Dissecting Linear and Conformational Epitopes on the Native Thyrotropin Receptor

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The TSH receptor (TSHR) is the primary antigen in Graves’ disease. In this condition, autoantibodies to the TSHR that have intrinsic thyroid-stimulating activity develop. We studied the epitopes on the native TSHR using polyclonal antisera and monoclonal antibodies (mAbs) derived from an Armenian hamster model of Graves’ disease. Of 14 hamster mAbs analyzed, five were shown to bind to conformational epitopes including one mAb with potent thyroid-stimulating activity. Overlapping conformational epitopes were determined by cell-binding competition assays using fluorescently labeled mAbs. We identified two distinct conformational epitopes: epitope A for both stimulating and blocking mAbs and epitope B for only blocking mAbs. Examination of an additional three mouse-derived stimulating TSHR-mAbs also showed exclusive binding to epitope A. The remaining nine hamster-derived mAbs were neutral or low-affinity blocking antibodies that recognized linear epitopes within the TSHR cleaved region (residues 316–366) (epitope C). Serum from the immunized hamsters also recognized conformational epitopes A and B but, in addition, also contained high levels of TSHR-Abs interacting within the linear epitope C region. In summary, these studies indicated that the natively conformed TSHR had a restricted set of epitopes recognized by TSHR-mAbs and that the binding site for stimulating TSHR-Abs was highly conserved. However, high-affinity TSHR-blocking antibodies recognized two conformational epitopes, one of which was indistinguishable from the thyroid-stimulating epitope. Hence, TSHR-stimulating and blocking antibodies cannot be distinguished purely on the basis of their conformational epitope recognition.

THE TSH RECEPTOR (TSHR), expressed on the basolateral membranes of thyroid epithelial cells, is the primary regulator of thyroid cell growth and thyroid hormone synthesis (1). The TSHR undergoes complex posttranslational processing including intramolecular cleavage and forms a two-subunit structure (extracellular α and transmembrane and cytoplasmic β) by losing the intervening region (residues 316–366) (Fig. 1) (2). In addition, the TSHR is the major autoantigen in Graves’ disease (GD) in which autoantibodies (Abs) to the TSHR act as TSH agonists and overstimulate the thyroid cells. TSHR-Abs tend to be oligoclonal (3, 4), and their serum levels are relatively low (<10 μg/ml) (5–8). In addition, the fact that each patient may have a mixture of TSHR agonistic and antagonistic abs (9, 10) has precluded the determination of conformational epitopes on the TSHR recognized specifically by stimulating TSHR-Abs. Based on the recent generation of animal models of GD (11–13), it is now known that the native conformation of the TSHR is of paramount importance in the induction of stimulating TSHR-Abs but not blocking TSHR-Abs. These animals models have also allowed the generation of pathophysiologically relevant monoclonal antibodies (mAbs) to the TSHR with stimulating activity (14–16) and the ability to induce thyroid stimulation in vivo (17).

The conformational binding site for TSHR-stimulating mAbs on the TSHR is located on the α-subunit (the first ~316 residues) (5, 7, 18, 19). Furthermore, the binding site for TSHR-stimulating mAbs derived from animal models of GD is identical, or closely related, to that for TSHR-Abs present in the sera from patients with GD when studied by binding competition assay (14, 20). However, the same TSHR binding site was also recognized by blocking TSHR-Abs in the sera of patients with atrophic thyroiditis (20, 21). Hence, TSHR-Abs of the stimulating and blocking varieties must share similar epitopes. Such information contrasted with earlier data derived from the binding of TSHR-Abs to TSHR mutant preparations, which suggested that stimulating and blocking antibodies could be distinguished by their binding epitopes (18, 22).

The small antigenic segments of the TSHR (linear epitopes) recognized by GD patients and animals immunized with TSHR antigen have been repeatedly confined to the N terminus domain, particularly the first approximately 20 residues of the TSHR (23, 24) and the TSHR-cleaved region (25, 26) between the α- and β-subunits (residues 316–366). However, antibodies to both these regions were not conformationally dependent and these epitopes were recognized using antibodies derived from TSHR antigen-immunized animals that failed to become hyperthyroid. Hence, the pathological significance of N terminus and cleavage region recognition by such antibodies, which act exclusively as TSHR blocking or TSHR neutral, is unclear. Furthermore, because most of the natural TSHR in normal thyroid cells and

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Abbreviations: Ab, Autoantibody; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GD, Graves’ disease; GFP, green fluorescent protein; mAb, monoclonal antibody; *mAb, labeled mAb; MFI, mean fluorescent intensity; TSHR, TSH receptor.

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tissues has already undergone cleavage (27, 28), the pathologic relevance of antibodies to this region is also unclear because their epitope would be markedly reduced (23).

We previously reported five TSHR-mAbs generated from two Graves’ Armenian hamsters (15). These were mAbs binding to the conformational epitope of the α-subunit of the TSHR, excluding the cleaved region (residues 316–366) (mAbs MS-1 and TAb-8), and mAbs binding to the TSHR cleaved region (mAbs TAB-4, -6, and -16) (15). In the present study, we characterized the conformational and linear epitopes of the native TSHR recognized by the antisera and TSHR-mAbs from a hamster model of GD (29). These mAbs have allowed us to define two distinct conformational epitopes, one of which was recognized by both stimulating and blocking mAbs (epitope A), and the second that was recognized exclusively by blocking abs (epitope B).

### Materials and Methods

#### Cells

Chinese hamster ovary (CHO)-TSHR, CHO cells stably expressing wild-type human TSHR (IP-09) (30), kindly provided by Dr. G. Vassart (Universite Libre de Bruxelles and Service de Genetique Medicale, Brussels, Belgium), CHO-NC35 cells (15) stably expressing human TSHR lacking residues 316–366 plus an amino acid substitution at 367–369 (31) (construct kindly provided by Dr. B. Rapoport, University of California, Los Angeles, CA) tagged with green fluorescent protein (GFP) at the C terminus of the TSHR, and control CHO cells were maintained as previously described (15).

#### Immunization of hamsters

We previously reported immunization of 10 Armenian hamsters with adenovirus vector incorporating human TSHR (29) [this adenovirus vector (13) was kindly provided by Dr. Y. Nagayama, Nagasaki University School of Medicine, Nagasaki, Japan]. Here we similarly immunized 10 additional female Armenian hamsters (6–8 wk old), purchased from Cytogen Research and Development, Inc. (West Roxbury, ME). One hamster from the present study was not available for the analysis. Four hamsters from the previous study showed signs of GD, such as hyperthyroidism, stimulating TSHR-Abs in serum, and thyroid hyper trophy (Graves’ hamster) (29). The hamsters from the present study did not develop GD (data not shown). Hamsters were studied in accordance with protocols approved by the Internal Review Board of Mount Sinai.

#### mAbs

TSHR-mAbs used in this study are summarized in Table 1. MS-1, TAB-4, -6, -8, and -16, which we previously reported (15), were generated from two Graves’ hamsters. RSR-12, -14, and -15 were mouse mAbs with TSHR-stimulating activity generated by DNA immunization protocol in NMR mice (14), kindly provided by Dr. B. Rees Smith (RSR Ltd., Cardiff, UK). An additional nine hamster mAbs were generated in the present study (Table 1). One hamster was chosen for hybridoma fusion based on strong binding of its serum to native TSHR antigen in CHO-TSHR cells.

### TABLE 1. A list of mouse and hamster mAbs used in this study

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<thead>
<tr>
<th>Clone</th>
<th>IgH</th>
<th>IgL</th>
<th>NC35</th>
<th>Fold stimulation</th>
<th>% Blocking</th>
<th>Epitope</th>
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The MS-1 mAb and the series of mAbs named TAB were reported previously by this laboratory (15), and the series of mAbs named RSR were mouse TSHR-stimulating mAbs previously reported by Sanders et al. (14). Other mAbs (9F4, 10C12, 10A11, 1C8, 7G10, 8C9-B, 9D9, 8A4, and 8B12) listed were produced in the present study. The definition of epitopes A, B, and C is described in Results. NC35 indicates that the antibody binding target were cells called CHO-NC35, which expressed a TSHR variant lacking TSHR residues 316–366 and included amino acid substitutions at 367–369 as reported by Tanaka et al. (31).
determined by isotonic fluorescence-activated cell sorter (FACS), as described below. Hamster-mouse heterohybridomas were generated as described previously (15), and 960 wells were screened by isotonic FACS as described below.

**Labeling of mAbs**

Hamster mAbs, purified using Protein G column (Amersham Bioscience, Piscataway, NJ), were labeled with green fluorescent dye, Alexa 488 (Molecular Probes, Santa Monica, CA). Labeled mAb (*mAb) was first tested by binding to CHO-TSHR and control CHO cells in the presence or absence of the corresponding mAb without labeling (mAb) to examine specific binding of *mAb and binding inhibition of *mAb by mAb to ensure that *mAb retained its original binding.

**Detection of TSHR-Ab by isotonic FACS**

Hamster serum (1:100) or hybridoma supernatant (1:10) were diluted with isotonic FACS buffer [PBS containing 0.1% BSA and 0.01% sodium azide (BSA and sodium azide were purchased from Sigma-Aldrich, St. Louis, MO)] and incubated with CHO-TSHR. CHO cells were used as negative control. Anti-Armenian hamster IgG conjugated with fluorescein isothiocyanate (FITC) (BD Bioscience Pharmingen, San Diego, CA) was used as the secondary Ab. The mean fluorescent intensity (MFI) was measured by FACScan flow cytometer (BD Bioscience Pharmingen). Specific TSHR-Ab binding was determined by positive binding to CHO-TSHR cells and negative to control CHO cells.

**Determination of mAb isotypes**

IgH isotypes of hamster mAbs were studied as previously described (15). IgL isotypes were determined by biotin conjugated anti-hamster IgLk antibody (BD Bioscience Pharmingen) in flow cytometry as described above. mAbs bound to CHO-TSHR cells were stained with this secondary antibody followed by streptavidin conjugated with phycoerythrin (Sigma-Aldrich). Positive binding was an indicator for k-light chain and negative for λ-light chain.

**Signal transduction**

cAMP assays were performed as previously described (15). In brief, CHO-TSHR cells were seeded at 4 × 10^4 cells/well on 96-well plates a day before the assay. Cells were stimulated with hybridoma supernatant (1:10 dilution), or purified mAb diluted with cell culture medium, containing 2 mM isobutylmethylxanthine (Sigma-Aldrich). The intracellular cAMP was measured by enzyme immunoassay according to the manufacturer’s protocol (McPherson [Amersham Bioscience]). Stimulation was expressed as the fold increase in cAMP based on basal cAMP production by media containing both mAb and 100 μM U/ml of βTSH (Sigma-Aldrich) divided by cAMP produced by medium containing 100 μM/ml of βTSH alone. When stimulating potency of four stimulating TSHR-mAbs were compared, stimulation was expressed as the percentage cAMP divided by cAMP at saturating concentration of each mAb.

**Linear epitopes study**

Human TSHR peptide ELISAs were performed as previously described (32). In brief, TSHR peptides (33) (kindly provided by Dr. J. Morris, Mayo Medical School, Rochester, MN) were used to coat the wells and incubated with hamster serum (1:500 dilution) or hybridoma supernatant (1:10), and bound IgG was probed with antihamster IgG conjugated with horseradish peroxidase (BD Bioscience Pharmingen). Each TSHR peptide corresponds to the following amino acid residues of human TSHR: no. 1 (22–41), no. 35 (52–71), no. 4 (67–86), no. 5 (82–102), no. 6 (97–116), no. 7 (127–146), no. 8 (142–161), no. 10 (157–176), no. 11 (172–191), no. 12 (187–206), no. 13 (202–221), no. 14 (217–236), no. 15 (232–251), no. 16 (247–266), no. 17 (262–281), no. 18 (277–296), no. 19 (292–313), no. 20 (307–326), no. 21 (322–341), no. 22 (337–356), no. 23 (352–371), no. 24 (367–386), no. 25 (382–401), and no. 26 (397–415). Western blotting was performed as previously described (15). CHO cells stably expressing the TSHR tagged with GFP at the C terminus of the TSHR (34) were used instead of CHO-TSHR cells as a source of TSHR antigen.

**Conformational epitopes study**

Cell binding competition assay. To study whether or not two mAbs (e.g. mAb-1 and mAb-2) share the conformational epitope, fluorescein-labeled mAb-1 (*mAb-1) and unlabeled mAb-2 (mAb-2), both at the saturating concentration, were simultaneously incubated with CHO-TSHR cells. MFI determined by isotonic FACS was a measure of *mAb-1 binding. Positive competition was determined by more than 50% reduction of *mAb-1 binding in the presence of mAb-2 based on *mAb-1 binding without mAb-2.

In the dose-dependent competition study, CHO-TSHR cells were simultaneously incubated with fixed saturating concentration of *mAb-1 and various concentrations of mAb-2. The competition was expressed as the percentage reduction of *mAb-1 binding in the presence of mAb-2 divided by *mAb-1 binding in the absence of mAb-2.

**Indirect mAb binding study.** To study the binding of mAb to the TSHR, mAb in varying concentrations was incubated with CHO-TSHR cells, and bound mAb was then probed with corresponding secondary antibody conjugated with FITC (antimouse IgG conjugated with FITC from Sigma-Aldrich). MFI measured by isotonic FACS was a measure of mAb binding. The mAb binding was expressed as the percentage MFI at the indicated concentration divided by MFI at the saturating concentration.

**Hypotonic FACS.** Hypotonic FACS was performed in a similar way to the isotonic FACS except we used hypotonic buffer (5.4 mM KCl, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 1.3 mM CaCl2, 0.6 mM MgSO4, supplemented with 220 mM sucrose, 5.6 mM glucose, and 10 mM HEPES), which also contained 0.1% BSA and 0.01% sodium azide. In brief, detached cells were washed twice with this buffer before the experiment. This buffer was used throughout the experiment including the incubation of cells with *mAb or washing of cells. Cells were incubated with the indicated concentration of *mAb for an hour on ice and washed twice with the buffer. Propidium iodide (5 μg/ml, Sigma-Aldrich) was added right before the assay, and dead cells were excluded from the analysis. *mAb binding was expressed as the percentage MFI divided by MFI at the saturating dose.

In the binding inhibition assays, cells were incubated with the indicated concentration of unlabeled mAb for 90 min on ice, and then fixed concentration of the corresponding *mAb was added and incubated for an additional 30 min. Cells were washed twice and *mAb binding was measured by MFI. The binding inhibition was expressed as the percentage reduction of *mAb binding in the presence of mAb divided by *mAb binding in the absence of mAb.

When hamster anti-TSHR sera were used for *mAb binding inhibition, cells were similarly incubated with serum (1:50 unless otherwise indicated), and then fixed concentration of *mAb was added. Preimmune sera at the same dilution were used as negative control. Binding inhibition was expressed as the percentage reduction of *mAb binding in the presence of anti-serum divided by *mAb binding in the presence of preimmune serum. More than 10% inhibition was considered positive. mAb-like activity in each serum was estimated from the binding inhibition curve of *mAb by the corresponding mAb without labeling as described above.

**Data analyses**

Data obtained in dose-dependent TSHR stimulation, cell-binding competition, and indirect mAb binding studies were analyzed and curve fitted using Prism 4 software (GraphPad Software, Inc., San Diego, CA) after pooling two to three separate experiments. The Student’s t test was used for statistical analysis. P < 0.05 was considered significant.

**Results**

**Identification of three distinct mAb epitopes on the native TSHR**

To examine whether MS-1 (a stimulating mAb) and TAB-8 (a blocking mAb) shared the same epitope, MS-1 was labeled...
with green fluorescent dye. This labeled MS-1 (*MS-1) was able to bind to the TSHR (Fig. 2A) and was inhibited by unlabeled MS-1 (Fig. 2B), indicating that the labeled molecule retained its properties. *MS-1 binding was not affected by the presence or absence of TAb-8 (Fig. 2C). This was confirmed by the reverse experiment using *TAb-8 and unlabeled MS-1 (data not shown). Thus, the MS-1 and TAb-8 binding sites were independent. Three additional mAbs recognizing the TSHR cleaved region did not show binding competition with *MS-1 or *TAb-8 (data not shown). Therefore, on the native TSHR, this repertoire of mAbs demonstrated two distinct conformational epitopes encompassing the α-subunit together with linear epitopes localized within the TSHR cleaved region.

**Confirmation of three epitopes on the native TSHR using additional mAbs**

To confirm the recognition of these TSHR epitopes, we generated an additional nine mAbs reacting to the native TSHR antigen (Table 1). These TSHR-Abs were all IgG2 and eight of nine had a γH9261-light chain. Of these nine additional mAbs, three were TSHR blocking antibodies and six were neutral. We then studied the epitopes of these mAbs in relation to the three epitopes previously identified. We first studied linear epitopes by their binding to TSHR peptides, and, if positive, Western blotting was also performed. mAbs negative for peptide binding were tested in cell binding competition assays with *MS-1 and *TAb-8.

As shown in Fig. 3, six of nine mAbs recognized TSHR peptides corresponding to the TSHR cleaved region (residues 316–366) and were positive on Western blotting (Fig. 4). No other linear epitopes on the TSHR were revealed. Supporting these findings, all six mAbs failed to bind to CHO-NC35 cells, which lacked the cleaved region (Table 1). Three mAbs did not react with TSHR peptides but showed good binding to CHO-NC35 cells, indicating their conformational epitopes were outside the TSHR cleaved region (Table 1). These mAbs were tested with cell binding competition assays against *MS-1 or *TAb-8. Two mAbs (9F4 and 10C12, both blocking mAbs) showed approximately 80% binding competition with *MS-1, and one mAb (10A11; blocking mAb) competed with *TAb-8 only (~70% competition).

Thus, all 14 mAbs, five from the previous study (15) and
nine from the present study, bound to: (1) the MS-1 binding site (n = 3) designated epitope A; (2) the TAb-8 binding site (n = 2) designated epitope B; or (3) the TSHR cleaved region (n = 9) designated epitope C. As described above, epitopes A and B were conformational epitopes shared with TSHR binding, and epitope C was linear rather than conformational.

Epitope A is also recognized by mouse stimulating TSHR mAbs

We hypothesized that mouse thyroid stimulating mAbs would also recognize epitope A like the MS-1 stimulating mAb. We tested the binding of three mouse stimulating mAbs (RSR-12, -14, and -15; see Table 1), generously supplied by Sanders et al. (14), to CHO-TSHR and CHO-NC35 cells. Each of these mAbs bound to both these cell lines (Table 1). To test whether they bound to epitopes A or B in the TSHR ectodomain, we studied binding competition with *MS-1 and *TAb-8. All three mouse mAbs showed good competition with *MS-1 but not with *TAb-8 (Fig. 5); therefore, we studied the dose-dependent competition with *MS-1 (Fig. 6A).

The binding competition of two hamster mAbs, MS-1 and 9F4, was approximately 10- to 20-fold greater than the mAbs from mice (EC50 was ~1.0 and ~0.7 mg/ml for MS-1 and 9F4 vs. ~11.8, ~18.6, and ~10.8 mg/ml for RSR-12, -14, and -15). This difference in competition could be explained by a difference in overlapping binding sites recognized by these mAbs or a direct difference in binding affinity. To clarify this, mAb binding to the native TSHR was studied using indirect binding assays (Fig. 6B). MS-1 and 9F4 had similar binding for the TSHR (EC50 ~220 and ~175 ng/ml for MS-1 and 9F4). However, binding of the mouse mAbs was 10–40 times lower (EC50 ~2, ~8.5, and ~1.9 mg/ml for RSR-12, -14, and -15). Thus, the increased amount of mouse antibody needed in the competition assays was explained by their lower binding affinity. When we compared the TSHR-stimulating potency of mouse mAbs with MS-1 (Fig. 7), the maximum stimulation was similar for RSR-12, RSR-14, and MS-1 (~10 times basal), but MS-1 was significantly more potent at low concentrations (EC50 was ~37 ng/ml for MS-1 and ~260 and ~780 ng/ml for RSR-12 and -14) in keeping with the competition and binding studies. RSR-15 did not stimulate as well as the other mAbs (3–4 times basal) (data not shown).

Epitope determination of anti-TSHR sera

To examine whether the repertoire of mAbs that we had studied represented the repertoire of TSHR-Abs found in the

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**Fig. 5.** Cell binding competition study of mouse-stimulating mAbs. CHO-TSHR cells were stained with a fixed concentration of fluorescence-labeled *mAb as indicated on the x-axis in the presence or absence of unlabeled mAb (10 μg/ml) shown in an each panel. The vertical broken lines indicate the MFI of the corresponding *mAb alone. In *MS-1 competition experiments (left), MS-1 showed almost complete blockade of *MS-1 binding, and the RSR-stimulating antibodies showed significant competition. However, on the *TAb-8 competition experiments (right), only TAb-8 showed any competition for *TAb-8 binding.

**Fig. 6.** Epitope A recognition of mouse and hamster TSHR-mAbs. A, Dose-dependent cell binding competition study of mAbs binding to epitope A. *MS-1 was used to study dose-dependent binding competition by five mAbs including four stimulating mAbs (MS-1 and RSR-12, -14, and -15) and one blocking mAb (9F4). A fixed concentration of *MS-1 and each mAb at the indicated concentration was incubated with CHO-TSHR cells and *MS-1 binding was studied. Results are expressed as percent competition of *MS-1 binding. B, Dose-dependent indirect mAb binding study. mAb binding to epitope A was detected with secondary antibody specific to mouse or hamster IgG. Results are expressing as percent binding of mAb. See Materials and Methods for data processing.
serum of our hamster model of GD, we examined the linear epitopes recognized by the polyclonal antisera from such immunized animals. Binding to TSHR peptides was studied using three serum specimens from animals that originated the hamster mAbs. All three antisera showed similar binding patterns to TSHR peptides, indicating potent recognition confined to the cleaved region (Fig. 8). This result was confirmed by three additional hamster sera with potent TSHR-Ab activity as determined by isotonic FACS (data not shown). Similarly, we studied immune sera that showed only background binding to CHO-TSHR cells in isotonic FACS assay. This serum did not bind to TSHR peptides (data not shown), excluding the possibility that the linear recognition of the TSHR cleaved region was merely an intrinsic activity by hamster serum. Thus, we concluded that the linear epitopes on the native TSHR in the hamster model were predominantly in the cleaved region of the TSHR as reflected in our repertoire of mAbs.

Second, we looked to see whether the hamster sera recognized the two conformational epitopes A and B. Cell binding competition between *mAb and hamster serum was unsuccessful, perhaps due to the low concentration of competing antibodies in the serum (data not shown). However, when we used hypotonic conditions known to improve the detection of TSHR-Abs (35, 36), the *mAbs bound with 10- to 20-fold higher binding affinity (Fig. 9A) and, therefore, improved the potential sensitivity of the detection assays (Fig. 9B). When we preincubated CHO-TSHR cells with hamster serum under these conditions, we observed a significant dose-dependent binding inhibition of *MS-1 by hamster sera from the animals that generated MS-1 and 9F4 (Fig. 9C). We also observed that epitopes A and B were commonly recognized in other hamster sera (Fig. 10) (11 of 19 serum specimens for epitope A, 13 of 19 for epitope B; statistically not significant between epitopes A and B). From the binding inhibition curves, we were also able to extrapolate that the serum levels of epitope A binding activity in Graves’ hamsters (MS-1-like stimulating antibodies + 9F4-like blocking antibodies) were less than 100 ng/ml. This was compatible with the serum levels of stimulating TSHR-Abs for optimal stimulation, estimated from earlier *in vivo and *in vitro studies (17). Epitope A binding activity in non-Graves’ hamsters (9F4-like blocking antibodies) was up to approximately 400 ng/ml, 4 times higher than those in hyperthyroid animals. *Tab-8 like blocking activity in the sera was up to approximately 100 ng/ml.

**Discussion**

We have demonstrated that hamster-derived TSHR-mAbs obtained by native TSHR immunization showed a clear tendency to bind only to three restricted epitopes on the TSHR. Among them, two (epitopes A and B) were conformational on the α-subunit, which is also important for TSH binding. However, the third region (epitope C) was linear and located in that part of the TSHR subject to intramolecular cleavage (summarized in Fig. 11). The major linear epitope in our
hamster model was epitope C, the cleaved region, as evidenced by the recognition profile of the antisera. Recognition of conformational epitopes A and B by antisera could also be detected. Additionally, we determined that all four stimulating TSHR-mAbs available to us, one from a hamster and three from mice, shared the same conformational binding site (epitope A) but with different binding affinities. However, some blocking mAbs also bound to epitope A, so this epitope could not be designated a stimulating epitope.

Our study supported previous reports indicating that stimulating antibody binding sites were confined to the α-subunit (5, 7, 18, 19) and likely to be very similar (14, 20, 21). Our data indicated that all four stimulating mAbs bound to the same epitope on the α-subunit. In keeping with their different binding affinities, TSHR activation at low concentrations differed markedly, with MS-1 being the most potent. Because binding of these antibodies to epitope A determined the subsequent functional status of the TSHR, we concluded that conformational epitope A was a pivotal site for activation and inactivation of the TSHR.

Using experimentally produced mAbs, Jeffreys et al. (37) recently identified a TSH binding pocket with three discontinuous TSHR residues involved in TSH binding. They showed that two of the three residues were located on the α-subunit of the TSHR and one on the β-subunit. However, mAbs used to determine this TSH binding pocket were TSHR-blocking mAbs with linear epitope recognition. Our study revealed at least two important conformational binding sites for TSHR-blocking antibodies on the native TSHR. 9F4 and TAb-8, the representative blocking mAbs that recognized these two conformational epitopes, did not show binding competition with the one mAb used by Jeffreys et al. and available to us (RSR-1, with an epitope at residues 381–385 (38)) (data not shown). This emphasized that TSH binding to the TSHR is both discontinuous and conformational. How the two conformational epitopes A and B are related to the TSH binding pocket defined by Jeffreys et al. remains uncertain.

The TSHR cleaved region has been previously found to be immunogenic (22, 24, 39–41). We demonstrated that the TSHR cleaved region was a major linear epitope recognized in the hamster model of GD. The mAbs binding to this epitope C were mostly of the neutral variety. The TSHR cleaved region is a hydrophobic part of the TSHR (24, 25) and
also unique when compared with similar G protein-coupled receptors for leutropin and folitropin (2). However, some studies have suggested that the cleaved region may also be a putative binding site for stimulating TSHR-Abs as evidenced by peptide binding of serum IgG from patients with GD (40–42). Our data indicated that this region was not involved in the binding of stimulating TSHR-mAbs.

Another issue concerning the antibody recognition of the TSHR cleaved region is the likely paucity of this epitope on the thyroid cell membrane. The major species of TSHR expressed in vivo is a cleaved receptor (27, 28), and therefore, epitope C should be found in only a minority of receptors. However, we generated several mAbs to this region, and the hamster antisera confirmed this region as a major linear TSHR epitope in vivo. Hence, the full-length holoreceptor must be highly immunogenic in this animal model despite, or because of, its lower concentration. In addition, cleavage may often be in progress or incomplete, leaving antigenic ends to stimulate an immune response.

In contrast to our findings here, it has been well shown that the N terminus of the TSHR is an important linear epitope after immunization with denatured TSHR as well as native TSHR (reviewed in Ref. 23). Because of the conserved recognition of this region unrelated to the development of thyroid overactivity it is doubtful that the N terminus epitope is important for stimulating TSHR-Abs. In support of this conclusion were the studies of the TSH binding pocket, which indicated that the N terminus of the TSHR was not important for TSH binding (37). Second, stimulating antibodies recognize the conformation of the TSHR and not a restricted segment of the antigen, such as the N-terminal region (14, 15, 20). Third, we have shown here that antisera derived from hyperthyroid hamsters did not recognize N terminus peptides. Thus, the N terminus region of the TSHR may be immunogenic, but antibodies recognizing this region would be neutral and/or blocking antibodies and not thyroid-stimulating antibodies.

In summary, our study indicated that epitopes on the native TSHR recognized in a hamster model of GD were restricted rather than broadly distributed. Furthermore, the binding sites for stimulating TSHR-Abs were highly conserved, and their stimulating potency was dependent on their binding affinity. However, blocking TSHR antibodies may bind to different conformational sites including the stimulating site. Their separation from stimulating antibodies cannot, therefore, be made on the basis of their epitope recognition.

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