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The Rat Asialoglycoprotein Receptor Binds the Amino-Terminal Domain of Thyroglobulin

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We have previously reported that the rat hepatic lectin-1 (RHL-1) subunit of rat asialoglycoprotein receptor (ASGPr), the endocytic receptor found on the basolateral surface of hepatocytes, was expressed in rat thyroid tissue and localized on the apical surface of polarized rat thyroid FRT cells. Here we show that PC Cl3 cells, a differentiated rat thyroid cell line, bound thyroglobulin (Tg) via ASGPr. In fact, both the bacterial recombinant carbohydrate recognition domain of RHL-1 ($rCRD_{RHL-1}$) and the anti-$rCRD_{RHL-1}$ antibody markedly inhibited ^{125}I-Tg binding to the cell surface of PC Cl3 cells. Ligand blot assays with deglycosylated Tg show that the $rCRD_{RHL-1}$ was able to interact with Tg even after remotion of sugars. The region of Tg involved in the binding to RHL-1 was investigated by ligand blot assays with biotinylated $rCRD_{RHL-1}$ on thermolysin-digested native and desialated rat thyroglobulin. It is shown that the $rCRD_{RHL-1}$ specifically recognized a thyroglobulin fragment with an apparent M_r of 68,000, corresponding to the amino-terminal part of the molecule. To our knowledge, this is the first report that attributes to the amino-terminal portion of Tg molecule, containing its earliest and major hormonogenic site, the function of binding to a cell surface receptor of the thyroid. Moreover, we show that oligosaccharides are not the only molecular signals for binding to RHL-1, but amino acidic determinants could also play a role. © 2000 Academic Press

Key Words: **asialoglycoprotein receptor; thyroglobulin; N-terminal domain.**

Thyroglobulin is secreted by thyrocytes into the follicular lumen, where it is stored as the major component of colloid. Thyroid hormone secretion requires endocytosis and delivery of Tg to lysosomes. Tg internalization, under intense thyrotropic hormone (TSH) stimulation, may result from pseudopod ingestion, but micropinocytosis is the usual route (1). Micropinocytosis of Tg may occur by non-selective fluid-phase intake or by receptor-mediated endocytosis (2). The presence of specific receptors, involved in thyroglobulin endocytosis, has been postulated (2). The evidences that the endocytosis of Tg is, at least in part, a receptor-mediated event have been recently strengthened. In fact, Marinò *et al.* (3) have reported that cultured FRTL-5 thyroid cells express megalin (gp330), an endocytic receptor capable of mediating high-affinity binding and endocytosis of Tg. In addition it has been previously shown (4) that asialo-thyroglobulin interacts with rat thyroid membrane preparations likely by binding to a galactose receptor, similar to liver ASGPr, on the surface of thyrocytes. Based on these observations and on the unusual complexity (two subunits, 330 kDa each one) of the Tg molecule, the question arises if a specific domain of Tg is involved in the interaction with the ASGPr. Liver ASGPr binds and internalizes serum desialated glycoproteins by receptor-mediated endocytosis (5), interacting with terminal residues of N-acetyl-galactosamine and/or galactose. Rat ASGPr is a dimer made up of two polypeptide chains, RHL-1 (42 kDa) and RHL-2 (49 kDa), which form an heterooligomeric complex (6). However, RHL-1 contains all the necessary signal motifs for binding, endocytosis and recycling (7). The receptor domain involved in sugar binding, the Carbohydrate Recognition Domain (CRD), is localized on the extracytoplasmic side of both RHL-1 and RHL-2 subunits (7). Recently, we have reported the expression of RHL-1 in rat thyroid tissue as well as in several thyroid cell lines (9, 11) and its localization on the apical surface of polarized FRT thyroid cell line

Abbreviations used: RHL, rat hepatic lectin; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, tris-buffered saline Tween 20; ECL, enhanced chemiluminescence; TSH, thyroid-stimulating hormone; PBS, phosphate-buffered saline.

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(11, 20). Furthermore, we have shown that the $rCRD_{RHL-1}$ binds both native and asialo-Tg in ligand blot assays (10, 12). Based on these previous findings, we report here that PC Cl3 cells, a differentiated rat thyroid cell line, bind Tg via RHL-1 and that oligosaccharide chains are not necessary for the binding. We also demonstrate that RHL-1 binds exclusively the N-terminal part of Tg molecule, which contains its earliest site of iodination and the major hormonogenic site (17).

MATERIALS AND METHODS

Expression and purification of the $rCRD_{RHL-1}$; anti-$rCRD_{RHL-1}$ antibodies production. The CRD region of the thyroid RHL-1 cDNA was amplified, subcloned in a bacterial expression vector and the recombinant protein ($rCRD_{RHL-1}$) was purified according to the procedures already described (9, 10). Polyclonal anti-$rCRD_{RHL-1}$ antiserum was developed in rabbits and its specificity evaluated by Western blot (10).

Cell cultures and cell binding assay. PC Cl3 rat differentiated thyroid cells were grown as previously described (13). PC Cl3 cells, plated in six-well dishes at a density of 0.5×10^6 cells/well, were washed three times in PBS additioned with 1 mg/ml ovalbumin (Sigma, St. Louis, MO) and stripped of surface-bound endogenous Tg by acid treatment. Briefly, cells were incubated for 3 min at 4°C in 50 mM glycine, 100 mM NaCl, pH 3.0; a half-volume of 50 mM Hepes, 100 mM NaCl, pH 7.4, was then added to reach neutral pH. After three washes in F12-ovalbumin, cells were incubated for 2 h at 4°C with ^{125}I-labeled Tg alone or in the presence of either unlabeled Tg or RHL-1 competitors, namely anti-$rCRD_{RHL-1}$ antiserum or purified $rCRD_{RHL-1}$ in F12-ovalbumin. After incubation, cells were washed three times with PBS-ovalbumin to remove non-specifically bound proteins and lysed with 0.1 M NaOH. Lysates were collected and the cell-bound radioactivity was measured.

Iodination and biotinylation of Tg and $rCRD_{RHL-1}$. Native thyroglobulin was purified from rat thyroids as previously described (4) and radiolabeled by Iodogen (Pierce Chemical Co., Rockford, IL) and $Na^{125}I$ (Amersham, Little Chalfont, UK). ^{125}I-Tg preparations were analyzed by 7% SDS–PAGE under reducing conditions and autoradiography. Native and deglycosylated rat Tg, as well as $rCRD_{RHL-1}$ were biotinylated *in vitro* using an N-hydroxysuccinimide-biotin ester (Amersham) as previously described (10).

Neuraminidase and N-glycosidase F digestion of rat Tg. Digestion of rat Tg with neuraminidase was performed as already described (10). The digestion with N-glycosidase F was carried out as follows: 10 μg of rat Tg were heated in boiling water bath for 3 min in the presence of 0.5% SDS and 1.6 mM dithiothreitol. Aliquots of the denatured samples (20 μl) were adjusted to include 200 mM sodium phosphate, pH 8.6, 1.5% Triton X-100, 10 mM EDTA, up to a volume of 60 μl. The samples were then incubated with or without 2 units of N-glycosidase F (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 20 h. The entity of deglycosylation was then evaluated by total carbohydrate labeling of proteins with biotin–hydrazide (Amersham). Biotinylated carbohydrates were evidenced with horseradish peroxidase-conjugated streptavidin and ECL, as described (10).

Thermolysin digestion of native and asialo-Tg. Limited proteolysis of rat Tg was performed as described (21). Under these conditions, rat Tg is cleaved in a limited number of fragments which have been already well characterized and altogether span the entire length of the polypeptide chain of Tg (21). A 100-μg of thermolysin-digested native and asialo-Tg were subjected to electrophoresis in SDS on 4–13% polyacrylamide gradient gels, electroblotted onto PVDF membranes and subjected to ligand blot. For NH_2-terminal peptide sequencing, 1 mg of thermolysin-digested native and asialo-Tg was subjected to SDS–electrophoresis as above in the presence of 100 mM sodium thioglycolate on 4–13% gradient gels and transferred onto PVDF membranes by semi-dry blotting in 25 mM Tris base, 10 mM glycine transfer buffer, at the constant current of 0.8 mA/cm^2 for 1 h. PVDF membranes were washed thoroughly in double-distilled water and stained for 3 min in Coomassie blue R-250 (Bio-Rad, Hercules, CA) in 50% (v/v) methanol followed by destaining in 50% methanol, 10% (v/v) acetic acid. Bands were cut and subjected to automated gas-phase NH_2-terminal microsequencing at the Protein Structure Laboratory, University of California at Davis, California.

Ligand blot. Filters containing 10 μg of purified $rCRD_{RHL-1}$ and recombinant 37 laminin receptor (r37LRP) (14) were subjected to ligand blot with 10^{-10} M biotinylated native or deglycosylated thyroglobulin, as described (10). Filters containing thermolysin-digested native and asialo-Tg were probed with 10^{-10} M biotinylated $rCRD_{RHL-1}$.

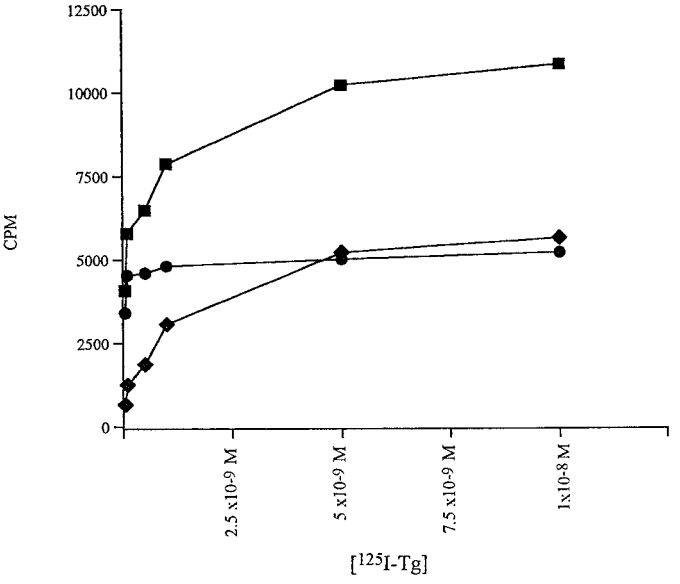

FIG. 1. Binding of ^{125}I-labeled Tg to PC Cl3 differentiated rat thyroid cells. PC Cl3 cells at a density of 0.5×10^6 cells per 35-mm well were incubated for 2 h at 4°C with various concentrations of ^{125}I-labeled Tg, alone or in the presence of a 200-fold molar excess of unlabeled Tg. The amount of Tg specifically bound to PC Cl3 cells (◆) was calculated by subtracting from total ^{125}I-Tg binding, in the absence of unlabeled Tg (■), the ^{125}I-Tg binding obtained in the presence of a 200-fold molar excess of unlabeled Tg (●).

RESULTS

Thyroglobulin binding to the surface-expressed RHL-1 of PC Cl3 thyroid cells. Binding experiments were designed in order to show that Tg binds PC Cl3 cells via the RHL-1 subunit of ASGPr. PC Cl3 cells were incubated for 2 h at 4°C with increasing amounts of ^{125}I-Tg alone or in the presence of a 200-fold molar excess of unlabeled Tg. As shown in Fig. 1, unlabeled Tg competed with ^{125}I-Tg for binding to PC Cl3 cells, demonstrating a specific and saturable Tg binding to the surface of PC Cl3 cells. Then, cell binding experiments were performed on PC Cl3 cells with a saturat-

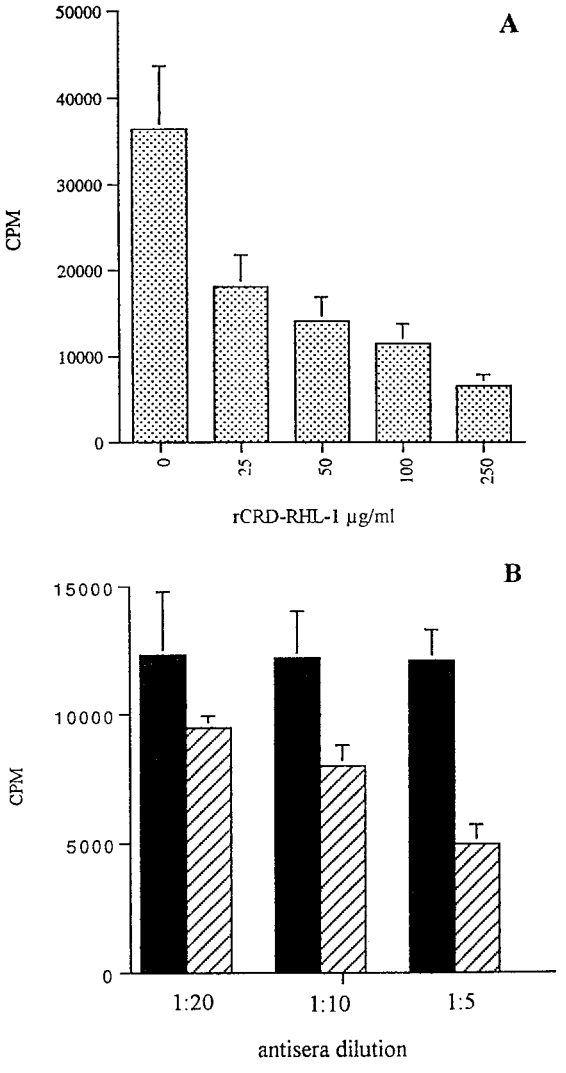

FIG. 2. Binding of ^{125}I-labeled Tg to RHL-1 on the surface of PC Cl3 thyroid cells. (A) PC Cl3 cells at a density of 0.5×10^6 cells per 35-mm well were incubated for 2 h at 4°C with ^{125}I-labeled Tg alone or in the presence of RHL-1 competitors. The $rCRD_{RHL-1}$ was added at concentrations ranging from 25 to 250 μg/ml and the cell-bound radioactivity was compared to that of untreated cells. (B) Anti-$rCRD_{RHL-1}$ antiserum was added at various dilutions (striped bars) and cell-bound radioactivity compared to that of cells treated with preimmune serum (filled bars) at the same dilutions.

ing concentrations of ^{125}I-Tg alone (5×10^{-9} M, see Fig. 1) or in the presence of increasing concentration of $rCRD_{RHL-1}$, as a competitor (Fig. 2A). The $rCRD_{RHL-1}$ competed for ^{125}I-Tg binding to PC Cl3 cells and Tg cell binding was inhibited, up to 82%, by $rCRD_{RHL-1}$ (Fig. 2A). To assess the specificity of Tg-RHL-1 interaction(s), we also performed experiments in which PC Cl3 cells were treated for 20 h with anti-$rCRD_{RHL-1}$ antiserum or with pre-immune serum and then incubated with 5×10^{-9} M ^{125}I-Tg. Rabbit anti-$rCRD_{RHL-1}$ serum significantly inhibited ^{125}I-Tg binding to PC Cl3 cells: the mean inhibition produced by anti-$rCRD_{RHL-1}$ was 22% when diluted 1:20, 37% when diluted 1:10 and 59% when diluted 1:5 in comparison with controls treated with pre-immune serum (Fig. 2B).

Binding of RHL-1 to deglycosylated Tg. Our previous results (10–12) show that thyroid RHL-1 binds asialo-Tg as well as native Tg, leading us to hypothesize that exposed galactose and/or galactosamine on the surface of Tg could not be the only molecular signal mediating the binding of Tg to CRD_{RHL-1}. To investigate the potential role of amino acid determinants on the Tg-RHL-1 interaction, ligand blot experiments with native (Fig. 3A, panel 3) and deglycosylated rat Tg (Fig. 3A, panel 2) on blotted $rCRD_{RHL-1}$ (Fig. 3A, panel 1) were performed. N-linked oligosaccharide units were removed from the Tg molecule by N-glycosidase F digestion and both Tg integrity and sugar removal

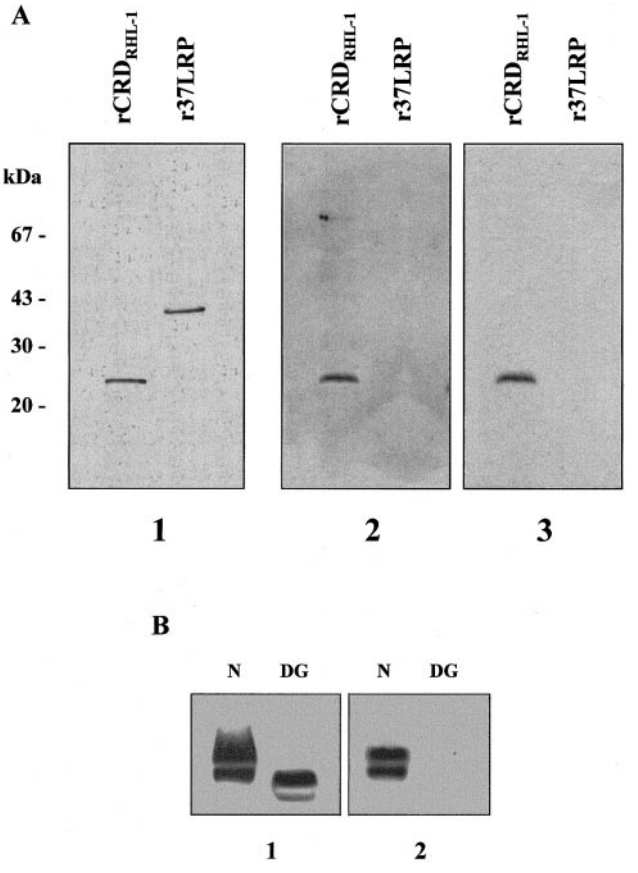

FIG. 3. $rCRD_{RHL-1}$ binding to rat deglycosylated thyroglobulin. (A) Panel 1, SDS–PAGE analysis of purified $rCRD_{RHL-1}$ and recombinant 37 laminin receptor (r37LRP) before blotting. Panel 2, ligand blot with biotin-labeled deglycosylated rat thyroglobulin. Panel 3, ligand blot with biotin-labeled native rat thyroglobulin. (B) Panel 1, biotin-labeled native (N) and deglycosylated (DG) rat thyroglobulin before ligand blot (1). Panel 2, total carbohydrate labeling with biotin hydrazide of native (N) and deglycosylated (DG) rat Tg (2). Note that deglycosylated rat Tg retains its ability to bind the $rCRD_{RHL-1}$ (A, panel 2), biotinylation does not alter Tg integrity (B, panel 1) and N-glycosidase-F removes all the N-linked sugars from the Tg molecule (B, panel 2).

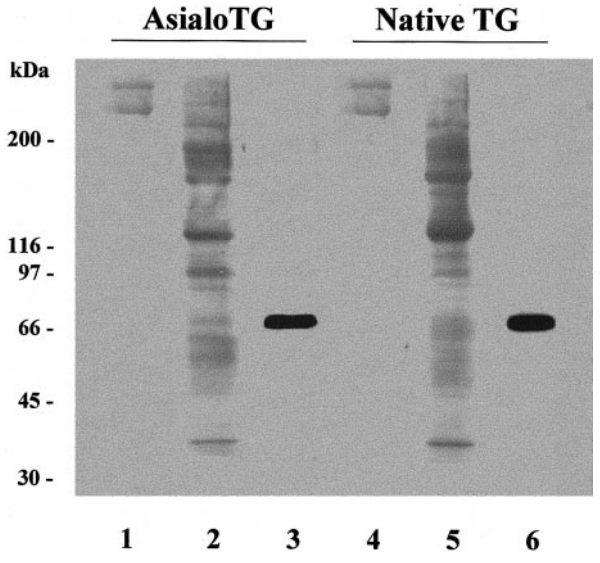

FIG. 4. $rCRD_{RHL-1}$ binding to thermolysin-digested native and desialated rat thyroglobulin. Lanes 1, 4, Coomassie stain of blotted purified desialated and native rat thyroglobulin. Lanes 2, 5, Coomassie stain of blotted desialated and native Tg fragments after thermolysin digestion. Lanes 3, 6, ligand blot with biotinylated $rCRD_{RHL-1}$ on blotted desialated and native Tg fragments after thermolysin digestion. Note that the $rCRD_{RHL-1}$ specifically recognizes a fragment with an apparent M_r of 68,000.

were checked (Fig. 3B, panels 1 and 2). Ligand blot experiments show that the $rCRD_{RHL-1}$ binds both native and deglycosylated rat Tg. The assay specificity was evidenced by the inability of thyroglobulin (either native or deglycosylated) to bind the r37LRP (Fig. 3A, panels 1, 2, and 3).

Binding of RHL-1 to the amino-terminal part of rat Tg. To localize, within the large Tg molecule, the domain(s) which is(are) recognized by RHL-1, rat native and desialated Tg (Fig. 4, lanes 4 and 1) was digested with thermolysin (Fig. 4, lanes 5 and 2). The resulting fragments were analyzed by ligand blot assay with biotinylated $rCRD_{RHL-1}$ (Fig. 4, lanes 6 and 3). As reported in Fig. 4, the $rCRD_{RHL-1}$ specifically bound a fragment with an apparent M_r of 68,000 (Fig. 4, lanes 6 and 3), derived either from thermolysin-digested native or asialo-Tg. This $rCRD_{RHL-1}$-binding fragment was excised from PVDF membranes and NH_2-terminal peptide sequencing was performed. In keeping with previous observations (21), the resulting sequences were XXFEXQVDAQ and X(I, D)FEXQVDAQ for asialo-Tg-derived and for native Tg-derived fragment, respectively, where X refers to unidentified amino acid residues. Both sequences were identified as the amino-terminal domain of rat Tg (Table I) (15, 21). Failure to identify Y at position 5 of both sequences most probably derived from its modification by iodination and/or coupling.

DISCUSSION

The secretion of thyroid hormones involves three successive steps, i.e., the internalization of thyroglobulin through an endocytotic process, the proteolytic cleavage of thyroglobulin within lysosomes and the transfer of the thyroid hormones to the bloodstream. With respect to the first of these steps it is known that follicular thyroglobulin is internalized into thyrocytes by macropinocytosis or micropinocytosis (1). While macropinocytosis does not require specific receptors, micropinocytosis, which occurs in the physiological thyroid state, is a process similar to receptor-mediated endocytosis (1). We have recently reported the expression of the RHL-1 subunit of ASGPr in rat thyroid tissue and in several thyroid cell lines (9–11). The presence of RHL-1 on the apical membrane of polarized FRT thyroid cells (11) and the finding that the thyroid $rCRD_{RHL-1}$ binds thyroglobulin *in vitro* (10–12) support the hypothesis that RHL-1 could mediate binding and endocytosis of thyroglobulin from the follicular lumen. In this report we show that PC Cl3 differentiated thyroid cells bind thyroglobulin via the RHL-1 subunit of ASGPr. Moreover, we have characterized the Tg-RHL-1 interaction by demonstrating that (i) the N-linked oligosaccharides chains of Tg exposing either sialic acid or galactose are not necessary for this interaction, and (ii) RHL-1 recognizes selectively the amino-terminal part of ~68 kDa of Tg. The property of the thyroid RHL-1 to bind intact, as well as deglycosylated thyroglobulin is similar to that of the liver receptor that interacts with deglycosylated lactoferrin (8). The present data provide evidences that oligosaccharides are not the only molecular signals for binding to RHL-1, but amino acidic determinants could also play a role. Thus, the ASGPr may bind the same or different ligands by carbohydrate-dependent and -independent mechanisms (16).

We also demonstrate that the RHL-1 binding site on rat Tg is restricted to the amino-terminal domain of the molecule, a fragment derived from thermolysin digestion and encompassing amino acids 1–500 (15, 21). This is the first demonstration that a specific domain of Tg is involved in its recognition by a surface receptor of

TABLE I

Alignment of Sequences from RHL-1 Binding Fragments of Desialated (a) and Native (n) Tg with the cDNA-Derived Sequence of Rat Tg

(a)	X	X	F	E	X	Q	V	D	A	Q
(n)	X	(I/D)	F	E	X	Q	V	D	A	Q
rat Tg	N	I	F	E	Y	Q	V	D	A	Q

Note. The sequence of RHL-1-binding fragments was obtained by N-terminal peptide sequencing. Amino acid numbering for rat Tg is as in (17) and X refers to unidentified amino acid residues. Boxed amino acids are identical.

the thyroid. Moreover, this finding could have relevant physiologic consequences. In fact, the NH_2 terminus of Tg contains, in correspondence of residue number 5, its earliest site of iodination and the major hormonogenic site (17). Furthermore, it contains both the acceptor Tyr5 and the donor Tyr130 sites (22, 23) and it is capable of forming T_4 by itself (22). Also, it has been recently shown that the thyroidal proteases cathepsin B and D are secreted in the follicular lumen, where they are functionally active. These cleave rabbit Tg at positions 532 and 551, respectively (18, 19). Therefore, the Tg fragment which binds RHL-1 can be generated at the apical surface of thyrocytes or in the follicle lumen. Thyrocytes are thus provided with a pathway of limited extracellular proteolysis of Tg (19), which, as suggested by the selective binding of the N-terminal part of Tg to RHL-1, may be followed by endocytosis via ASGPr for subsequent processing by lysosomes to release free T4.

These findings suggest that the role of RHL-1 in thyroid could not be restricted to Tg endocytosis, but, as recently reported (20), Tg can suppress the expression of thyroid specific genes by interacting with RHL-1. Therefore, the role of RHL-1 in thyroid could be multifunctional: it could internalize intact Tg or its amino-terminal fragment providing a physiological, TSH-regulated mechanism for selective hormone release, or it could bind Tg to exert regulatory actions of the gland functions.

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