serum IgG to the surface of L2 cells, a rat yolk sac carcinoma cell line that expresses abundant megalin (74). Specificity of binding was demonstrated in inhibition and immunoprecipitation experiments. The possible role of these autoantibodies in the pathogenesis of thyroid autoimmune diseases and their clinical significance remain to be investigated.

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