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GANGLIOSIDE DEPENDENT RETURN OF TSH RECEPTOR FUNCTION IN A RAT THYROID TUMOR WITH A TSH RECEPTOR DEFECT

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SUMMARY: The 1-8 rat thyroid tumor line with a thyrotropin and cholera toxin receptor defect and a deficiency in higher order membrane gangliosides is shown to regain both receptor functions with the in vivo resynthesis or the in vitro reconstitution of higher order gangliosides. Reconstitution was achieved by exposing primary cell cultures of the tumor to preparations of gangliosides from thyroid cells with functional thyrotropin receptor activity.

Studies of thyrotropin (TSH)[1/] binding to thyroid membranes have implicated higher order gangliosides as a potential component of the thyrotropin receptor (1). One important study supporting this possibility showed that membrane preparations from the 1-8 rat thyroid tumor with a TSH receptor defect were coincidentally devoid of higher order ganglosides (2). The TSH receptor defect was expressed as low TSH binding and no TSH stimulated adenylate cyclase activity despite normal thyroid functional responses to prostaglandins or dibutyryl cyclic AMP (3).

Recent studies involving monoclonal antibodies to the TSH receptor have supported a ganglioside role in TSH receptor structure and function (4-6). Thus a group of antireceptor monoclonals (4,5) which are, depending on the TSH concentration, competitive agonists or antagonists of TSH-stimulated adenylate cyclase activity or DNA synthesis were shown to interact with membrane ganglioside

1/ Abbreviations: TSH, thyrotropin; G_{M3}, N-acetylneuraminylgalactosylglucosylceramide; G_{M1}, galactosyl-N-acetylgalactosaminyl (N-acetylneuraminyl) galactosylglucosylceramide; cAMP, adenosine 3':5'-cyclic monophosphate. Ndase; neuraminidase.

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preparations. Monoclonal antibodies characterized as being directed against the glycoprotein component of the TSH receptor (6), i.e., that component believed important in the initial binding of TSH to the cell (1) were, in contrast, competitive inhibitors of both TSH binding and TSH enhanced functional responses rather than TSH mimetic (5,6).

The present report provides additional support for an important ganglioside role in TSH receptor function. Thus it shows that the presence of higher order gangliosides can "reconstitute" TSH receptor function in the 1-8 rat thyroid tumor with a TSH receptor defect.

MATERIALS AND METHODS

Cholera toxin was from Calbiochem; TSH was a purified preparation (25± 2 U/mg) previously characterized (7). Both were radioiodinated using a stoichiometric chloramine-T procedure (7). Forskolin was provided by Dr. K. Seamon, NIADDK, NIH, Bethesda, Md. Plasma membranes from normal rat thyroids and from rat 1-8 thyroid tumors were prepared as before (2,3).

Gangliosides were extracted and purified after membrane preparations were sonicated (Ultrasonics Inc. Model W185 D cell disrupter; 3 min.; output 3) under a stream of nitrogen; the procedure utilized was that of Yu and Ledeen (8). The final fraction was obtained using a Sep Pak™ cartridge as described by Williams and McCluer (9). The purified fractions, dried under a stream of nitrogen and dissolved in methanol, were chromatographed on silica gel 60 HP-TLC plates (E. Merck, Darmstadt, Germany). The running buffer was chloroform: methanol: KCl 2.5 mg/ml (120:70:16 by vol) (10); the gangliosides were visualized with resorcinol (11). Gangliosides that bind ^{125}I-cholera toxin were detected by autoradiography after chromatography on HP-TLC aluminia sheets (12). Neuraminidase treatment of ganglioside preparation was for 24 hours with a mixture of *Cholera vibrio* and *Arthrobacter ureafaciens* enzymes (13); reactions were terminated by 10 fold dilution with methanol; and ganglosides were reconstituted in buffer after drying with a stream of nitrogen. Lipid bound and free sialic acid were quantitated fluorometrically (14).

^{125}I-TSH binding and adenylate cyclase assays were performed as previously reported (2, 15). Protein was measured colorimetrically with crystalline bovine albumin as the standard (16); membrane preparations were first solubilized in 0.3 M NaOH.

Primary cultures of 1-8 rat thyroid tumors were prepared by a described technique (17); $1x10^6$ cells were plated in each chamber of a 24 well tissue culture dish, either in the presence or absence of 10 mU/ml TSH. For reconstitution experiments, cells were plated and maintained in the presence or absence of appropriate ganglioside extracts (0.1 mg/ml) for a 16 hour period before the TSH was added; techniques were otherwise the same as for reconstitution studies in NCTC 2071 cells (18). After 48 hours, cells were washed twice with a Hanks balanced salt solution and cAMP levels were measured (19). In experiments evaluating cholera toxin stimulated adenylate cyclase activity, the toxin was added for 1 hour prior to the cAMP assay to washed cells that had never been exposed to TSH.

The 11E8 and 22A6 monoclonal antibodies to the TSH receptor were the same preparations previously described (4-6); their ability to interact with thyroid cell or membrane preparations was detected by ^{125}I-protein A after washing excess antibody from the cells or membranes (4-6).

RESULTS

As originally described, the 1-8 rat thyroid tumor had a limited ability to bind TSH (2,3); in addition, despite normal fluoride sensitivity, it had no TSH stimulated adenylate cyclase activity (3). The original 1-8 tumor also had no cholera toxin stimulated adenylate cyclase response despite the ability of forskolin to activate adenylate cyclase activity (Table 1 top). During the course of 10 years of tumor passage, it was noted that a 1-8 variant evolved, 1-8N, which regained some TSH binding activity and both TSH and cholera toxin responsive adenylate cyclase activity (Table 1, Top).

The defect in TSH and cholera toxin stimulated adenylate cyclase activity in the original 1-8 tumor line had been associated with a defect in the synthesis of higher order ganglosides (2), i.e., only G_{M3} could be detected in its membrane fraction by thin layer chromatographic analysis (Fig. 1B Lane 2 and Ref. 2). Analysis of the 1-8N tumor which had regained cholera toxin and TSH receptor sensitivity indicated that there were now small amounts of higher order ganglosides chromatographically detectable in the G_{M1} region (Fig. 1 B Lane 1) and that at least some of these were higher order as measured by their ability to interact with ^{125}I-cholera toxin (Fig. 1A, lane 4). Although these data were compatible with the conclusion that the return of cholera toxin and TSH sensitive adenylate cyclase activity were coincident with the regained ability of the tumor cells to synthesize higher order gangliosides, they did not exclude the requirement for a glycoprotein component to have full expression of TSH receptor activity (1). Thus trypsinized 1-8N membranes lost TSH binding and TSH stimulated adenylate cyclase activity (Table 1, top) in the same manner as described earlier for trypsinized bovine, dog, and human thyroid membrane preparations (7,20). They also lost the ability to bind the 11E8 monoclonal antibody to the glycoprotein receptor component as measured by ^{125}I-protein A binding (4-6): 1250 $\pm$ 40 cpm as opposed 310 $\pm$ 50 cpm before and after trypsinization, respectively.

Incubation of a ganglioside extract from the 1-8N tumors with primary cultures of the original 1-8 tumor resulted in return of TSH and cholera toxin stimulated adenylate cyclase activity (Table 1 bottom). No reconstitution of

TABLE I

RECONSTITUTION OF TSH RECEPTOR EXPRESSION IN 1-8 RAT THYROID TUMORS

Thyroid Membrane or Cell Preparation	^{125}I-TSH Binding Activity[a] *cpm/20μg membrane protein*		Adenylate Cyclase Activity[b] *p moles cAMP/μg membrane protein/10 min.*					
	pH 7.4, 37° 50 mm NaCl	pH 6.0, 0°	Basal	Forskolin $1x10^{-5}$M	Cholera Toxin $1x10^{-9}$M	$1x10^{-8}$M	TSH $1x10^{-9}$M	$1x10^{-8}$M
Normal Rat	1890	20,980	11	140	46	80	28	54
1-8 Tumor (original)	320	4,050	10	135	11	8	10	12
1-8 N Tumor (variant)	1716	10,900	8	120	20	43	14	25
1-8N Tumor after trypsin/[c] treatment	415	1,400	11	125	-	-	10	9

	cAMP Content[b] *p moles cAMP/μg DNA*				
1-8 Tumor Cells (original)					
no addition	0.8	0.4	0.5	0.6	0.5
+ 1-8N (variant) gangliosides	0.6	4.4	6.4	3.5	5.6
+ Ndase treated 1-8N gangliosides	0.7	1.5	1.8	0.9	0.2
+ 1-8 (original) gangliosides	0.5	0.7	0.7	0.5	0.6
+ G_{M3}	0.8	0.7	0.6	0.7	0.8
+ Mixed brain gangliosides	0.8	3.1	4.8	1.1	1.3
+FRT_{L5} thyroid cell gangliosides	0.7	3.8.	5.8	5.2	7.2

a) TSH binding was for 1 hour in the assay buffer noted; assays were in triplicate. Average values are presented. In no case did the standard deviation exceed ± 10%.

b) Assays were performed in triplicate; results are the average of at least three separate experiments. In no case did the standard deviation of any value exceed ± 10%.

c) Trypsin treatment of membrane preparations used the procedures detailed in Reference 7.

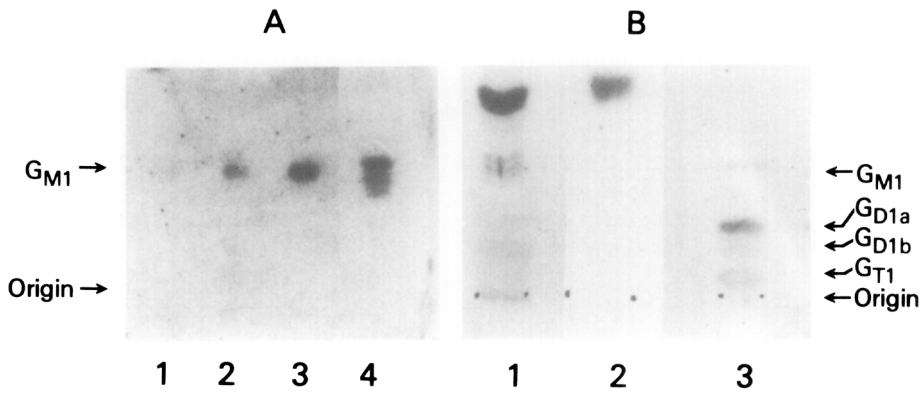

Figure 1: Thin layer chromatograms of ganglioside extracts from 1-8 thyroid tumors evaluated by (B) resorcinol staining or (A) autoradiography after ^{125}I-cholera toxin binding (12). In B: lane 1, 1-8 N tumor variant; lane 2, 1-8 original tumor; lane 3, mixed brain ganglioside standard. In A; Lane 1, G_{M3} standard; lane 2, G_{M1} standard; lane 3, mixed brain ganglioside standard; lane 4, 1-8 N tumor variant. Chromatography and analyses were performed as described in Materials and Methods.

TSH receptor function occurred in control reconstitution incubations containing no added 1-8N ganglioside extract; a ganglioside extract from the original 1-8 tumor (G_{M3}); mixed brain gangliosides; or the 1-8N ganglioside extract treated with a mixture of neuraminidases capable of converting 92% of the sialic acid residues from a lipid bound to free form. Reconstitution could be effected by gangliosides from cultured rat FRT_{L5} thyroid cells which have a functional TSH receptor sensitive to the monoclonal antireceptor stimulators which interact with gangliosides (4, 19).

Incorporation of the gangliosides from the 1-8N tumor variant or from FRT_{L5} thyroid cell preparations into the 1-8 original tumor cells was monitored by reactivity with the 22A6 stimulating monoclonal antireceptor antibody; this antibody has been shown to react with thyroid ganglioside preparations (4,5). Thus, as measured by ^{125}I-protein A, 22A6 binding to the no addition cells, to cells incubated with G_{M3}, or to cells incubated with neuramindase treated 1-8N gangliosides was 170 $\pm$ 50 cpm/mg membrane protein. The 22A6 binding to cells incubated with 1-8N or FRT_{L5} gangliosides, was 480 + 50 and 610$\pm$ 40 cpm respectively (p values < 0.01 compared to the controls above).

DISCUSSION

The present experiments support previous suggestions (1,2,4,5, 10) that gangliosides play a functional role in TSH receptor expression. They show that in vivo resynthesis of higher order gangliosides by a variant of the 1-8 thyroid tumor line with a TSH receptor defect is associated with return of TSH as well as cholera toxin stimulted adenylate cyclase activity. The "higher order" nature of the gangliosides is inferred from their migration on thin layer chromatograms and their ability to interact with cholera toxin, the G_{M1} specificity of which is well documented; their exact structure remains, however, to be characterized. The possibility that the reconstitution studies may require only a minor ganglioside component of the 1-8N mixture must be considered given previous results of Mullin et. al. (10) and current studies showing that monoclonal stimulating antibodies reactive with gangliosides (4) interact with a minor component of the total ganglioside preparation from either human thyroids or rat thyroid FRT_{L5} cells (unpublished observations).

A recent study of cultured rat FRT_L thyroid cells (21) has questioned the role of gangliosides since only small amounts of higher order gangliosides were detectable in that line. The ability of FRT_L ganglioside extracts to reconstitute adenylate cyclase activity in the 1-8 tumor primary cultured cells (Table 1 bottom) would suggest that these small amounts are, nevertheless, physiologically relevant. This conclusion is consistent with previous arguments showing that levels of G_{M1} detectable only by radioactive labeling procedures are sufficient for expression of cholera toxin sensitive adenylate cyclase activity (18, 22). The present data should, however, in no way be construed to negate the importance of the glycoprotein component of the TSH receptor (1, 4-6) as noted from the trypsin sensitivity data in Table 1, top. Rather the data must be taken a prelude to questions concerned with the mechanisms by which the interaction of the glycolipid and glycoprotein components of the TSH receptor yield a physiologic receptor response.

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