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INTERACTION OF THE THYROTROPIN RECEPTOR ON RAT FRTL-5 THYROID CELLS WITH THYROTROPIN AND A THYROTROPIN-STIMULATING AUTOANTIBODY FROM GRAVES' PATIENTS

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FRTL-5 rat thyroid cells were either surface-labeled with ^{125}I or biosynthetically labeled with [^{3}H]N-acetylglucosamine, solubilized by lithium diiodosalicylate and immunoprecipitated after sequential exposure to bovine thyrotropin and anti-bovine thyrotropin. Autoradiography of polyacrylamide gels run under denaturing conditions and in the presence of a reducing agent revealed two prominant bands with approximate molecular weights of 66-70 kDa and 47 kDa. Immunoprecipitation of the same radiolabeled and solubilized membrane preparations with a Graves' disease IgG having thyroid stimulating but no thyrotropin-binding inhibiting activity revealed only one major band, migrating near the 47 kDa component reactive with thyrotropin. No bands were immunoprecipitated in control incubations using normal human IgG or substituting radiolabeled, solubilized membranes from a rat thyroid cell line with no thyrotropin receptor activity. Thin layer chromatography of Folch extracts of the [^{3}H]-N-acetylglucosamine-labeled immunoprecipitates obtained by either procedure indicated that a specific thyroid ganglioside was coprecipitated with the immunoprecipitated proteins in both cases.

A consensus view of the thyrotropin (TSH) receptor structure may be emerging. Solubilization and gel filtration (1,2) or lectin affinity columns (3) have identified a large (~300 kDa) TSH binding component. Cross-linking studies from two laboratories (4,5) have defined a ~70 kDa TSH binding component with 50 ± 5 kDa and 20 ± 5 kDa subunits, the former subunit being hydrophilic (5) and able to bind TSH (4,5). Target analysis studies (6)

Abbreviations: TSH, thyrotropin; TSAB, thyroid stimulating antibody; TBIAb, thyrotropin binding inhibiting antibody; NIgG, normal human IgG; G_{M3}, N-acetylneuraminygalactosyl-glucosylceramide; G_{M2}, N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide; G_{M1}, galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide; G_{D1a}, N-acetylneuraminyl- galactosyl-N-acetyl galactosaminyl-[N- acetyl-neuraminyl]-galactosylglucosylceramide; G_{D1b}, galactosyl-N-acetyl-galactosaminyl-[N-acetyl-neuraminyl-N- acetylneuraminyl]- galactosyl-glucosylceramide; G_{T1}, N-acetylneuraminyl- galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl-N-acetylneuraminyl]- galactosylglucosylceramide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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identified both a large, >250 kDa, and a 70 kDa TSH binding unit as did recent studies using monoclonal antibodies to the TSH receptor (7,8). The latter studies (7,8) further indicated that the 50 ± 5 and 20 ± 5 kDa subunits appear to result from TSH-induced turnover of the ~300 kDa and 70 kDa units.

Unfortunately, studies which have tried to use autoantibody preparations from patients with Graves' disease (9-13) have not supported these data, but rather have identified numerous other sized proteins. One possible explanation for this discrepancy is that patients with Graves' disease actually have a wide spectrum of autoantibodies to the TSH receptor in their serum, for example, thyroid-stimulating antibodies (TSAbs) and thyrotropin binding inhibiting antibodies (TBIAb), as well as antibodies unrelated to the TSH structure, for example antimicrosomal and antithyroglobulin antibodies (14). Another problem in defining the TSH receptor structure has emerged in studies of monoclonal antibodies to the TSH receptor (7,8,15,16). Mixing experiments (17,18) have suggested that TSAbs and TBIAbs are directed at different determinants of the physiological TSH receptor structure and have supported the concept that a specific thyroid ganglioside may be a component of the receptor in addition to a glycoprotein capable of binding TSH with high affinity (4,7,8,15-20).

The present study attempted to address the question as to whether TSH and TSAbs interacted with different determinants of the TSH receptor by direct evaluation of their interaction with radiolabeled membrane components of FRTL-5 thyroid cells. It also attempted to address the problem of the diversity of proteins immunoprecipitated by Graves' autoantibody preparations. To accomplish this we used a rigorously prescreened Graves' IgG with TSAb activity, but no anti-thyroglobulin, anti-microsomal or TBIAb activities. This type of IgG is extrememly rare, since it was found only in 1 out of >600 patients tested. We compared the ability of this TSAb IgG and sequential treatment with TSH and anti-TSH to immunoprecipitate proteins related to the TSH receptor.

MATERIALS AND METHODS

The FRTL-5 cells (ATCC CRL #8305) are a continuous line of functioning rat thyroid cells (21). The FRT cells are a rat thyroid cell line which has lost its glycoprotein receptor component (22) and TSH-related functions, i.e., the ability to bind TSH and to respond TSH with respect to growth or function (22,23).

Bovine TSH was a purified preparation provided from the National Pituitary Agency, NIH, USA. Anti-bovine TSH was a generous gift of Dr. J.M. Hershman, University of California, Los Angeles, USA.

IgG was prepared by a previously described method (24) from the serum of a normal subject (NIgG) or of a patient with Graves' disease. The particular Graves' IgG used in these studies was selected for these properties: strong positivity in the thyroid adenylate cyclase stimulation assay, using either a membrane or a whole cell system; negativity in the TSH-binding inhibition assay, using either crude thyroid plasma membrane preparation or a solubilized TSH "receptor"; negativity for antimicrosomal and antithyroglobulin antibodies by hemagglutination. None of these activities could be detected either in the

original serum or in the IgG preparation up to a concentration of 1 mg/ml. This IgG was designated as TSAb IgG.

After presaturation with 0.1M KI, in order to avoid cellular uptake of ^{125}I, FRTL-5 and FRT cells were labeled with ^{125}I by a lactoperoxidase method (18). Alternatively, they were radiolabeled by incubation with 10 μCi/ml [^{3}H]-N-acetylglucosamine. Radiolabeled FRT and FRTL-5 cells were solubilized by 0.1M lithium diiodosalicylate (1,25) and TSH receptor was immunoprecipitated as follows. Aliquots of solubilized radiolabeled FRT and FRTL-5 cells were incubated overnight at 4°C with 10μg NIgG in a total volume of 1 ml of 0.05 M Tris-HCl, pH 7.6, containing 15 M NaCl, 0.02 M EDTA, 1% Triton X-100 and 200 U/ml aprotinin. Anti-gamma globulin (10μg) was then added at 37°C for 3 h. Nonspecific immune complexes were precipitated by the addition of 150 μl protein A and discarded. The supernatants after this preprecipitation were collected and incubated with 10 μg TSH, TSAb IgG or NIgG. After incubation as above, immunoprecipitations were carried out by the addition of anti-TSH or anti-gamma globulin, as appropriate. The immunoprecipitates were washed several times with 0.02 M Tris-HCl, pH 7.6, containing 0.6 M NaCl, 0.02 M EDTA, 1%, Triton X-100, and 200 U/ml aprotinin.

Immune complexes were solubilized and reduced by incubation at 100°C for 5 min in a solution of 1% SDS, 5% 2-mercaptoethanol, 0.02 M EDTA, 20% sucrose, 0.1M Tris-HCl, pH 7.6, and 0.01 M bromphenol blue. Protein A was removed by centrifugation, the supernatants were collected and applied to slab gels with a 5% polyacrylamide stacking gel and 7.5% resolving gel, both containing 0.1% SDS. Electrophoresis was carried out in 0.05 M Tris-glycine, pH 8.9, with 0.1% SDS and 1 μM 2-mercaptoethanol. Labeled or unlabeled molecular weight markers were added in each run. After fixing with 10% trichloroacetic acid, gels were stained with Coomassie Blue R-250, sliced into 2-mm sections, and the radioactivity in each section counted. Alternatively, gels were dried and analyzed by autoradiography.

Gangliosides were extracted and purified as previously described (26,27). Immune complexes obtained after labeling cells with [^{3}H]-N-acetylglucosamine were sonicated (Ultrasonics Inc., Model W 185 D cell disrupter, 3 min, output 3) under a stream of nitrogen. The final ganglioside fraction, obtained using a Sep Pak^{TM} cartridge, was dried under a stream of nitrogen, dissolved in methanol and 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 volume); the gangliosides were visualized by resorcinol or by autoradiography.

RESULTS

Radiolabeled and solubilized membrane material from FRTL-5 cells was incubated with TSH, TSAb IgG or NIgG and immunoprecipitated as described in Materials and Methods. The immunoprecipitated radioactivity was analyzed, after SDS-polyacrylamide gel electrophoresis, by autoradiography or by gamma-counting of 2-mm gel sections. Figure 1 shows the radioactivity pattern of the immunoprecipitated material from FRTL-5 cells by both methods. After incubation with TSH, a first, smaller peak containing a protein with an apparent molecular weight of approximately 66-70 kDa, and a second, larger peak with a protein having an apparent molecular weight of ~ 47 kDa were evident in both analyses (Figure 1). After incubation with TSAb IgG, only one peak was observed, migrating near the 47 kDa peak obtained with TSH (Figure 1). A faint band with a very high molecular weight (~280 kDa) was observed in both cases. No radioactivity could be detected by this system in the precipitates obtained with NIgG (Figure 1B). After immunoprecipitation of FRT membranes, labeled and

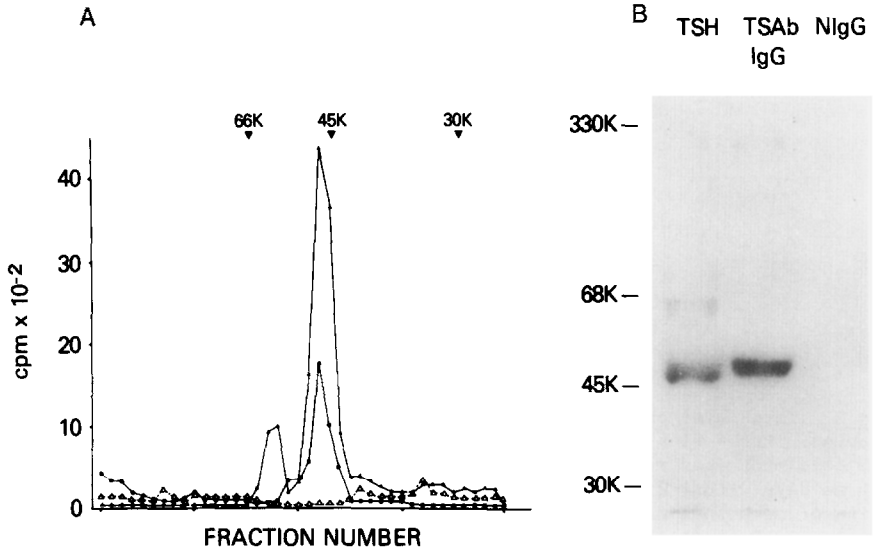

Figure 1. Immunoprecipitation of solubilized ^{125}I-labeled FRTL-5 or FRT cells with TSH, TSAb IgG, or normal IgG (NIgG). Precipitation of ^{125}I-labeled FRTL-5 cells with TSH (▲----▲) or TSAb IgG (●----●) and of ^{125}I-labeled FRT material with TSH (△----△) was as described in Materials and Methods. In A, Immunoprecipitates were applied to 7.5% SDS-PAGE, the gel was sliced into 2-mm sections and radioactivity was counted. Arrows indicate, from left to right, molecular weight markers of 68 kDa, 45 kDa and 30 kDa. In B, analysis is by SDS-PAGE and autoradiography of immunoprecipitated products. Lane 1 depicts immunoprecipitation of ^{125}I-labeled solubilized FRTL-5 cells with TSH; lane 2 is immunoprecipitation of the same material with TSAb IgG; lane 3 is immunoprecipitation of the same material with NIgG.

solubilized identically to FRTL-5 cell membranes, no radioactivity was found in immunoprecipitates on gels analyzed by autoradiography (data not shown) or gamma-counting of 2-mm slices (Figure 1A).

The immunoprecipitates were subjected to Folch extraction and the Sep Pak-treated ganglioside fraction was analyzed by thin layer chromatography. Figure 2 shows that a ganglioside is present in the immunoprecipitates obtained from FRTL-5 cells with both TSH and TSAb IgG (lane 3 and 4, respectively), but not from FRTL-5 cells immunoprecipitated with NIgG (lane 2). The ganglioside is specific relative to the total ganglioside pool (lane 5) and migrates near a G_{M2} standard (lane 1). Unlike a G_{M1} standard, it did not react with cholera toxin after thin layer chromatography and exposure of the plates to labeled cholera toxin (data not shown).

DISCUSSION

The present report, using a TSH/anti-TSH immunoprecipitation procedure, confirms cross-linking studies (4,5) in identifying an ~ 70 kDa and an ~50± 5 kDa TSH binding component on thyroid membranes. This data thus supports the conclusion of those (4,5) and other (7,8) studies using monoclonal antibodies to

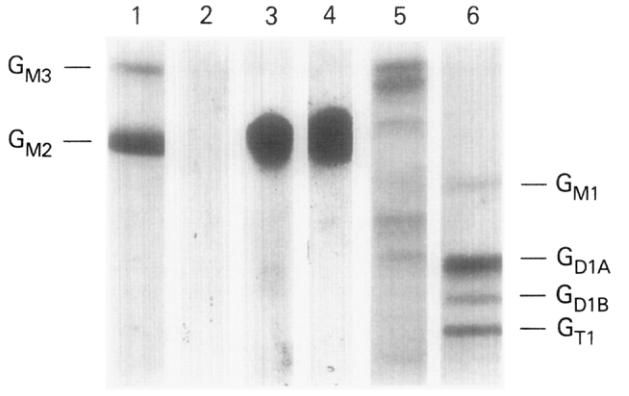

Figure 2. Thin layer chromatography of Folch-extracted pellets immunoprecipitated with TSH (lane 3), TSAb IgG, (lane 4) or NIgG (lane 2). FRTL-5 cell membranes were radiolabeled both by i) preincubation of the cells for three days with [^{3}H]-N-acetylglucosamine (10 μCi/ml), and ii) surface labeling of membranes using the radioiodination procedure described under Materials and Methods. The solubilized membranes were immunoprecipitated as in Figure 1. An aliquot of the resuspended pellet was subjected to SDS-PAGE as in Figure 1. The remainder was Folch-extracted (see Materials and Methods) and the ganglioside-containing fraction analyzed by thin layer chromatography. Based on ^{125}I radioactivity, the Folch extracts contained no significant protein (<75 cpm/extract). Autoradiography of the pellet precipitated with TSH or TSAb IgG from FRTL-5 cells (lanes 3 and 4, respectively) revealed one major tritiated spot migrating slightly above a G_{M2} standard. Pretreatment of the pellet or extract with pronase did not alter this result. No such spot was evident in NIgG pellet (lane 2). A resorcinol stained chromatogram of the total FRTL-5 ganglioside extracted from membranes is presented for comparison (lane 5), as are two chromatograms of brain gangliosides (lane 1 and 6). After scraping, the spots showed only tritium radiolabel, with no ^{125}I cpm. A mixture of neuraminidases caused both a change in migration of the spots similar to that described by Mullin et al. (27) and released free sialic acid as evidenced by chromatography with unlabeled standard.

the TSH receptor that these bands are relevant components of the physiologic TSH receptor. The present study amplifies this conclusion by showing that the bands are absent in a cell line which does not have a functional TSH receptor glycoprotein as assessed by monoclonal antibodies (22) and function (22,23). Since this study avoids cross-linking reagents, it seems to provide a control for those results (4,5). The high molecular weight band seen in the present study also affirms the idea that it too is a component of the receptor (1-3,6).

Studies which have attempted to use Graves' IgG preparations to immunoprecipitate TSH receptor components have complicated the problem since these preparations contain a multiplicity of antibodies to the TSH receptor and other thyroid antigens (7-14). In this study we have been careful to ensure, within the limits of current detection methods, that we used a preparation with only TSAb activity. There was no detectable TBIAb activity nor detectable levels of antibodies to thyroglobulin or the microsomal antigen. This contrasts with other studies where either no care was taken in the characterization of the IgG preparation or emphasis was on the TBIAb (10,11). It is in this perspective that the specificity and significance of individual bands must be interpreted.

It would appear that, as predicted in mixing experiments (17,18), the TSAb immunoprecipitate is different from the of TSH immunoprecipitate and that this is not simply a quantitative difference.

Further and in the context of the mixing experiments (17,18), the primary TSH binding component would be presumed to be the ~70kDa comonent whereas the primary TSAb binding the ~50kDa component. Since TSH also interacts with the 50 kDa component, competitive agonism between TSH and TSAbs (17-20) could be explained if there was TSH interaction both with the 70 kDa and the 50kDa components.

Emphasis has always been on techniques wherein the immunoprecipitate is subjected to gel electrophoresis and specific proteins are identified. It is evident herein that these protein precipitates contain a glycolipid and that this glycolipid is not a nonspecific contaminant of the total ganglioside pool. In the context of studies which have suggested that a ganglioside is a component of the TSH receptor and that TSAbs interact with a specific thyroid ganglioside (7,8,15-20), these data suggest that there is a specific portion of the glycoprotein receptor component of the TSH receptor with a molecular weight of ~50 kDa which binds the glycolipid. This observation would be compatible with the hydrophilic properties of the ~ 50 kDa fragment (5).

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