A Monoclonal Antibody with Thyrotropin (TSH) Receptor Inverse Agonist and TSH Antagonist Activities Binds to the Receptor Hinge Region as Well as to the Leucine-Rich Domain

Chun-Rong Chen, Sandra M. McLachlan, and Basil Rapoport

Autoimmune Disease Unit, Cedars-Sinai Research Institute and University of California Los Angeles School of Medicine, Los Angeles, California 90048

Monoclonal antibody CS-17 is a TSH receptor (TSHR) inverse agonist (suppresses constitutive activity) and a TSH antagonist. Elucidation of the CS-17 epitope will provide insight into TSHR structure and function. Present information on its epitope conflicts with recent data regarding another TSHR inverse agonist antibody. To characterize further the CS-17 epitope, we exploited the observation that CS-17 does not recognize a chimeric receptor with TSHR hinge region residues 261–289 replaced with homologous rat LH receptor residues (13 mismatches). We generated individual and double TSHR mutations corresponding to these mismatches. On flow cytometry, only T273L/R274V reduced CS-17 recognition. No mutation affected TSH-stimulated cAMP generation. Because the immunogen for CS-17 generation was highly glycosylated, we also investigated whether the glycan moiety at N198, topologically adjacent to Y195 (a previously identified epitopic component), could contribute to the CS-17 epitope. Elimination of this N-linked glycan (mutations of N198 and T200) abrogated CS-17 binding without altering TSH responsiveness. However, studies with tunicamycin suggested that these mutations affected CS-17 binding by altering the polypeptide backbone rather than eliminating the glycan moiety. TSHR residues N198 and T200, like Y195, are on the convex facet of the leucine-rich domain. In summary, the present data indicate that the discontinuous epitope of CS-17, a TSHR inverse agonist and TSH antagonist, includes a component in the hinge region as well as the convex surface of the TSHR leucine-rich domain. These findings expand our present concept of glycoprotein hormone binding and function. (Endocrinology 150: 3401–3408, 2009)

The TSH receptor (TSHR) plays a critical role in thyroid function in health and disease (reviewed in Refs. 1 and 2). The physiological negative feedback mechanism between thyroid hormones and TSH secretion by the pituitary contributes to the maintenance of euthyroidism. This equilibrium is subverted by thyroid-stimulating autoantibodies that activate the TSHR in Graves’ disease leading to hyperthyroidism (3, 4). More rarely, competitive antagonists of TSH binding to its receptor cause hypothyroidism (5). The TSHR also maintains relatively high activity even in the absence of ligand (6). Such constitutive activity may reduce the efficacy of TSH suppressive therapy for metastatic well-differentiated thyroid carcinomas. TSHR somatic (7) or germ line (8) mutations can up-regulate constitutive activity leading to “toxic” thyroid adenomas or familial, nonautoimmune hyperthyroidism.

Besides agonists (thyroid-stimulating autoantibodies) and antagonists (TSH blocking antibodies), some TSHR antibodies function as inverse agonists, defined as the ability to reduce ligand-independent TSHR constitutive activity. However, in general pharmacological terms, these functions are not clearly separable. A weak agonist can be an antagonist, and many antagonists are not “neutral” but also have inverse agonist activity (reviewed in Ref. 9). Two monoclonal TSHR antibodies with inverse agonist activity have recently been reported: murine CS-17 (10) and human 5C9 (11). Both are also TSH antagonists. TSHR inverse agonists may be of clinical value, e.g. in well-differentiated thyroid carcinoma (10). Because of ligand-independent constitutive activity, even total suppression of TSH with high doses of T4 can only partially reduce TSHR activity in re-
sidual thyroid carcinoma cells. Understanding the mechanism by which TSHR antibodies with inverse agonist activity suppress constitutive activity can also provide valuable insight into the TSHR structure-function relationship. To attain this goal, it is necessary to identify the epitopes for the TSHR inverse agonist antibodies; not an easy task because, like most antibodies to large proteins (12), both CS-17 and SC9 have conformational, discontinuous epitopes that cannot be localized by binding to synthetic peptides (10, 11). Although TSHR mutagenesis studies have identified a few amino acid residues likely to be involved in the binding sites for both monoclonal antibodies (mAb) (11, 13), much additional work is required to more fully delineate these epitopes.

The approximate location of the CS-17 epitope on the TSHR ectodomain has been deduced from several lines of evidence. First, CS-17 was generated by immunization with TSHR residues 22–289 (residues 1–21 being the signal peptide) (10). This immunogen includes the entire TSHR leucine-rich domain (LRD) and the proximal portion of the hinge region, also known as the signaling specificity domain (SSD) (14). Together, the LRD and proximal SSD comprise the TSHR A subunit, formed by intramolecular cleavage of TSHR on the cell surface into disulfide linked A and B subunits, with deletion of an imprecisely defined loop (C-peptide region) (reviewed in Ref. 2). Second, studies on CS-17 recognition of chimeric receptors, in which segments of the human TSHR are substituted with the homologous regions of the rat LH receptor (LHR), further narrowed the CS-17 epitope to residues 170–289 (10). Finally, based on the fortuitous observation that CS-17 recognizes the human but not the porcine TSHR, replacing human with porcine amino acids within the residue 170–289 “window” revealed TSHR Y195 to be important for CS-17 binding (13). Mutation of residues Q235 and S243 together (but not individually) reduced CS-17 binding to a much lesser extent.

Identification of TSHR residue Y195 in the epitope of mAb CS-17 had interesting implications for understanding TSHR structure and function. This residue is on the convex surface of the LRD. In contrast, binding of mAb SC9 (also a TSH binding antagonist and a functional inverse agonist) was diminished by mutagenesis of residues on the opposite, concave, facet of the TSHR LRD (11). The present study reinforces the deduction that CS-17, a TSH antagonist, binds to the convex surface of the TSHR LRD, an observation that poses questions regarding the prevalent concept for the location of the glycoprotein hormone binding sites (15, 16). Moreover, we provide the first evidence that a TSHR inverse agonist interacts with the enigmatic SSD (or hinge region) linking the TSHR LRD with the hepta-helical membrane-spanning segment.

Materials and Methods

**TSHR mutation and expression**

Introduction of the wild-type TSHR cDNA (17) (with the H601 polymorphism converted to Y601) into the vector pDNA5/FRT (Invitrogen Corp., Carlsbad, CA) was described previously, as was the TSHR with the combined Q235Y/S243P mutation (13). The mutations described below in the text (Results) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA). All mutations were confirmed by nucleotide sequencing. Although the vector is designed for stable transfections, because of difficulty in attaining this goal, we transiently transfected Chinese hamster ovary (CHO) cells with FuGENE HD according to the protocol of the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). Cells were cultured in Ham’s F12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamycin (50 μg/ml), and Fungizone (2.5 μg/ml; University of California San Francisco cell culture facility, San Francisco, CA), passaged the day after transfection and tested the following day.

**Flow cytometry**

CHO cells were harvested from six-well plates using 1 mM EDTA, 1 mM EGTA in PBS. After washing twice with PBS containing 10 mM HEPES (pH 7.4), 2% fetal bovine serum, and 0.05% NaN₃, the cells were incubated for 30 min at room temperature in 100 μl of the same buffer containing 1 μg of either normal mouse IgG or the indicated mAb. After rinsing, the cells were incubated for 45 min with 100 μl fluorescein isothiocyanate-conjugated goat-antimouse IgG (1:100) (Caltag, Burlingame, CA), washed, and analyzed using a Beckman FACScan flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Cells stained with propidium iodide (1 μg/ml final concentration) were excluded from analysis.

**Cultured cell cAMP assays**

CHO cells transiently transfected with the wild-type TSHR or TSHR mutants were transferred into 96-well plates approximately 24 h after transfection and 24 h before assay. For bioassay, the culture medium described previously was replaced with F12 medium supplemented with 1 mM isobutyl methylxanthine, 10 mM HEPES, and, where indicated in the text, bovine TSH (bTSH) (Sigma-Aldrich Corp., St. Louis, MO). Mock-transfected CHO cells were included as controls. After 60 min at 37 C, the medium was aspirated, and intracellular cAMP was extracted with 0.2 ml 95% ethanol. The extracts were evaporated to dryness, resuspended in 0.1 ml PBS (pH 7.5), and samples (12 ml) were assayed using the LANCE cAMP kit according to the protocol of the manufacturer (PerkinElmer, Shelton CT). The effective dose of TSH required for half-maximal stimulation of intracellular cAMP levels (EC₅₀) was calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

**TSHR mAb**

The generation and purification of murine mAb CS-17 have been described previously (10, 13). Nonfunctional murine mAb 2C11 and 4C1 (18) were purchased from Serotec (Oxford, UK).

Results

**Contribution of the TSHR hinge region to the CS-17 epitope**

Previously, based on CS-17 recognizing the human but not the pig TSHR, TSHR LRD amino acids 195, and to a lesser extent 235 and 243, were identified as contributing to the CS-17 epitope (13). However, identity in the human and pig TSHR amino acid sequences further downstream of residue 243 (up to residue 289, the C terminus of the immunogen) precluded further analysis of the CS-17 epitope based on a human-pig TSHR difference. On the other hand, the inability of CS-17 to recognize chimeric receptors TSH-LHR-6 (10) (Fig. 1A) provided an alternative approach to identifying potential CS-17 epitopic residues between TSHR residues 261 and 289. Within this segment, homology between the rat LHR and human TSHR is relatively poor, with 13 mismatches in 29 amino acid residues (Fig. 1B). It is not
Flow cytometric detection of TSHR mutants by mAb CS-17. CHO cells transiently transfected with plasmids expressing TSHR with the indicated mutations [on a background of TSHR Q235Y/S243P (13); modified wild-type TSHR (Mod-wt)] were tested in the same experiment with mAb CS-17 and, as a control, mAb 4C1, whose epitope includes residues 381–384 (18), far downstream of TSHR residue 289 (18) (Fig. 1B). Neither did any of the other mutations within the TSHR segment 261–289 (Fig. 1B) reduce TSH responsiveness (data not shown). Therefore, although CS-17 is a also competitor for TSH binding and function (10), some of the amino acids deduced to be within the CS-17 epitope are not significantly involved in TSH function.

**Potential role of the TSHR N-linked glycan in the CS-17 epitope**

TSHR-289, the immunogen used to generate mAb CS-17, is heavily glycosylated, with glycan comprising nearly half of its mass (22). Of the six N-linked glycans in the TSHR ectodomain, that at N198 is adjacent to Y195 on the convex surface of the LRD (Fig. 3). As mentioned previously, mutation of Y195 substantially reduces CS-17 binding, as detected by flow cytometry without affecting TSH binding (13). Therefore, the question occurred whether a glycan moiety could contribute to the CS-17 epitope. To address this question, we mutated wild-type TSHR residue N198, as well as N177 slightly further upstream, to eliminate the N-linked glycans at these sites. On flow cytometry with transfected CHO cells, TSHR mutants N177Q and N198Q were expressed on the cell surface when detected with mAb 2C11 (Fig. 4). However, CS-17 recognized only N177Q and not N198Q, suggesting that the latter residue contributed to the CS-17 epitope.

The N198Q mutation could have its effect by altering the TSHR polypeptide backbone or by abolition of the N-linked glycan. To distinguish between these two possibilities, we mutated TSHR N198 to a number of other residues; N198A, N198D, and N198T. All of these mutations behaved identically to N198Q, namely loss of CS-17, but not 2C11 recognition on flow cytometry (Fig. 4). Mutation of T200, the third residue in the N-linked glycan motif to a residue other than serine would also abolish N-linked glycosylation at N198 while leaving the N198 residue intact. Indeed, a T200A mutation in the TSHR abolished CS-17 but not 2C11 recognition (Fig. 4). The EC50 for
TSH stimulation of intracellular cAMP levels was unaffected by the N198 and T200 mutations relative to the wild-type TSHR (Fig. 5), as were the EC<sub>50</sub> values for the other mutations shown in Fig. 4 (data not shown).

Despite this evidence that a glycan moiety at N198 contributed to the CS-17 epitope, the possibility remained that the N198 and T200 residues themselves, and not glycan, were part of the CS-17 epitope. Therefore, we incubated CHO cells stably expressing the wild-type TSHR for 1, 2, or 3 d with a high concentration of tunicamycin (5 μg/ml), conditions known to greatly reduce or abolish N-linked glycosylation in cells expressing B glycoprotein hormone receptors (23, 24). A higher concentration or longer duration was not attempted because at the end of 3 d, cells were clearly sick. As expected there was a time-dependent decrease in flow cytometric detection of TSHR on the cell surface using mAb 2C11 as a control (epitope without a glycan component). Contrary to providing evidence for a glycan epitopic component, the CS-17 flow-cytometric signal did not decrease more rapidly over 3 d than the signal of 2C11.

Discussion

TSHR mAb CS-17 is a remarkable reagent in that it represents one of the few antibodies known to possess inverse agonist activity for members of the superfamily of G protein-coupled receptors. Such antibodies have also been described for the b2-adrenergic (25) and M2-muscarinic acetylcholine (26) receptors. Because of the very small extracellular regions of the latter receptors, these inverse agonist antibodies interact with the extracellular loops. In contrast, both mouse mAb CS-17 (10) and human mAb CS-17 (11) bind to the very large ectodomains of the TSHR (397 amino acid residues after signal peptide removal). Because of the importance of TSHR constitutive activity in disease, the characterization of the CS-17 and 5C9 epitopes is of mechanistic interest.
The present data carry the novel message that an inverse agonist for the TSHR, CS-17, interacts with a portion of the receptor’s SSD that links the LRD to the membrane-spanning region. We prefer the term SSD rather than hinge, the former coined by Moyle et al. (14) because it conveys a ligand binding and signaling (not inert) role for this vital component of the glycoprotein hormone receptors. The SSD has largely been ignored in recent years, perhaps because its three-dimensional structure is unknown, unlike the LRDs for the FSH receptor (FSHR) (15) and TSHR (16), whose crystal structures have been determined, and their serpentine transmembrane domains can be modeled on the crystal structures of other G protein-coupled receptors (reviewed in Ref. 28). Indeed, based on the crystal structure of FSH in complex with the FSHR LRD (15), and TSH binding modeled on the crystal structure of the TSHR LRD (29), the glycoprotein hormone receptor SSDs have been largely overlooked despite much experimental data implicating this region in ligand binding and function (reviewed in Refs. 30 and 31). Fortunately, the immunogen for CS-17 included the proximal portion of the TSHR SSD, and we were consequently able to identify TSHR residues T273 and R274 as contributing to the CS-17 epitope. Although the present lack of a structural model for the TSHR SSD precludes the topographical localization of these residues, our data are consistent with increasing evidence that the SSD plays an important role in modulating TSHR constitutive activity (e.g. Refs. 30 and 32). We suggest that CS-17 binding (at least in part) to the SSD stabilizes the TSHR, thereby suppressing constitutive activity.

In addition to implicating the TSHR SSD as contributing to the CS-17 epitope, the present data provide additional insight into its epitopic component in the TSHR LRD. Previous data provided evidence for the involvement of TSHR LRD residues Y195, and to a much lesser extent residues Q235 and S243 (13). These data for CS-17 raised questions regarding the present concept of the glycoprotein hormone receptor binding sites. Besides being an inverse agonist, CS-17 (as well as its Fab fragment) is also an antagonist for TSH binding (10), and TSHR Y195 is present on the convex surface of the TSHR LRD (Fig. 3). This observation requires reconciliation with the present concept of the TSH binding site (the concave surface of the LRD) deduced from the crystal structure of FSH in complex with the FSHR LRD (15), from molecular modeling of TSH in combination with the crystallography-determined TSHR LRD (29), as well as from mutagenesis studies (33). Adding to the uncertainty is a recent report that another TSHR inverse agonist mAb, 5C9 (unlike CS-17), binds to the concave surface of the TSHR LRD (11).

Fortuitously, our focus on a potential glycan component to the CS-17 epitope strengthens the evidence that CS-17 does, indeed, bind to the convex surface of the TSHR LRD. Thus, mutation of either TSHR residues N198 or T200, both on the convex surface of the LRD and adjacent to Y195 (Fig. 3), abrogates CS-17 binding. Nevertheless, TSHR N198 (and even more so T200) is not diametrically opposed to the convex surface but is clustered toward the edge of the convex facet. Therefore, it is possible that as yet unidentified residues in the CS-17 epitope do overlap with the TSH binding site. Alternatively, there is a greater likelihood of steric hindrance between the CS-17 Fab and TSH. However, it should
be appreciated that the epitope of a Fab, including M22 (a stimulatory TSHR antibody whose crystal structure in complex with the TSHR LRD has been determined), contains approximately 16–24 amino acid residues (12, 16). In contrast, the FSH (15) and (modeled) TSH (29) “footprints” on their respective receptors are much larger (45–50 residues). Furthermore, whereas a Fab is an elongated cylindrical molecule that binds via its smaller end facet (e.g., Fig. 3A in Ref. 16), the elongated glycoprotein hormones bind via their flat surfaces. Therefore, despite a Fab having nearly twice the mass of TSH, the binding site of the former is more “focused” and may not readily result in steric hindrance with TSH. Unlike for CS-17, inverse agonist activity for 5C9 has not, to our knowledge, been tested using its Fab component. Therefore, such activity may occur indirectly, e.g., via its Fc region. Assuming that further studies confirm 5C9 binding to the concave surface of the LRD, it could then be inferred that suppression of constitutive activity need not require antibodies with similar epitopes. In this case it will also be of interest to determine whether the 5C9 epitope, like CS-17, has an epitopic component in the TSHR SSD.

TSHR residues N198 and T200 comprise an N-linked glycan motif (NXS/T), and TSHR N198 is known to be glycosylated (34). Whether the glycan at N198 does, indeed, contribute to the CS-17 epitope remains uncertain. Besides a glycan epitope being a rare phenomenon, eliminating N-linked glycosylation by mutagenesis also alters the amino acid structure. It is not possible to enzymatically remove N-linked glycan from mature TSHR on the cell surface without destroying the cells and denaturing the protein. Enzymatically inhibiting glycan incorporation during TSHR synthesis with tunicamycin greatly reduces or abolishes the trafficking of functional TSHR to the cell surface (24). With this background, our goal in using tunicamycin was to determine whether the time course of TSHR disappearance from the cell surface would be more rapid when assessed using CS-17 vs. a mAb (2C11), whose epitope does not contain glycan. Such an observation would support an N-linked glycan contributing to the CS-17 epitope. Indeed, the opposite occurred. The decay in flow cytometric detection of cell surface TSHR was more rapid with 2C11 than CS-17. This observation is consistent with the visible toxicity of tunicamycin on the cells. TSHR cleavage and loss of the C-peptide region (the 2C11 epitope) are accelerated with “unhealthy” cells in culture (35).

The TSHR, like other glycoprotein hormone receptors, can exist as multimers (probably dimers) in the plasma membrane (36, 37). There are conflicting data as to whether TSH binding influences receptor multimerization (37, 38). However, TSH
binding to TSHR homodimers can generate allosteric changes, leading to reduced affinity for ligand (negative cooperativity) (37). Previously, even in the absence of ligand, a progressive increase in the number of TSHR expressed on the cell surface was observed to be associated with a reciprocal decrease in the affinity for ligand. Because this phenomenon suggested allosteric effects consequent to TSHR-TSHR interactions, it was also termed negative cooperativity (39). With this background it is interesting to consider whether CS-17 antagonism of TSH binding and inverse agonist properties are related to TSHR multimerization. It should be noted that monomeric CS-17 Fab and divalent IgG molecules have equimolar potency for both properties (13). However, we cannot exclude the possibility that CS-17 competition for TSH binding or suppression of constitutive activity are, by an unknown mechanism, related to TSHR dimerization.

Parenthetically, an explanation is required as to why we have depicted relevant TSHR amino acids on the three-dimensional structure of the FSHR rather than the TSHR LRD (Fig. 3). Although the latter structure has been reported (16), the coordinates have only been provided in a patent application (40) rather than in the National Center for Biotechnology Information (NCBI) Structure database. From this information, we (and others) are unable to generate a three-dimensional model of the TSHR LRD. Nevertheless, the highly similar structures of the TSHR and FSHR LRD regions (15, 16) make TSHR amino acid localization using the FSHR structure quite accurate.

In conclusion, the present study provides novel information on the epitope of mAb CS-17, a TSHR inverse agonist and TSH antagonist. Interaction between an inverse agonist antibody with the TSHR SSD (or hinge region) has not been previously reported. Furthermore, our data provide additional strong evidence that the CS-17 epitope includes a discontinuous segment.

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Address all correspondence and requests for reprints to: Basil Rapoport, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Suite B-131, Los Angeles, California 90048. E-mail: rapoportb@cshs.org.

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