Engineering the Human Thyrotropin Receptor Ectodomain from a Non-secreted Form to a Secreted, Highly Immunoreactive Glycoprotein That Neutralizes Autoantibodies in Graves’ Patients’ Sera*  

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Gregorio D. Chazenbalk, Juan Carlos Jaume, Sandra M. McLachlan, and Basil Rapoport†‡  

From the Thyroid Molecular Biology Unit, Veterans Administration Medical Center and the University of California, San Francisco, California 94121  

Previous attempts to generate autoantibody-reactive, secreted thyrotropin receptor (TSHR) ectodomain in mammalian cells have failed because of retention within the cell of material with immature carbohydrate. We have overcome this difficulty by performing progressive carboxyl-terminal truncations of the human TSHR ectodomain (418 amino acid residues including signal peptide). Three ectodomain variants (TSHR-261, TSHR-289, and TSHR-309) were truncated at residues 261, 289, and 309, respectively. Unlike the full ectodomain, ectodomain variants were secreted with an efficiency inversely proportional to their size. Secreted ectodomain variants contained ~20 kDa of complex carbohydrate. TSHR-261 was chosen for further study because it was secreted very efficiently and neutralized autoantibodies in Graves’ patients’ sera. This ectodomain variant was partially purified using sequential lectin and nickel-chelate chromatography, permitting the first direct visualization and quantitation of the mammalian TSHR. Most important, very small (nanogram) quantities of this material neutralized 70–100% of TSHR autoantibody activity in all 18 Graves’ sera studied.  

In summary, carboxyl-terminal truncation of the human TSHR ectodomain generates a secreted protein with complex carbohydrate that neutralizes autoantibodies in Graves’ patients’ sera. Antigenically active TSHR will be valuable for future studies on the diagnosis, pathogenesis, and immunotherapy of Graves’ disease.

Graves’ disease is a very common (~1% prevalence) (1), organ-specific autoimmune disease, affecting only humans. Unlike in diabetes mellitus, type I, a less common organ-specific disease, there is no spontaneous animal model for Graves’ disease. Also in contrast to diabetes mellitus, type I, one specific antigen is unequivocally and directly involved in the pathogenesis of Graves’ disease, namely the thyrotropin receptor (TSHR). Thus autoantibodies to the TSHR activate the receptor, leading to thyroid overactivity and thyrotoxicosis (reviewed in Ref. 2). The interaction between autoantibodies and the TSHR is, therefore, of interest from the theoretical, diagnostic, and (potentially) therapeutic points of view.  

Because of the importance of the TSHR as an autoantigen, a large effort has been made in the 7 years since the molecular cloning of its cDNA (3–5) to generate this protein in various expression systems, including bacteria (6–11), insect cells (12–16), stably transfected mammalian cells (3, 17–21), and cell-free translation (22), as well as by peptide synthesis (23, 24). However, the generation of effective TSHR antigen has been extraordinarily difficult. Thus, despite this major effort and although some of these approaches have appeared promising, it is remarkable that a direct assay for TSHR autoantibodies using recombinant TSHR antigen has not supplanted the indirect, TSH binding-inhibition assay with porcine thyroid extracts (25) in use for nearly 2 decades. Moreover, lack of effective antigen has hampered mechanistic and structural studies of autoantibody-TSHR autoantigen interactions.  

An important factor contributing to this difficulty is that TSHR autoantibodies, like TSH, predominantly recognize discontinuous, highly conformational epitopes (26–28). Recombinant TSHR expressed on the surface of mammalian cells are conformationally intact and are unquestionably recognized by autoantibodies in patients’ sera (29–31). Moreover, large numbers of TSHR-expressing mammalian cells can be produced in fermentors (20) and TSHR overexpression in Chinese hamster ovary (CHO cells) has been achieved by transgenome amplification (32). However, the seven membrane-spanning segments of the TSHR do not facilitate purification. Contrary to expectations, when the 418-amino acid residue, autoantibody-binding TSHR ectodomain is expressed in CHO cells without its serpentine region, it is not secreted but is retained within the cell (33, 34), largely in a form containing high mannose carbohydrate (34). Moreover, this TSHR ectodomain with immature carbohydrate is not recognized by autoantibodies in patients’ sera (34).  

We now report that, in contrast to the entire TSHR ectodomain, progressive carboxyl-terminal truncations lead to the secretion by CHO cells of a modified TSHR ectodomain with mature, complex carbohydrate. Further, by epitope-tagging this autoantigen and by amplifying its transgenome in CHO cells, we report the first direct visualization and quantification of TSHR of mammalian cell origin. Most important, this material can neutralize all or most TSHR binding activity in patients’ sera. Antigenically active TSHR will provide a major impetus for future studies on the pathogenesis of Graves’ disease.
Materials and Methods

Plasmid Constructs

We generated three plasmids for expression in mammalian cells of limited TSHR ectodomain truncations (Fig. 1). TSHR-261—Plasmid TSHR-5TR-NEO-ECE (35) contains an AflII site at codon 260 and an Xhol site in the vector at the 3 end of the insert. The AflII-Xhol fragment was excised and replaced with a cassette coding for 6 histidine residues (His6), followed by 2 stop codons. The cassette was created by annealing two oligonucleotides: sense, 5'-TTA-ACTATCCACCAACCCATCATCGTAAAT-3', antisense, 5'-CTAGGATCATGATGTTGGTGATG-3'. Ligation at the AflII site generated an Asn residue upstream of the His6, hence the nomenclature "261." TSHR-289—A cDNA fragment including the AflII site at codon 260 continuing to codon 289 followed by an Xhol site was generated by polymerase chain reaction using Pla DNA polymerase (Stratagene, San Diego, CA). This fragment was used to replace the AflII-SpeI segment in TSHR-5TR-NEO-ECE (the SpeI site is at codon 418) (26). Subsequently, an oligonucleotide cassette coding for His6 followed by 2 stop codons with SpeI and Xhol adhesive ends was inserted into the same sites of the intermediate construct (sense, 5'-CTAGCCATCACCCACCCACCGTAAAT-3'; antisense, as described above for TSHR-261).

TSHR-309—Construction used the identical strategy to that used for TSHR-289, except that the AflII-SpeI cDNA fragment generated by polymerase chain reaction extended up to codon 309.

After confirmation of the nucleotide sequences of the relevant areas, the TSHR-261, TSHR-289, and TSHR-309 cDNAs were excised with SaII and Xhol and transferred to the vector pSV2-ECE-dhfr (36).

Expression of TSHR Ectodomain Variants

Cell lines, stably transfected with the above TSHR ectodomain cDNA variants were established in CHO dhfr- cells (CHO-DG44; kindly provided by Dr. Robert Schimke, Stanford University, Palo Alto, CA), using procedures described previously (34). Transgene amplification was achieved by progressive adaptation to growth in methotrexate (final concentration 10 µM) (34).

Detection of TSHR Ectodomain Variants in Medium and in Cells

CHO cells to be tested for TSHR ectodomain variant expression were metabolically labeled with [35S]methionine/cysteine, exactly as described previously (1-h pulse and overnight chase) (32). The medium was harvested for immunoprecipitation of secreted TSHR protein, and the cells were processed further for analysis of intracellular TSHR protein, as described previously (32), with the following modifications. Cells were lysed in buffer containing 1% Triton X-100, were centrifuged for 45 min at 100,000 × g prior to preclarifying with mouse IgG and protein A, followed by immunoprecipitation using mouse monoclonal antibody (mAb) A10 (37) (kindly provided by Dr. Paul Banga, King's College, London, United Kingdom; epitope at amino acid residues 22–35; final concentration 1:1000) and transferred to the vector pSV2-ECE-dhfr (36).

Immunoblots of TSHR Ectodomain Variants

Lectin Adsorption

Binding of TSHR in conditioned medium was determined for three Sepharose-linked lectins: wheat germ agglutinin, Bandeiraea simplicifolia, and concanavalin A (ConA) (Pharmacia Biotech Inc.). Medium (40 ml) was slowly stirred for 2 h at room temperature with 0.4 ml of Sepharose lectin. The beads were then extensively washed in batch with 10 ml Tris, pH 7.5, 150 mM NaCl, and adsorbed material released (tumbling for 45 min at room temperature) with 3 ml of the same buffer supplemented with 0.25 M N-acetylglucosamine (wheat germ agglutinin), 20 mM α-methylgalactopyranoside (B. simplicifolia), and 0.5 M α-methylmannoside (ConA). Material (3 ml) was diluted as indicated in the text and spotted on nitrocellulose filters (Schleicher & Schuell). After air drying, the filters were incubated (45 min) in 50 mM Tris buffer, pH 7.5, and 150 mM NaCl (Tris-buffered saline) containing 5% skim milk powder, rinsed and incubated (2 h at 37°C) in Tris-buffered saline containing mAbA10 (1:1000) and 0.05% bovine serum albumin. The filters were rinsed, incubated (1 h at room temperature) with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G, and the signal developed as described previously (34).


c-terminal residue of TSH receptor ectodomain variants

FIG. 1. Schematic representation of three TSHR ectodomain variants truncated at their carboxyl termini. The serpentine transmembrane and cytoplasmic portions of the holoreceptor (764 amino acid residues including signal peptide) are not shown. Six histidine residues (6H) followed by a stop codon are inserted into the indicated TSHR residues. Insertion of a stop codon at residue 418 has previously been shown to generate an ectodomain containing predominantly high mannose carbohydrate that is largely retained within the cell and is not recognized by TSHR autoantibodies in Graves' patients' sera (34). As shown in the present study, progressive carboxy-terminal truncations of the TSHR ectodomain lead to a high level of secretion of material with mature, complex carbohydrate that can completely neutralize TSHR autoantibody activity.

Assay for Neutralization of TSHR Autoantibodies in the Serum of Graves' Patients

TSHR autoantibody kits were purchased from Kronus, San Clemente, CA. The principal of this assay is the ability of autoantibodies to compete for 125I-TSH binding to TSHR solubilized from porcine thyroid glands ("TSH binding inhibition," or TBI assay) (25). In brief, solubilized TSHR (50 µl) are preincubated (15 min) with patient's serum (50 µl). Buffer containing 125I-TSH is then added (2 h at room temperature). Solubilized TSHR complexed with TSH is precipitated by polyethylene glycol. Antibody activity is measured as percent inhibition of 125I-TSH binding relative to a standard serum from a normal individual. TSH binding assay by methods previously been shown to generate an ectodomain containing predominantly high mannose carbohydrate that is largely retained within the cell and is not recognized by TSHR autoantibodies in Graves' patients' sera (34). As shown in the present study, progressive carboxy-terminal truncations of the TSHR ectodomain lead to a high level of secretion of material with mature, complex carbohydrate that can completely neutralize TSHR autoantibody activity.

Immunoblots of TSHR Ectodomain Variants

Lectin-bound TSHR-261, TSHR-289, and TSHR-309 were eluted (see above), and Laemmli sample buffer (38) with 0.7 M (final concentration) β-mercaptoethanol was added (30 min at 45°C). Enzymatic deglycosylation with N-glycosidase F and endoglycosidase H was as described previously (34). After electrophoresis on SDS, 10% polyacrylamide gels, proteins were electrophoretically transferred to PVDF membranes, which were then processed as described above with the exception that incubation in mAbA10 was overnight and the second antibody was added for 1–2 h. In some experiments (e.g. Fig. 6), the immunoblots were developed using the BioMax-CD5-PRO kit (Kodak) according to the protocol of the manufacturer. Autoradiography was with Hyperfilm ECL (Amersham). TSHR-261 Partial Purification

Conditioned medium was harvested from CHO cells expressing TSHR-261 cultured in non-selective F-12 medium containing 10% fetal calf serum, antibiotics and 5 mM sodium butyrate (39). Medium (2 liters) was applied to a 70-ml concanavalin A-Sepharose column. After washing with 10 ml Tris, pH 7.5, 150 mM NaCl, bound material was eluted with 80 ml of 0.15 M α-methyl-mannoside in the same buffer. The eluted material was made up to 50 ml imidazole, pH 7.2, and applied to two 5-ml His-Trap columns in series (Pharmacia). Elution was with buffer containing 10 ml Tris, pH 7.4, 50 mM NaCl, and 100 mM EDTA. The sample was concentrated and the buffer exchanged to 10 ml Tris, pH 7.5, 50 mM NaCl using a Centriprep 30 (Amicon, Beverly, MA). At all stages, TSHR-261 recovery was monitored by
RESULTS

Secretion of Truncated TSHR Ectodomain Variants—CHO-DG44 cells were stably transfected with plasmids coding for TSHR ectodomain variants truncated at amino acid residues 261, 289, and 309 (Fig. 1). Individual clones were obtained by limiting dilution, and transgenome amplification was performed by progressive adaptation to growth in methotrexate (final concentration 10 μM). One clone of each TSHR ectodomain variant, selected for high level of TSHR expression, was expanded and used for further studies.

The ectodomain variant with the greatest degree of COOH-terminal truncation (TSHR-261) was entirely secreted into the medium, as detected by immunoprecipitation after an overnight chase, with no receptor remaining in the cells (Fig. 2). TSHR-289, truncated to a lesser extent, was secreted to an intermediate degree. The receptor remaining within the cells was present in multiple forms, the dominant band having a molecular weight lower than the secreted form.

Finally, for TSHR-309, the least truncated ectodomain, secretion into the medium was relatively inefficient. Thus, proportionately less receptor was secreted than remained within the cells, the latter primarily in lower molecular weight form. Expression of the 6 His residues at the carboxyl termini of the ectodomain variants was confirmed by nickel-NTA resin purification of precursor-labeled material secreted into the culture medium (Fig. 2). In contrast, the TSHR variants could not be clearly identified within the cell because the nickel-NTA resin bound to a large number of labeled intracellular proteins (data not shown).

Interaction of TSHR with Autoantibodies in the Serum of Graves’ Patients—Because our main purpose in generating a secreted form of the TSHR ectodomain was to obtain material suitable for study with TSHR autoantibodies in the serum of patients with Graves’ disease, it was important to test the secreted TSHR ectodomain variants for this property. We used a TBI assay to test whether conditioned medium from cultured cells expressing TSHR-261, TSHR-289, and TSHR-309 could neutralize autoantibody activity in a Graves’ patient’s serum. Of these, TSHR-261 and TSHR-289 were clearly active in terms of reversing the inhibition by TSHR autoantibodies of 125I-TSH binding (Fig. 3).

Adsorption of TSHR Ectodomain Variants to Lectins—Although the Ni-NTA resin was effective in purifying radiolabeled TSHR secreted by CHO cells into tissue culture medium (Fig. 2), we were unable to purify unlabeled TSHR protein from medium using this approach. The Ni-NTA bound to many unlabeled proteins despite attempts to minimize nonspecific interactions with imidazole and adsorption at lower pH (data not shown). We, therefore, attempted partial purification of TSHR ectodomain variants from conditioned medium using lectins. TSHR-261 in conditioned medium bound poorly to wheat germ agglutinin and B. simplicifolia (Fig. 4). Almost all of this material remained in the “flow-through,” and minimal amounts could be recovered by elution with specific sugar. In contrast, ConA was effective in extracting TSHR-261 from the medium. Because the non-secreted, full-length TSHR ectodomain that

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**Fig. 2.** Relative secretion into the culture medium of TSHR ectodomain variants. CHO cells stably expressing TSHR ectodomain variants truncated at amino acid residues 261, 289, and 309 were precursor labeled for 1 h followed by a chase of 16 h (see “Materials and Methods”). TSHR in medium (M) and cells (C) was then immunoprecipitated with a murine mAb (A10) to amino acid residues 22–35 (37). TSHR in medium was also recovered using Ni-NTA resin that binds to the 6 histidine residues inserted at the C termini of the ectodomain variants. Autoradiography in the experiment shown was for 12 h.

**Fig. 3.** Recognition of TSHR ectodomain variants by TSHR autoantibodies in Graves’ disease serum. The assay involves the ability of autoantibodies to compete for 125I-TSH binding to TSHR in solubilized porcine thyroid membranes (25) (see “Materials and Methods”). *Left panel,* in the absence of conditioned medium from CHO cells, serum from a Graves’ patient, unlike serum from a normal individual, reduces 125I-TSH binding by ~60%. *Right panel,* conditioned medium from a non-relevant cell culture secreting thyroid peroxidase (TPO) has no effect on TBI activity. In contrast, conditioned medium from TSHR-261 and TSHR-289 cell cultures (unlike TSHR-309) nearly completely reverses the TBI activity. Bars indicate the mean ± range of duplicate determinations. See Fig. 9 for data on 18 Graves’ sera using TSHR-261 after partial purification from conditioned medium.
did not interact with TSHR autoantibodies contained immature, high mannose carbohydrate and bound strongly to ConA (34), we were concerned that the ConA was extracting an inactive, high mannose component of TSHR-261, perhaps released from disintegrating cells. Fortunately, this was not the case. Thus, immunoblotting of ConA-enriched TSHR-261 showed that, like the material immunoprecipitated from conditioned medium with mAb A10 (Fig. 2), the secreted receptor was endoglycosidase-resistant and endoglycosidase F-sensitive (complex carbohydrate) (Fig. 5). Indeed, this pattern was similar to the smaller amounts of TSHR-261 that could be recovered from conditioned medium using wheat germ agglutinin (Fig. 5). Most important, the ConA-enriched material was highly active in neutralizing autoantibody TBI activity in patients' sera (data not shown for these experiments; see data below on TBI activity in more extensive studies using multiple Graves' sera; Fig. 8).

In addition to TSHR-261, TSHR-289 and TSHR-309 were also extracted from culture medium using ConA. Immunoblotting indicated that TSHR-289 and TSHR-309, like TSHR-261, contained only mature, complex carbohydrate (Fig. 6). Remarkably, TSHR-261 contains ~20 kDa of N-linked glycosylation, 40% of its mass. The apparent molecular masses of the deglycosylated proteins (~30, 32, and 34 kDa for TSHR-261, TSHR-289, and TSHR-309, respectively) were slightly (~2 kDa) greater than predicted from their known amino acid sequences (including His6 tags). A similar phenomenon was observed previously with the deglycosylated TSHR ectodomain (residues 1–418) (34).

Autoantibody Neutralization by Partially Purified TSHR-261—In the preceding studies, the secreted TSHR variants could be detected qualitatively by immunoprecipitation, immunoblotting, or autoantibody neutralization. However, it was important to determine quantitatively the amount of receptor that was interacting with TSHR autoantibodies in patients' sera. No TSHR standards are available for this purpose. Indeed, the TSHR of mammalian cell origin has never been purified sufficiently for direct visualization on a polyacrylamide gel. We selected TSHR-261 for further study because, of the three ectodomain variants, it was secreted to the greatest extent (Fig. 2) and because its "bioactivity" in terms of autoantibody recognition appeared equal to that of TSHR-289 (Fig. 3). As monitored by autoantibody neutralization, ConA chromatography provided an initial purification of ~100-fold. Subsequently, and in contrast to its use as an initial capture system, nickel-chelate chromatography was quite effective in generating sufficient TSHR-261 for direct visualization and quantitation by Coomassie Blue staining (Fig. 7, left panel). Enzymatic deglycosylation confirmed the immunoblot evidence for a ~30-kDa polypeptide backbone with ~20 kDa of complex carbohydrate (Fig. 7, right panel) and also provided the best means to quantitate the amount of receptor recovered. In three separate preparations from 2 liters of conditioned medium, recovery of TSHR-261 (corrected for a 40% glycan component) was 0.3–0.4 mg/liter. Amino acid sequencing of the 30-kDa deglycosylated
FIG. 7. Direct visualization and quantitation of TSHR-261. Left panel, polyacrylamide gel electrophoresis and Coomassie Blue staining of material recovered after capture from the conditioned medium with concanavalin A (first lane) and after the subsequent nickel-chelate chromatography step (second lane). Right panel, estimation of the TSHR-261 concentration by polyacrylamide gel electrophoresis of enzymatically deglycosylated, Coomassie Blue-stained material. Deglycosylation yields a sharper, more intense band than the glycosylated protein. The recombinant endoglycosidase F present in the reaction provides an internal standard of 0.7 μg of protein. The TSHR-261 polypeptide represents 60% of the mass of the glycosylated protein (see also Figs. 5 and 6).

FIG. 8. Titration of TSHR autoantibody neutralization by TSHR-261. Serum from a Graves’ patient with moderate TBI activity was assayed using the commercial autoantibody kit (see “Materials and Methods”) in the presence of increasing concentrations of partially purified TSHR-261. Serum from a normal individual does not inhibit 125I-TSH binding to solubilized porcine thyroid TSHR (hatched bar). In the absence of TSHR-261, TSH binding is reduced to ~40% of maximum. Incubation volume in the assay is 0.2 ml. Bars indicate the mean ± range of duplicate determinations.

FIG. 9. Neutralization of TSHR autoantibodies by TSHR-261 partially purified from conditioned medium. TBI activity in the sera of 18 Graves’ patients was measured using a commercial kit (see “Materials and Methods”). These sera, unlike two sera from normal individuals, compete for 125I-TSH binding to solubilized membranes from porcine thyroids (hatched bars). Inclusion of TSHR-261 (50 ng/tube) neutralizes all or most of the autoantibody activity in the 18 sera. Bars indicate the mean ± range of duplicate determinations.

Secretd TSHR Ectodomain

Advances in the diagnosis and potential immunotherapy of Graves’ disease are dependent on the availability of relatively large amounts of immunoreactive, recombinant antigen. Many studies have explored the interaction of Graves’ sera with TSHR material generated in bacteria, insect cells, cell-free translates or as synthetic peptides (see Introduction). Most of these studies involved detection of recombinant material by immunoblotting, immunoprecipitation, or enzyme-linked immunoassay, procedures that do not evaluate the ability of this material to interact with functional autoantibodies. Trace amounts of “functional” TSHR ectodomain material are present in insect cells infected with recombinant baculovirus containing the TSHR cDNA (15), as well as in stably transfected CHO cells (33, 34). Very recently, there has been confirmation that the TSHR ectodomain generated in a baculovirus system can neutralize TBI activity in Graves’ patients’ sera (16). However, this material was largely insoluble, the active component was not identified or quantitated, and the nature of the carbohydrate (complex versus high mannose) was not determined.
In our experience, the recombinant TSHR of mammalian cell origin is the most effective in interacting with autoantibodies. The strongest testimony to this conclusion is that crude porcine thyroid extracts are still used in the standard clinical TBI assay. TSH holoreceptor overexpression in CHO cells (32) can generate a crude detergent extract that rivals, or surpasses, the efficacy of porcine thyroid extracts (41). However, this membrane-associated material cannot be used for large scale purification for structural studies and will be difficult to use in a future direct (as opposed to an indirect TBI) assay for TSHR autoantibodies.

We have now overcome the previous inability (33, 34) to generate in mammalian cells a secreted, soluble, complex carbohydrate-containing form of the TSHR ectodomain. Unlike the non-secreted, high mannose-containing ectodomain (34), secreted, COOH-terminal truncated TSHR ectodomain variants are recognized by autoantibodies. Whether the complex carbohydrate comprises part of the epitope(s) for TSHR autoantibodies or whether lack of autoantibody recognition of the “high mannose” ectodomain is secondary to incorrect polypeptide folding (and, hence, retention in the endoplasmic reticulum) (42, 43) is presently unknown. It is of interest (and paradoxical) that COOH-terminal truncation of the LH/CG receptor ectodomain at a position (amino acid residue 294) similar to the TSHR variants generates a non-secreted protein (44). On the other hand, the LH/CG receptor truncated at residue 329 or further downstream is secreted to a limited extent (45, 46). Alternately spliced truncated forms of TSHR mRNA have been detected in thyroid tissue (47, 48); however, whether these transcripts are actually expressed and, if so, secreted by thyrocytes is unknown.

The lectin specificity of TSHR-261 is consistent, in part, with previous data on the extraction of TSH holoreceptor activity from detergent-solubilized thyroid membranes. In this earlier study, B. simplificifolia was effective for bovine, but not for human, TSHR (49). Unlike the human TSHR ectodomain variants, the bovine TSH holoreceptor was also bound well by wheat germ agglutinin and was irreversibly bound by concanavalin A (49). Lectin chromatography by itself was insufficient for TSHR-261 purification. In the future, single-step affinity purification with a mAb will be a preferable approach. At present, murine IgG class mAb have been generated by immunization with TSHR of prokaryotic or insect cell origin (7, 37, 50, 51). Unfortunately, most mAb (including the 6 that we have tried from two different laboratories) (7, 37) do not recognize the native, mature mammalian TSHR that is necessary for immunological studies in Graves’ disease. Future immunization with complex carbohydrate-containing TSHR-261, or with mammalian cells expressing the TSHR in association with MHC class II molecules (52), may overcome this difficulty.

Autoantibody reactivity of TSHR-261 is reminiscent of the observation nearly 3 decades ago that freezing and thawing thyroid tissue releases a water-soluble factor (long acting thyroid stimulator absorbing activity; LAA) that neutralizes TSHR autoantibodies (53, 54). LAA (like TSHR-261) was estimated to be ~50 kDa (54). More recently, a fragment of the TSHR released by trypsin has been observed to neutralize TSHR autoantibodies (55). However, the segment of the TSHR with LAA activity, produced either by freeze-thawing or with trypsin, is unknown.

There are no previous studies on the quantitative neutralization of TSHR autoantibodies in Graves’ patients’ sera using defined amounts of mammalian antigen. The very small (up to 50 ng) amounts TSHR-261 required for autoantibody neutralization is orders of magnitude lower than those used in studies with synthetic peptides (24). This quantitative information will be useful as a future benchmark by which to judge the potency of purified TSHR of bacterial and insect cell origin. A corollary of the minute amount of antigen necessary for TSHR autoantibody neutralization is that the autoantibody concentration in patients’ sera is very low, consistent with previous flow cytometry data (40). The large difference between TSHR and thyroid peroxidase autoantibody concentrations is of potential significance in understanding the pathogenesis of Graves’ disease (56).

The TSHR ectodomain variant TSHR-261, despite its potent autoantibody neutralizing activity, does not bind TSH. The TSH binding site on the TSHR is discontinuous and involves multiple segments throughout the entire ectodomain, including segments downstream of residue 261 (26, 57). The TSHR autoantibody epitope(s) may, therefore, be more limited than the TSH binding site. Support for this notion is provided by data from most (9, 15, 33, 34), but not all (58, 59), laboratories that TSH binding to the full TSHR ectodomain is negligible or absent. Remarkably, in contrast to TSH, hCG binds with high affinity to the isolated ectodomain of its cognate receptor, even when the latter lacks carbohydrate (reviewed in Ref. 60). The reason for this major difference between such closely related receptors is an enigma. The present data with TSHR-261 must also be reconciled with previous evidence obtained using chimeric TSH-LH/CG receptors that the epitope(s) for autoantibodies are also discontinuous and extend downstream of residue 261 (27). One possible explanation for this paradox is that TSHR-261 contains the dominant portion of a discontinuous epitope, sufficient to neutralize most TSHR reactivity in Graves’ patients’ sera.

In summary, progressive carboxyl-terminal truncations lead to the secretion by CHO cells of modified TSHR ectodomains with mature, complex carbohydrate. Most important, TSHR-261 is highly potent in interacting with TSHR autoantibodies. Antigenically active TSHR will provide a major impetus for future studies on the diagnosis, pathogenesis, and (possibly) immunotherapy of Graves’ disease.

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