Direct evidence that ganglioside is an integral component of the thyrotropin receptor

(thyroid-stimulating hormone/glycolipid/Limax flavus agglutinin/sialidases/ceramide glycanase)

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ABSTRACT Gangliosides were extracted from purified human and porcine thyrotropin (TSH) receptors (TSH-R) and were detected by probing with an 125I-labeled sialic acid-specific lectin, Limax flavus agglutinin. Gangliosides copurified with human and porcine TSH-R migrated between monosialo-ganglioside GM1 and disialoganglioside GD1a. Ceramide glycanase digestion of the purified human TSH-R-associated glycolipid confirmed its ganglioside nature. It was resistant to Vibrio cholerae sialidase, which digests all gangliosides except GM1, but was sensitive to Arthrobacter ureafaciens sialidase, which digests all gangliosides including GM1. These findings indicate that the human TSH-R contains ganglioside that belongs to the galactosyl(β1 → 3)-N-acetylgalactosaminyl(β1 → 4)-[N-acetyleneuraminyl(α2 → 3)galactosyl(β1 → 4)glucosylβ1 → 1]ceramide (GM1) family. Its intimate association with receptor protein implies a key role for ganglioside in the structure and function of the TSH-R.

The thyrotropin (TSH) receptor (TSH-R) mediates the actions of TSH on the thyroid and is believed to be the target of autoantibodies in Graves hyperthyroidism (1, 2). The TSH-R was recently cloned from dog and human thyroid cDNA libraries (3-5) and, like the lutropin/choriogonadotropin receptor (LH/CG-R) (6, 7), contains seven transmembrane domains characteristic of the guanine nucleotide-binding protein (G protein)-coupled family of receptors. However, the TSH-R is distinct by virtue of its long extracellular domain containing five putative N-glycosylation sites. Indirect evidence suggests that ganglioside (sialic acid-containing glycosphingolipid) is a component of the TSH-R (8-12). The demonstration that the B subunit of cholera toxin, which binds to GM1 ganglioside, 1 could inhibit TSH binding to thyroid membranes (9) and that a homologous amino acid sequence exists in the subunits of TSH and cholesterol toxin (10) suggested that ganglioside was an integral component of the receptor. Based on the separation of two TSH-binding components, protein and lipid, it was proposed that the TSH-R was a complex of glycoprotein and ganglioside, each of which contributed to receptor structure and function (11). The restoration of 125I-labeled TSH (125I-TSH) binding in a ganglioside-deficient rat thyroid tumor cell line after ganglioside incorporation into cell membranes (14) provided further evidence that the TSH-R is a glycolipid-glycoprotein complex. However, direct evidence for ganglioside in the TSH-R has been lacking because of the limited availability of purified receptor and the insensitivity of ganglioside detection by resorcinol staining or anti-ganglioside antibody binding.

Several thyroid membrane gangliosides have been identified, some of which decrease the binding of 125I-TSH to thyroid membranes (12). In the human thyroid, 70% of the gangliosides are GM3 and GD3, together with several monosialilated and disialilated gangliosides and GT1b and GQ1b (15). Porcine thyroid gangliosides, while not as well characterized, include GD3, GM1, and GD1a but not GM3 (16).

We have purified and characterized the human (2) and porcine (17) TSH-R by sequential wheat germ agglutinin (WGA) and TSH affinity chromatography. Purified human (from Graves and multinodular thyroid glands) and porcine TSH-R have a similar glycoprotein structure comprising proteins of M, 70,000, 50,000, and 35,000 in the nonreduced state. Immunoblotting or immunoprecipitation with Graves disease immunoglobulins demonstrated an autoprotein in the M, 50,000 protein of both human and porcine TSH-R (18). In addition, we recently described (19) a highly sensitive technique for the detection of gangliosides (less than 1 ng or 0.5 pmol) by direct binding of 125I-labeled Limax flavus agglutinin (125I-LFA) to TLC plates.

The direct demonstration of ganglioside in the TSH-R would have significant implications for receptor structure and function. We asked, therefore, whether affinity-purified human and porcine TSH-R that bind TSH and react with Graves disease immunoglobulins contain ganglioside.

MATERIALS AND METHODS

Materials. Partially purified bovine TSH (10 international units per vial), Triticum vulgare WGA, N-acetyl-b-glucosamine, Triton X-100, leupeptin, phenylmethylsulphonyl fluoride, dithiothreitol, polyvinylpyrrolidone (M, 40,000), and bovine serum albumin (RIA grade) were purchased from Sigma. Iodo-Gen (1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycouril)- and 1,1′-carbonyldiimidazole-activated 6% cross-linked beaded agarose (CDI-agarose, Reacti-Gel (6×)) were obtained from Pierce. Poly-Prep chromatography columns were purchased from Bio-Rad.

LFA; bovine brain gangliosides GM2, GD1a, GD1b, GT1b, and GQ1b; and bovine erythrocyte GM3 at least 98% pure as assessed by TLC, were from Calbiochem. GM1 was from Supelco. Sialidase (EC 3.2.1.18) from Vibrio cholerae was from Behring Diagnostics, and that from Arthrobacter ureafaciens was from Boehringer Mannheim. Ceramide glycanase (endoglycoceramidase) from Macrobrachia decora (leech) was from Boehringer Mannheim. High-performance TLC plates (silica gel 60, aluminium backed no. 5574) were from Merck. Polyisobutyl methacrylate) of high molecular weight.

Abbreviations: TSH, thyrotropin; TSH-R, TSH receptor; LH/CG-R, lutropin/choriogonadotropin receptor; LFA, Limax flavus agglutinin; WGA, wheat germ agglutinin (Triticum vulgare).

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1 Gangliosides are named according to the nomenclature system of Svennerholm (13) in which G = ganglioside, M = monosialo, D = disialo, T = triosialo, Q = tetrasialo, and arabic numbers indicate the sequence of TLE migration.

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weight was from Aldrich. All other chemicals were purchased from BDH and were of analytical grade.

The Iodo-Gen method (20) was used to radiolabel affinity-purified porcine or human TSH-R with 125I to a specific activity of \( \approx 2 \muCi/\mug \) (1 \( \muCi = 37 \) kBq) and bovine TSH to a specific activity of \( \approx 10 \muCi/\mug \). LFA was iodinated by the chloramine-T method (21) to a specific activity of 25 \( \approx 5 \muCi/\mug \) and was stable for at least 3 weeks at 4°C.

**Preparation of Membranes and Purified TSH-R.** Human thyroid tissue was obtained with consent from patients undergoing surgery for multinodular goitre or Graves disease and human whole adrenal gland (control tissue) from a kidney donor subject. Porcine thyroids were obtained within 30 min after slaughter. Tissues were transported on ice and stored at -70°C. Frozen tissues were solubilized, and TSH-R was purified as described (2). All procedures were performed at 4°C. Briefly, tissue was homogenized in 10 mM Tris, pH 7.4/50 mM sodium chloride (Tris/NaCl) in the presence of protease inhibitors (leupeptin at 20 \( \mug/ml \), aprotinin at 1000 kalikrein inhibitory units/ml, bacitracin at 100 units/ml, and phenylmethylsulfonyl fluoride at 2 mM). Particulate membranes were solubilized in Tris/NaCl containing Triton X-100 at 10 g/liter and protease inhibitors and were centrifuged at 100,000 \( \times g \) for 1 hr. Receptors in solubilized thyroid membranes were affinity-purified on WGA-agarose, and fractions eluted from WGA-agarose that contained 125I-TSH-binding activity were adsorbed onto TSH-agarose. Purified receptors were eluted with 3 ml of 0.3 NaCl/10 mM Tris/HC1, pH 7.4/1 g of Triton X-100 per liter and were dialyzed against Tris/NaCl containing Triton X-100 at 1 g/liter. Adrenal tissue was also sequentially purified on WGA- and TSH-agarose.

The protein concentration of membrane preparations was determined by the method of Bradford (22), and the amount of purified receptor was estimated by silver staining of SDS/PAGE gels. 125I-TSH binding assays were performed as described (3). Sequential purification produced a 1250- and 800-fold increase in 125I-TSH binding activity over that observed in human and porcine membranes, respectively.

**Ganglioside Detection in Porcine and Human Thyroid Tissue.** Gangliosides were extracted by phase partition from equivalent quantities (=3 g) of human and porcine thyroid tissue—i.e., thyroid particulate membranes, solubilized membranes, WGA-agarose eluate, and TSH-agarose eluate as described elsewhere (19). Both upper and lower phases were dried in a HetoVac VR-1 vacuum centrifuge. The residues were reconstituted in chloroform/methanol/water, 60:30:4.5 (vol/vol), and centrifuged at 5000 \( \times g \); the supernatants were stored at -20°C without further purification prior to TLC. To reduce diffusion and increase the glycolipid concentration applied to the TLC plate, glycolipid extracts were spotted onto the TLC plate at one point only and migrated as dots. Plates were developed in chloroform/methanol/KCl aqueous solution (2.5 g/liter), 5:4:1 (vol/vol). After drying, the silica on the plates was prevented from detaching by soaking in hexane containing 1 g of polyisobutyl methacrylate per liter. The TLC plates were incubated with 125I-LFA, washed in phosphate-buffered saline containing 10 g of polyvinylpyrrolidone per liter, dried, and exposed to Amersham hyperfilm (MP) for 48–72 hr at -70°C.

With the exception of purified receptors, all of the other thyroid membrane preparations examined were unlabeled, and gangliosides were detected by using the overlay technique described above. Purified porcine and human TSH-R was analyzed either unlabeled or labeled with 125I. Gangliosides from the latter were extracted and analyzed by TLC and autoradiography.

Gangliosides were also extracted from human adrenal tissue.

Enzymatic Analysis of Human TSH-R Gangliosides. To further characterize the structure of the ganglioside copurified with Graves thyroid TSH-R, glycolipids were extracted from 40-ng lots of purified receptor, transferred to polypropylene tubes, dried under a stream of nitrogen, and subjected to separate enzymatic treatments.

**Ceramide glycanase digestion.** Glycolipids were dissolved in 200 \( \mul \) of 50 mM sodium acetate buffer (pH 5.0) containing 1 \( \mug \) of sodium cholate per \( \mul \), 5 milliunits (100 \( \mul \)) of enzyme was added, and the mixture was incubated for 6 hr at 37°C. The reaction was terminated by the addition of 1.2 ml of methanol/chloroform, 2:1 (vol/vol), and lipids were extracted by phase partition in glass tubes.

**V. cholerae sialidase digestion.** Glycolipids were dissolved in 2 ml of 50 mM sodium acetate, pH 5.5/4 mM CaCl2 buffer containing 200 \( \mug \) of bovine serum albumin. One unit (1 ml) of sialidase was added, and the mixture was incubated for 15 hr at 37°C. The reaction was terminated by the addition of 12 ml of methanol/chloroform, 2:1 (vol/vol), and glycolipids were extracted as above.

**RESULTS**

Gangliosides Detected during Purification of the Human TSH-R. Glycolipids were extracted at each step during the purification of human TSH-R from multinodular and Graves thyroid tissue. The major gangliosides detected in particulate and solubilized membrane preparations of multinodular thyroid tissue migrated near GM3, GD3, and GD1a (Fig. 1, lane A) and near GM3 and GD1a (Fig. 1, lane B), respectively. The major ganglioside detected in the WGA-agarose eluate migrated at the position of GM1 (Fig. 1, lane C). When the extraction was performed on receptors eluted from TSH-agarose in 3 M NaCl, which selectively elutes a \( M_1 \) 50,000 TSH-R protein (2), a single ganglioside was detected migrating between GM1 and GD1a (Fig. 1, lane D). Gangliosides identified during purification of the TSH-R from Graves thyroid tissue were identical to those from multinodular goitre. When adrenal tissue was carried through the same procedures, gangliosides at GM3 and GM1 were eluted from

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**Fig. 1.** Gangliosides extracted from human multinodular thyroid tissue. Lanes (each lane is equivalent to the ganglioside in 3 g of whole tissue): A, thyroid particulate membranes; B, solubilized thyroid membranes; C, WGA affinity eluate; D, TSH affinity/NaCl eluate; St. standards. Unlabeled arrows indicate detected gangliosides.
**FIG. 2.** Gangliosides extracted from porcine thyroid tissue. Lanes (each lane is equivalent to the ganglioside in 3 g of whole tissue): A, thyroid particulate membranes; B, solubilized thyroid membranes; C, WGA affinity eluate; D, TSH affinity/NaCl eluate; St, standards. Unlabeled arrows indicate detected gangliosides. Lane C is from the plate on the left, and lane D is from the plate on the right.

WGA-agarose but were not detected after subsequent TSH-agarose affinity chromatography (data not shown).

**Gangliosides Detected During Purification of the Porcine TSH-R.** The major ganglioside in porcine thyroid particulate membranes migrated just below GD1a (Fig. 2, lane A), while in solubilized membranes the major ganglioside migrated at the position of GM1 (Fig. 2, lane B). After elution from WGA-agarose a ganglioside was also detected at the GM1 position (Fig. 2, lane C). When the porcine receptor was eluted from TSH-agarose in 3 M NaCl, a single ganglioside was detected migrating between GM1 and GD1a (Fig. 2, lane D). The broad band below GD1a in lane D was not considered to be specific because it was present across the TLC plate as seen in the adjacent “St” lane.

**Extraction of 125I-Labeled Purified Receptors.** To assess the separation of ganglioside from receptor protein, human receptors purified from a multidisciplinary thyroid gland were labeled with 125I, and glycolipids were extracted as described above. In these experiments both the upper (glycolipid-containing) and lower (protein-containing) phases recovered after the partition were analyzed by TLC and autoradiography. As expected, almost all of the 125I radioactivity remained in the lower protein-rich phase (Fig. 3, lane B). However, a small amount of radioactivity in the upper phase migrated as a single spot to a position near GM1 (Fig. 3, lane A). This radioactivity is presumably lipid-associated and may be incorporated in the double bond of the ceramide moiety of ganglioside because it was labile and could not be detected after a 12-hr exposure of the dried TLC plate to air at room temperature.

**Characterization of TSH-R-Associated Gangliosides.** The glycolipid extracted from purified Graves thyroid TSH-R was treated with ceramide glycanase which, in the presence of cholact, cleaves the bond between the ceramide moiety and the glucose of the glycan chain (23). This resulted in release of the carbohydrate chain and an inability to detect ganglioside on the TLC plate (Fig. 4, lane A), confirming the glycosphingolipid nature of TSH-R-associated glycolipid. In addition, this finding excluded galactose as the first sugar bound to the ceramide moiety. Carbohydrate released by enzymatic treatment was not detected by the LFA-3 sialic acid probe at the plate front because the digest was reextracted before TLC and probing.

TSH-R ganglioside was further characterized by treatment with V. cholerae and A. ureafaciens sialidases. V. cholerae sialidase hydrolyzes ketosidic bonds in all gangliosides except GM1 (24). A. ureafaciens sialidase releases sialic acid attached to an internal galactose in any ganglioside, including GM1 (25). When TSH-R ganglioside was treated with V. cholerae sialidase, there was no change in its mobility or intensity compared with untreated ganglioside (Fig. 4, lane B). However, it was no longer visualized after A. ureafaciens sialidase treatment (Fig. 4, lane D). These findings suggest that the ganglioside is a member of the GM1 family, even though it does not migrate at the exact location of the bovine brain GM1 standard on TLC plates. The variation in mobility on TLC of the TSH-R-associated ganglioside (compare lane D in Fig. 1 with lane C in Fig. 4) may be explained by its modification—e.g., by deacetylation or delactonization. The acidic conditions used for the sialidase experiments (Fig. 4, lane C) can relactonize ganglioside or prevent the loss of acetyl groups prior to TLC. That the receptor-associated ganglioside can be modified is also evident by its alkali lability. Thus, when ganglioside on the TLC plate was exposed to ammonia vapor for 5 hr and then developed, it migrated below a GM1–GD1a cluster (data not shown).

**DISCUSSION**

We have shown that specific gangliosides are sequentially copurified with human and porcine TSH-R. In addition, a
single ganglioside was identified that coeluted with the Mγ 
50,000 TSH-R protein purified from human multinodular 
and Graves thyroid tissue and from normal porcine thyroid tissue. These findings 
represent direct evidence for the intimate 
association of ganglioside with the TSH-R. 

The ability to detect receptor-specific gangliosides is at-
tributed to the bulk purification of receptor and the use of 
125I-LFA as a probe. The binding of LFA to sialic acids within the 
oligosaccharide chains of glycoprotein and glycolipids 
allows gangliosides to be detected with a TLC overlay 
method. As little as 1 ng (0.5 pmol) of bovine ganglioside 
standard can be detected by this technique, a sensitivity 3 
orders of magnitude greater than that with the traditional 
resorcino1 and thiobarbituric acid methods for sialic acid (26). 

Salt, organic acids, and simple saccharides present in the 
upper phase after extraction do not interfere with binding of 
125I-LFA (19). Furthermore, the results with 125I-TSH-R 
show that the glycoprotein remains in the lower (protein-rich) 
phase. 

The copurification of ganglioside with TSH-R glycoprotein 
after two sequential chromatography steps indicates that 
ganglioside and glycoprotein are linked by strong noncovalent 
forces. Strong noncovalent association between lipid and protein 
has been described for the vitronectin receptor on melanoma cells (27). Analysis of the recently published 
sequences of the human Graves (4, 5) and dog (3) TSH-R 
reveals a possible site for glycolipid binding. The dog and 
human receptors contain an 11-amino acid sequence in the 
extracellular domain, 62 amino acids towards the N terminus 
from the first transmembrane domain, with >80% and 45% 
homology, respectively, to the sequence of peanut agglutinin 
(Fig. 5). This lectin binds the sugar sequence of Galβ1 → 3-
GalNAc in glycoproteins and in only one ganglioside, GM1 (28). Our findings indicate that a ganglioside from the GM1 
family is copurified with the human TSH-R. This lectin-like 
sequence in the TSH-R could serve as a putative binding site 
for the ganglioside. Interestingly, analysis of the LH/CGR 
sequence (6, 7) also reveals a similar putative ganglioside 
binding site. The LH/CGR contains a sequence with partial 
homology to another lectin, soybean (Glycine max) agglutinin 
(Galβ1 → 3-GalNAc), which is specific for GalNAc residues. GM2 is the only 
ganglioside that binds to soybean agglutinin (28). Although 
gangliosides have been implicated in LH (29) and CG (30) 
binding, a definitive LH/CGR-associated ganglioside has not 
been identified. 

Calcium may play a crucial role in the formation and/or 
activation of the TSH-R glycoprotein-ganglioside complex. Calcium has been directly implicated in the association 
with proteins (31), and both peanut and soybean lectins require calcium for binding (32). Calcium-binding 
proteins contain amino acid sequences in which alternate residues within a 9- to 12-residue loop provide 
sequence homology for metal binding (33). It may be 
relevant, therefore, that the dog and human TSH-R contain 
specific residues within the putative ganglioside-binding site 
that could donate oxygen ligands for calcium binding (Fig. 5). 

However, most of the carbohydrate and calcium-binding 
proteins identified to date contain separate carbohydrate 
and calcium-binding sites (32). As the amino acid sequence shown 
in Fig. 5 could represent a calcium-binding site, we searched 
the human TSH-R sequence for another putative ganglioside-
binding site. It has been shown that tryptophan is critical for 
the binding of the B subunit of cholera toxin to GM1 (34). The 
human TSH-R contains two extracellular tryptophan resi-
dues (4). The first, at amino acid residue 237, is not present 
in the LH/CGR-R sequences (6, 7), while the second, at amino acid residue 467, is in the short loop between the second 
and third transmembrane domains. Other charged residues 
potentially important for carbohydrate-protein interaction (35) 
are also present within this loop. 

If calcium were required for ganglioside binding to the 
TSH-R glycoprotein, one might predict that calcium-specific chelating agents would dissociate the complex. In prelimi-
nary experiments, we have obtained evidence to support this 
prediction, using TSH-R purified from the rat thyroid cell 
line FRTL-5. TSH-R was eluted in 3 M NaCl from TSH affinity 
coupled to blue dye beads (32). The receptor was extensively 
prewashed with 10 mM EGTA. Receptor eluted without prewashed EGTA treatment had a Mγ of 55,000, compared with 50,000 after EGTA 
treatment of the column. When both receptors were 
extracted, a ganglioside was identified only in the former, 
suggesting that EGTA dissociated ganglioside from receptor 
protein bound to the TSH column, which was then eluted 
with a slightly lower molecular weight in 3 M NaCl. 

The ganglioside copurified with the human TSH-R 
migrated between GM1 and GD1α on TLC. It was not 
sensitive to V. cholerae sialidase but was sensitive to A. ureafaciens 
sialidase, which implies that it belongs to the GM1 family 
of gangliosides. This family, taking into consideration the car-
bohydrate moiety only, includes GM1, GM1b, Fuc-GM1, 
and LM1 (lacto-series ganglioside), O-acetylated gangliosides 
(4,7,9-O-acetylated from each of the above groups), and 
O-methylated and sulfated gangliosides. Additionally, sialic 
acid can be linked via 2→3 or 2→6 bonds to galactose and 
possibly to galactosamine (36). Because the exact structure 
of the TSH-R ganglioside is not known, we can only speculate 
about its carbohydrate composition. It is of interest that 
fucosylated GM1 has been described in bovine (37) and 
human (26, 38) thyroids as a minor ganglioside, as were 
O-acetylated gangliosides. O-acetylated gangliosides are 
stronger immunogens than parent ganglioside molecules (39) 
and have been implicated as putative autoantigens in human 
melanoma cells (27) and in diabetes mellitus (40). The human 
TSH-R ganglioside may also be a lactone of a polysialylated 
ganglioside, which can be detected by LFA (W.K., unpublished data). GD1b was originally found to be the strongest 
bovine ganglioside inhibitor of TSH binding (10), and GD1b 
lactone migrates below GM1 (41). Ganglioside lactones have 
been shown to occur naturally in the nervous system of 
rodents (42) and in human brain (41). GD1b lactone would 
also be resistant to V. cholerae sialidase because of its 
blockade by carboxyl group mimetic to the α-keto-cyclic linkage, 
but in contrast to our findings with the purified TSH-R ganglio-
side, it should be resistant to A. ureafaciens sialidase (41). 

There are several reports (9–12, 14) that ganglioside mix-
tures inhibit the binding of TSH to solubilized receptor 
preparations and that some ganglioside fractions contain 
TSH-binding activity. Initially, a ganglioside from bovine 
thyroid membranes, containing two sialic acids and migrating 
at GM1, was shown to inhibit TSH binding (12). A GM2 
ganglioside from solubilized rat thyroid cell line (FRTL-5) 
membranes was then coprecipitated with a TSH-R glycopro-
tein by monoclonal antibodies that mimicked the bioeffect of 
TSH (43). These studies did not identify a specific receptor-
associated ganglioside because they employed impure recep-
tor preparations and relatively insensitive methods for gan-

![Fig. 5. Sequence similarities between dog and human TSH-R and peanut agglutinin (PNA). Amino acids are in the one-letter code. Asterisks above the dog TSH-R sequence indicate identical residues in the dog receptor and PNA; asterisks below the human TSH-R sequence indicate identical residues in the human receptor and PNA. Residues that could donate oxygen ligands for calcium binding are boxed.](image-url)
glioside detection. The present direct evidence that ganglioside may be an integral component of the TSH-R and the evidence that gangliosides modulate signal transduction events at the cell membrane (44, 45) imply a key role for ganglioside in TSH-R structure and function.

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