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A Mouse Monoclonal Antibody to a Thyrotropin Receptor Ectodomain Variant Provides Insight into the Exquisite Antigenic Conformational Requirement, Epitopes and *in Vivo* Concentration of Human Autoantibodies*

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ABSTRACT

We used the secreted TSH receptor (TSHR) ectodomain variant TSHR-289 (truncated at amino acid residue 289 with a 6-histidine tail) to investigate properties of TSHR autoantibodies in Graves' disease. Sequential concanavalin A and Ni-chelate chromatography extracted milligram quantities of TSHR-289 (~20–40% purity) from the culture medium. Nanogram quantities of this material neutralized the TSH binding inhibitory activity in all 15 Graves' sera studied. We generated a mouse monoclonal antibody (mAb), 3BD10, to partially purified TSHR-289. Screening of a TSHR complementary DNA fragment expression library localized the 3BD10 epitope to 27 amino acids at the N-terminus of the TSHR, a cysteine-rich segment predicted to be highly conformational. 3BD10 preferentially recognized native, as opposed to reduced and denatured, TSHR-289, but did not interact with the TSH holoreceptor on the cell surface. Moreover, mAb 3BD10 could extract from culture medium TSHR-289 nonreactive with autoantibodies, but not the lesser amount (~25%) of TSHR-289 molecules capable of neutralizing autoantibodies. Although the active form of TSHR-289 in culture medium was stable at ambient temperature, stability was reduced at 37 C, explaining the mixture of active and inactive molecules in medium harvested from cell cultures.

In conclusion, studies involving a TSHR ectodomain variant indicate the exquisite conformational requirements of TSHR autoantibodies. Even under "native" conditions, only a minority of molecules in highly potent TSHR-289 preparations neutralize patients' autoantibodies. Therefore, Graves' disease is likely to be caused by even lower concentrations of autoantibodies than previously thought. Finally, reciprocally exclusive binding to TSHR-289 by human autoantibodies and a mouse mAb with a defined epitope suggests that the extreme N-terminus of the TSHR is important for autoantibody recognition. (*J Clin Endocrinol Metab* **84:** 702–710, 1999)

GRAVES' disease is caused by autoantibodies that usurp the function of TSH by activating the TSH receptor (TSHR) (reviewed in Ref. 1). The availability of recombinant TSHR is, therefore, an important goal from the theoretical, diagnostic, and (potentially) therapeutic points of view. Most effort toward this goal has involved the use of prokaryotic and insect cell expression systems, synthetic peptides, and cell-free translation. However, in our experience, the highly conformational nature of TSHR autoantibody epitopes (reviewed in Ref. 1) makes the TSHR of mammalian cell origin the most effective antigen for recognition by human autoantibodies.

It is now feasible to generate large amounts of mammalian TSHR by using fermenters to propagate TSHR-expressing myeloma cells (2), by human TSHR complementary DNA (cDNA) transgenome amplification in CHO cells (3), and by vaccinia virus infection of HeLa cells (4). However, the hydrophobic, serpentine membrane-spanning region of the TSHR as well as the need to harvest cells rather than culture medium make purification of the holoreceptor difficult. Unfortunately, the TSHR ectodomain, truncated at its entry into plasma, is not secreted, but is largely retained within mammalian cells (5, 6) in a form containing immature, high mannose carbohydrate that is not recognized by patients' autoantibodies (6). Whether autoantibody binding requires mature complex carbohydrate or whether it is incorrect folding of the truncated ectodomain that affects both autoantibody binding and normal intracellular trafficking of the ectodomain is presently unknown. It is now appreciated that correct TSHR ectodomain trafficking can be attained by attaching to its C-terminus a membrane-anchoring tail (7–9). However, another approach that we employed to achieve secretion of a highly potent, conformationally intact TSHR ectodomain was to progressively truncate the ectodomain at sites predicted to be in the vicinity of the carboxyl-terminus of the a-subunit (10).

Of the three truncated ectodomain variants that we generated, the most efficiently secreted is TSHR-261 (amino ac-

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ids 22–261, after signal peptide removal) (10). Although TSHR-289 (residues 22–289) is less well secreted than TSHR-261, we have since observed that its autoantibody-neutralizing activity in cell culture medium is more stable, making TSHR-289 a better candidate for studies of TSHR autoantibodies in Graves' disease. The present report describes our experience with this remarkable molecule. Although stable when partially purified, TSHR-289 disintegrates on purification or even on concentration of the semipurified form. However, a mouse monoclonal antibody (mAb) generated to partially purified TSHR-289 provides information of pathophysiological importance regarding the antigenic conformational requirement, epitopes, and the *in vivo* concentration of human autoantibodies.

Materials and Methods

Expression of truncated ectodomain variant TSHR-289

The construction and expression of plasmid TSHR-289 have been described previously (10). In brief, the cDNA for TSHR residues 1–289 (1–21 representing the signal peptide) was inserted into the vector pSV2-ECE-dhfr (11). A double stranded oligonucleotide cassette coding for six histidine residues followed by two stop codons was introduced after the codon for TSHR residue 289. CHO-DG44 dhfr$^-$ cells (provided by Dr. Robert Schimke, Stanford University, Palo Alto, CA) were stably transfected with TSHR-289 cDNA (6). Transgenome amplification was achieved by progressive adaptation over approximately 1 yr to growth in methotrexate (final concentration, 10 μmol/L) (6).

Assay for neutralization of TSHR autoantibodies in the serum of Graves' patients

TSHR autoantibody kits were purchased from Kronus (San Clemente, CA). Our modification of this TSH binding inhibition (TBI) assay (12) to measure autoantibody neutralization has been reported previously (10). In brief, 25 μL serum from Graves' patients were preincubated (30 min at room temperature with 25 μL conditioned medium containing TSHR-289 or with partially purified TSHR-289 (see below). Solubilized porcine TSHR (50 μL) was then added followed by radiolabeled TSH (total volume, 200 μL; 2 h at room temperature), after which TSHR-TSH complexes were precipitated with polyethylene glycol. As controls, we used serum from normal individuals and conditioned medium from CHO cells secreting a truncated form of thyroid peroxidase (11). Autoantibody activity was expressed as the percent inhibition of [^{125}I]TSH binding relative to that of a standard serum from a normal individual without autoantibodies.

Partial purification of TSHR-289 by concanavalin A and Ni-chelate chromatography

Conditioned medium was harvested three times per week from CHO cells expressing TSHR-289 cultured in Ham's F-12 medium containing 10% FCS, antibiotics, and 2.5 mmol/L sodium butyrate (13). Medium (2 L) was applied to a 70-mL concanavalin A-Sepharose (Pharmacia Biotech, Piscataway, NJ) column. After washing with 10 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl, bound material was eluted with about 80 mL 0.25 mol/L α-methylmannoside in the same buffer. The eluted material was made up to 50 mmol/L imidazole (pH 7.2) and applied to two 5-mL His-Trap columns in series (Pharmacia Biotech). Elution was performed with buffer containing 10 mmol/L Tris (pH 7.4), 50 mmol/L NaCl, and 100 mmol/L ethylenediamine tetraacetate. The sample was concentrated, and the buffer was changed to 10 mmol/L Tris (pH 7.4) and 50 mmol/L NaCl using a Centriprep 30 (Amicon, Beverly, MA). At all stages, TSHR-289 recovery was monitored by the TBI neutralization described above.

Mouse mAb to TSHR-289

Six BALB/c mice were immunized sc with approximately 60 μg partially purified TSHR-289 (see above) in complete Freund's adjuvant, followed by two further injections (at 3.5 and 9 weeks) in incomplete Freund's adjuvant. Spleen cells (3 days after the final boost) were fused with SP2/0 mouse myeloma cells using polyethylene glycol by standard techniques, and selection of resistant clones was performed in hypoxanthine-aminopterin-thymidine medium (Sigma Chemical Co., St. Louis, MO). Wells were screened by enzyme-linked immunosorbent assay (ELISA) for IgG production and for antibodies to TSHR-289. For the latter, ELISA plates were coated with partially purified TSHR-289, and detection was performed with antimouse IgG conjugated to horseradish peroxidase (Sigma Chemical Co.). The specificity of IgG-secreting, positive clones was determined by immunoblotting under native and denaturing conditions (described below). In addition, we performed flow cytometry, as previously described (14), using TSHR expressed on the surface of TSHR-10,000 cells (3) and, as second antibody, affinity-purified goat anti-mouse IgG (0.8 μL; fluorescein isothiocyanate-conjugated; Caltag, South San Francisco, CA).

Determination of TSHR-289 mAb epitopes

We screened a size-selected (200–500 bp) TSHR cDNA fragment library in the bacteriophage vector λ-Zap (Stratagene, La Jolla, CA; Nagayama, Y., and B. Rapoport, unpublished data) using ascites containing three newly isolated mAb to TSHR-289 (see below). Screening was performed as described previously (15), with minor modifications. BB4 cells (optical density of 1.0 in 10 mmol/L magnesium sulfate) were infected with the bacteriophage λ-Zap library. After 4 h at 37 C, about 3×10^4 plaque-forming units/150-mm diameter petri dish were overlayed with nitrocellulose filters soaked in 10 mmol/L isopropyl-thio-β-D-galactopyranoside and incubated overnight at room temperature. Filters were washed in Tris-buffered saline (TBS) buffer (10 mmol/L Tris, pH 7.4, and 150 mmol/L NaCl) containing 0.05% Tween, incubated for 30 min in 3% milk powder in TBS at room temperature, rinsed, and then incubated with the antibodies (ascites diluted 1:500) for 2.5 h at room temperature. After washing with 10 mmol/L Tris, pH 7.4, and 150 mmol/L NaCl containing 0.05% Tween, peroxidase-conjugated affinity-purified sheep antimouse IgG (Sigma Chemical Co.; 1:500) was applied to the filters for 3 h at room temperature. Color was developed with 2.8 mmol/L 4-chloro-1-naphthol, 10 mmol/L imidazole, and 0.0125% H_2O_2. Positive plaques were rescreened three or four times until clonal. The nucleotide sequences of TSHR cDNA inserts in plaque-purified clones were determined by the dideoxy-nucleotide method (16) after rescue of pBS double stranded plasmids in XL1-blue bacteria using the helper phage R408 according to the protocol of the manufacturer (Stratagene).

Affinity purification of TSHR-289

Conditioned medium (2–3 days of culture, stored at −80 C) was thawed and filtered (0.22 μm pore size), and 1–2 L were applied (1.5 mL/min at room temperature) to a column with 5 mL Sepharose-linked mouse mAb 3BD10. After extensive washing with phosphate-buffered saline, pH 7.4, the protein was eluted (1.5 mL/min) with 0.2 mol/L glycine, 0.15 mol/L sodium chloride, and 0.02% sodium azide, pH 2.3. Fractions (2 mL) were immediately neutralized with 0.4 mL 2 mol/L Tris, pH 8.0. Fractions with an optical density greater than 0.1 were pooled; dialyzed against 10 mmol/L Tris (pH 7.4), 50 mmol/L NaCl, and 0.02% sodium azide; and concentrated with a Centriprep 30 (Amicon, Beverly, MA). Aliquots were applied to polyacrylamide gels and stained with Coomassie blue or were assayed for their ability to neutralize TSHR autoantibodies in patients' sera (see above).

Immunodepletion of TSHR-289

Mouse mAb 3BD10 to the N-terminus of the molecule (see below) or Penta-His to the six histidines at the C-terminus of the molecule (Qiagen, Chatsworth, CA; 2.5 μg each) were added (16 h at 4 C) to 0.45 mL of 2-day conditioned medium from CHO cells secreting TSHR-289. Samples were then diluted to 2.0 mL in 10 mmol/L Tris-HCl (pH 7.4) and 50 mmol/L NaCl and applied to a 1-mL HiTrap protein G column (Pharmacia Biotech). After discarding the first 1.5 mL of the flow-through (diluted in column buffer), the final 0.5-mL fraction was kept to determine its ability to neutralize the BI activity of autoantibodies in Graves' serum (see above).

Immunoblotting of TSHR-289

For immunoblotting under native conditions, TSHR-289 in 10 mmol/L Tris (pH 7.4) and 50 mmol/L NaCl was added to native sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and applied to 10% two-dimensional well polyacrylamide gels without SDS (Bio-Rad Laboratories, Inc.). For immunoblotting under denaturing conditions and reducing conditions, TSHR-289 was added to Laemmli sample buffer (17) with 2% SDS and 0.7 mol/L (final concentration) β-mercaptoethanol (30 min at 45 C) and applied to SDS-polyacrylamide gels (Bio-Rad). Enzymatic deglycosylation with *N*-glycosidase F (New England Biolabs, Beverly, MA) was performed as described previously (6). After electrophoresis, proteins were electophoretically transferred to polyvinyldifluoride membranes (Bio-Rad Laboratories, Inc.). After blocking (45 min) in TBS containing 5.0% skim milk powder, membranes were rinsed and incubated (overnight at 4 C) in TBS-containing mouse mAb 3BD10, A9, or A10 (1:1000; the latter two were provided by Dr. Paul Banga, London, UK) (18) and 0.5% BSA. The filters were rinsed, incubated (1–2 h at room temperature) with alkaline phosphatase-conjugated goat antimouse IgG, and the signal was developed as described previously (6).

Immunoprecipitation of TSHR-289

CHO cells expressing TSHR-289 were metabolically labeled with [^{35}S]methionine/cysteine, exactly as described previously (1-h pulse and overnight chase) (3). For comparison of native *vs.* denatured TSHR-289 antigen, an aliquot of medium harvested after the chase was frozen at −80 C. Another aliquot underwent reduction and alkylation using dithiothreitol and iodocetamide, exactly as described previously (19), was dialyzed against 10 mmol/L Tris (pH 7.4) and 50 mmol/L NaCl, and was frozen. Aliquots (1 mL) of each were then thawed and simultaneously subjected to immunoprecipitation as previously described (3, 10). Immunoprecipitates were dissolved in Laemmli buffer with 2% SDS and 0.7 mol/L β-mercaptoethanol and electrophoresed on 10% polyacrylamide-SDS gels. Radiolabeled proteins were visualized by autoradiography on Kodak BioMax MS x-ray film (Eastman Kodak Co., Rochester, NY).

Results

Partial purification of soluble TSHR-289 by lectin and Ni-chelate chomatography

Although TSHR-261 is secreted into the culture medium to a greater extent than the less truncated TSHR ectodomain variants TSHR-289 and TSHR-309 (10), we observed suboptimal stability of TSHR-261 in culture medium in terms of its ability to interact with TSHR autoantibodies. Therefore, with the goal of purifying a soluble, immunologically active TSHR ectodomain variant, we turned to TSHR-289, which retained its bioactivity for at least 16 h at room temperature (data not shown). Initially, we applied the same two-step approach previously used for TSHR-261, namely concanavalin A lectin chromatography followed by Ni-chelate chromatography (10) (TSHR ectodomain variants were constructed with six histidines at their carboxyl-termini). This procedure yielded about 0.4–0.6 mg TSHR-289 glycoprotein of approximately 20–40% purity/L conditioned medium (Fig. 1). Shown in the same figure for comparison is TSHR-261 partially purified by the same method. Of note, the loss of TSHR-261 immunoactivity (see above) was not associated with any visible change in the appearance of the glycoprotein on Coomassie blue staining.

Biological activity of TSHR-289. Partially purified TSHR-289 was very potent in terms of its ability to neutralize TSHR autoantibodies in the sera of patients with Graves' disease. For 15 Graves' sera, 50 ng TSHR-289/assay tube (0.2 mL) neutralized essentially all TBI activity (Fig 2). Thus, TSH binding by the individual sera was inhibited by 52.2 ± 20.9% (±SD; range, 23.1–80.4%) in the absence and by 10.8 ± 4.0% (range, 5.0–16.4%) in the presence of TSHR-289 (by paired *t* test, $P < 0.0001$).

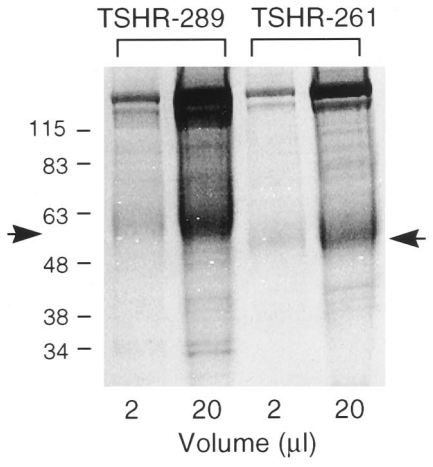

FIG. 1. Partial purification of truncated ectodomain variant TSHR-289. Conditioned medium (~2 L) was sequentially subjected to concanavalin A and Ni-chelate chromatography (10), followed by buffer exchange and concentration in 10 mmol/L Tris (pH 7.4) and 50 mmol/L NaCl to about 4 mL. Aliquots (2 and 20 μL) were subjected to 10% PAGE and staining with Coomassie blue. The *arrows* indicate TSHR-289 and, for comparison, TSHR-261 partially purified by the same method.

Instability of TSHR-289 on further purification or at high concentration

Although TSHR-289 activity was quite stable for up to 24 h at ambient temperature during the lectin and Ni^{2+}-chelate chromatography steps, this material was remarkably recalcitrant to further purification. The major contaminating proteins following the Ni-chelate chromatography step were of very high (>120 kDa) molecular mass (see Fig. 1). N-Terminal sequencing of this material was uninformative and did not indicate that it represented aggregated TSH-289. The wide size difference between TSHR-289 and the high molecular mass contaminant(s) suggested that a final gel filtration chromatographic step would be straightforward. Application of approximately 30% pure TSHR-289 to a Sephacryl S-100 column led to the recovery of high molecular mass material in the void volume, but only trace amounts of TSHR-289. The latter was replaced by very low molecular fragments lacking bioactivity (data not shown). Mono-Q ion exchange fast protein liquid chromatography similarly resulted in the loss of all bioactivity as well as the absence of a TSHR-289 peak (data not shown). Attempts at separation of TSHR-289 from the high molecular mass contaminant(s) by centrifugation through a membrane with a 100-kDa pore led to total loss of TSHR-289 protein in terms of both bioactivity and detection by Coomassie blue. Even further concentration of partially purified TSHR-289 to more than approximately 1 mg/mL resulted in the total loss of bioactivity and detectable protein.

Generation of mAb to TSHR-289

Concomitantly with the attempts at TSHR-289 purification, mouse mAb to this material were produced by immunization with TSHR-289 partially purified by lectin and Ni-chelate chromatography. Clones were initially screened by ELISA using partially purified TSHR-289 (logistical reasons precluded screening by flow cytometry using intact TSHR-expressing CHO cells). Approximately 50 IgG-producing clones that recognized the TSHR-289 preparation were subsequently screened by immunoblotting under native and denaturing conditions as well as by flow cytometry. Three clones (3BD10, 3BE12, and 1CE1) as well as a positive control mAb (A9) (18) interacted with partially purified TSHR-289 on immunoblotting under both native and denatured/reduced conditions as well as after enzymatic deglycosylation with endoglycosidase F (Fig. 3). The remaining clones interacted with the high molecular mass contaminating protein(s) and were not studied further. None of these clones recognized the TSH holoreceptor on flow cytometry, performed as previously described (14). For example, median fluorescence with 3BD10 (3.3 U) was similar to that with a nonspecific monoclonal antibody (2.6 U). The mAb A9 provided a slightly higher value (6.5 U), far lower than with a potent Graves' serum (181.0 U). 3BD10 ascites also did not inhibit [^{125}I]TSH binding to solubilized porcine TSHR (94% and 100% binding relative to normal mouse IgG; each value is the mean of duplicate determinations).

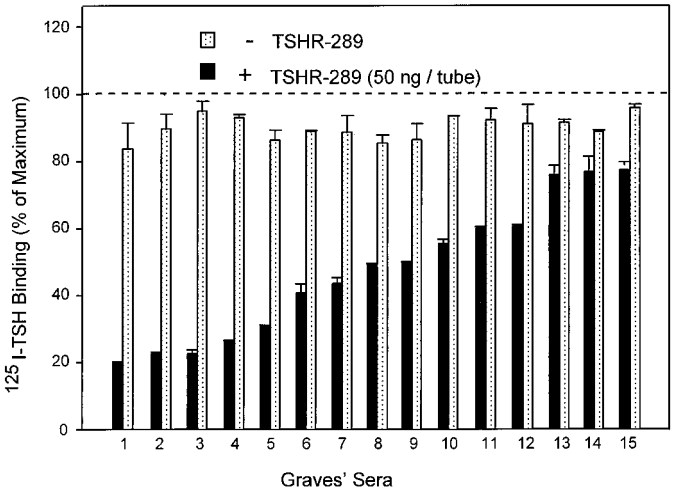

FIG. 2. Neutralization of TSHR autoantibodies by TSHR-289 partially purified from conditioned medium. The TBI activity of autoantibodies in the sera of 15 Graves' patients was measured using a commercial kit (see *Materials and Methods*). Values (*black bars*) are expressed as a percentage of maximum TSH binding observed in the presence of a normal serum (*horizontal dashed line*). The ability of TSHR-289 (50 ng/tube) to neutralize TBI activity is indicated by the *speckled bars*. *Bars* indicate the mean ± range of duplicate determinations.

Immunoprecipitation of metabolically labeled, secreted TSHR-289 revealed to a much greater extent than the immunoblots that mAb 3BD10 (the ascites with the highest titer) preferentially recognized the native molecule. Thus, mAb 3BD10 interacted strongly with native TSHR-289 in culture medium, but not at all with the same material after reduction and alkylation, even at very high mAb concentrations (Fig. 4). In contrast, mouse mAb A10 (obtained from Dr. P. Banga) (18) recognized both native and denatured TSHR-289.

The discrepancy between mAb 3BD10 recognition of soluble TSHR-289 under native conditions and its inability to

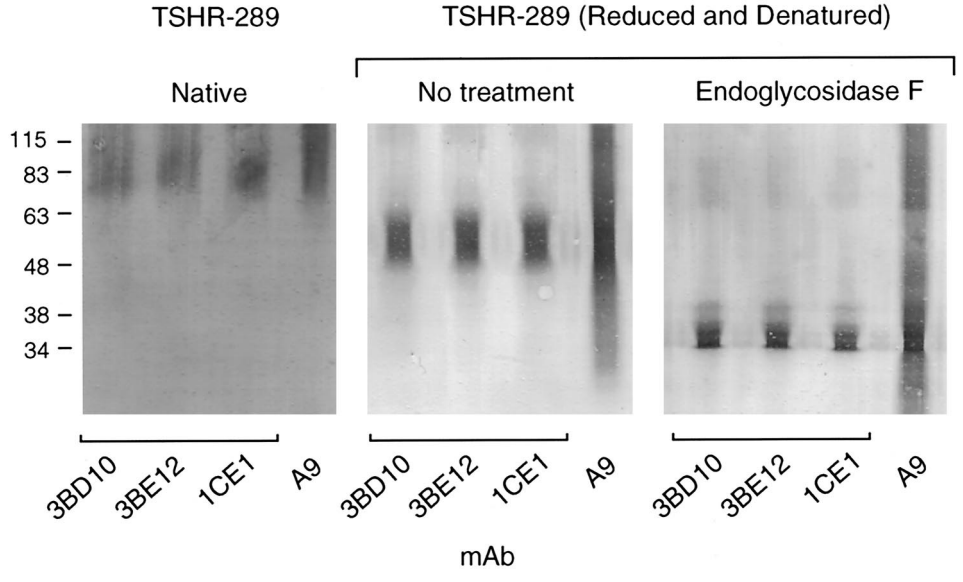

FIG. 3. Mouse mAb to TSHR-289. Mice were immunized with partially pure (~30%) TSHR-289 (see Fig. 1). IgG-producing clones positive for TSHR-289 by ELISA were used for immunoblotting against the partially purified antigen after electrophoresis under native conditions (absence of SDS and β-mercaptoethanol in sample and gel; *left panel*) and under denaturing and reducing conditions (samples treated with 2% SDS, 0.7 mol/L β-mercaptoethanol, and electrophoresis on SDS-polyacrylamide gels; *middle* and *right panels*). Note the very slow migration of TSHR-289 under native relative to denatured and reducing conditions. The mAb were also tested for recognition of TSHR-289 enzymatically deglycosylated with endoglycosidase F (*right panel*). Shown are the three mAb (3BD10, 3BE12, and 1CE1) that recognized TSHR-289 rather than the high molecular mass contaminating protein (see Fig. 1). Included as a control is mouse mAb A9 to the TSHR (from Dr. Paul Banga, London, UK) (18).

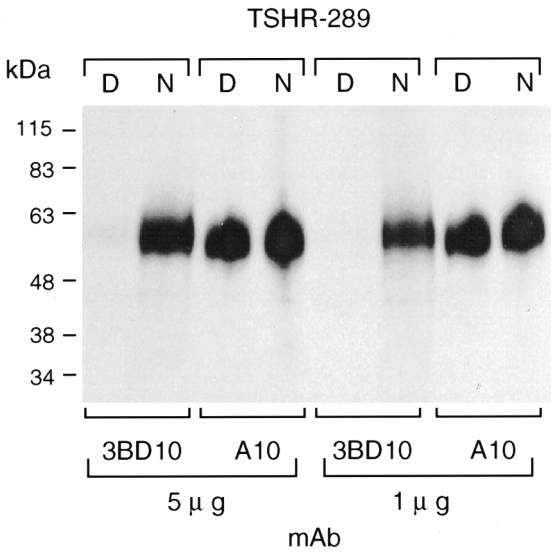

FIG. 4. Immunoprecipitation of secreted, precursor-labeled antigen with mouse mAb to TSHR-289. CHO cells expressing TSHR-289 were precursor labeled with [^{35}S]methionine/cysteine, and immunoprecipitations were performed, as described in *Materials and Methods*, on material secreted into the culture medium after a 16-h chase. The mAb 3BD10 was chosen for this study because its titer in ascites was the highest of the three mAb generated, all with the same epitope. The mAb A10 (from Dr. P. Banga) (18) was included as a control. For comparison of native (N) *vs.* denatured (D) TSHR-289 antigen, medium was harvested after the chase and half of the sample was frozen at −80 C (N). The other half of the conditioned medium underwent reduction and alkylation using dithiothreitol and iodocetamide, exactly as described previously (19), followed by freezing (D). Aliquots of N and D radiolabeled TSHR-289 were then thawed and simultaneously subjected to immunoprecipitation in 300 mM NaCl, 20 mm Hepes, pH 7.2, 0.1% SDS 0.5% NP-40 buffer. Autoradiograms were performed for 16 h.

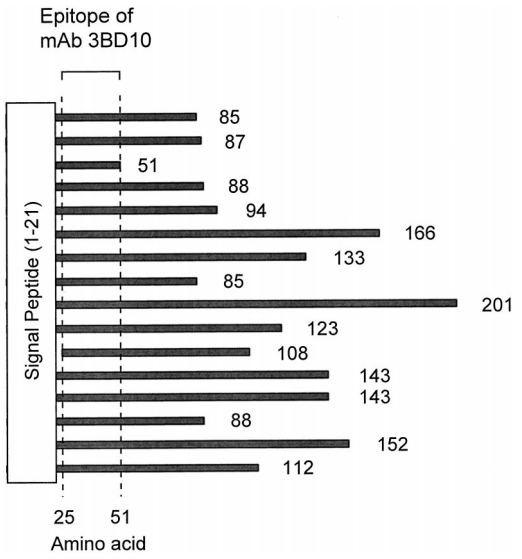

FIG. 5. Determination of the TSHR-289 mAb epitope. A size-selected (200- to 500-bp) TSHR cDNA fragment expression library was screened with three mAb (3BD10, 3BE12, and 1CE1; see *Materials and Methods*). All mAb recognized the same clones and, therefore, had identical epitopes. Nucleotide sequencing of 16 clones narrowed this epitope to amino acid residues 25–51. Note that only 1 of the 16 cDNA clones sequenced lacked residues 22–24. The signal peptide motif corresponds to residues 1–21.

interact with the native holoreceptor on flow cytometry with intact cells raised the possibility of steric hindrance by either the plasma membrane or more downstream components of the TSHR. Determination of the epitopes for 3BD10 (as well as 3BE12 and 1CE1) could, therefore, provide insight into the structure of the TSHR and its interaction with patients' autoantibodies. Although TSHR-289 is nearly 50% carbohydrate (10) (see Fig. 3), the three mAb interacted with its polypeptide component, making epitope mapping feasible.

Screening with the three mAb of a size-selected (200–500 bp) TSHR cDNA fragment expression library yielded numerous clones. Nucleotide sequencing of 16 clones and cross-screening of individual clones with the three mAb revealed that their epitopes were the same, all at the N terminus of the TSHR. Despite characterizing this large number of reactive TSHR polypeptide fragments, we were unable to define a linear segment of less than 27 amino acids (residues 25–51; Fig. 5). Indeed, the sequences of 15 of the 16 3BD10-reactive clones included residue 22, the first amino acid in the mature protein (residues 1–21 being the signal peptide).

Affinity purification of TSHR-289

Preferential recognition by mAb 3BD10 of the native form of TSHR-289 indicated the feasibility of affinity purification of this antigen. Passage of liter quantities of conditioned medium (2–3 days) from CHO cells secreting TSHR-289 over a 3BD10-Sepharose column readily purified TSHR-289 (~0.5 mg/L) to near homogeneity as determined by PAGE of the freshly isolated sample (data not shown). However, this affinity-purified material was devoid of bioactivity (ability to neutralize TBI autoantibodies), and, as after Sephacryl S-100 gel filtration, it was no longer detectable when reanalyzed a few days later by PAGE.

Although the 3BD10-Sepharose affinity column effectively purified TSHR-289 (at least in the short term), we were interested in determining the efficiency of this procedure for extracting immunologically active protein. For this purpose, we applied a relatively small volume (50 mL) of conditioned medium from TSHR-289 CHO cell cultures to the high capacity 3BD10-Sepharose column (10 mg 3BD10). The estimated concentration of TSHR-289 in conditioned medium is less than 1 μg/mL. As anticipated given the vast excess of mAb, a large proportion (74.5%) of TSHR-289 in the medium was extracted after a single passage over the column (Fig. 6A). Quantitation was performed by densitometric analysis of immunoblots of reduced and denatured TSHR-289 detected with mAb A10 that strongly reacts with denatured TSHR. Surprisingly, however, despite the large excess capacity of the affinity column, two additional applications of the same flow-through did not increase the extent of TSHR-289 extraction (74.3% and 73.2%, respectively). Moreover, even though the affinity column extracted three quarters of the TSHR-289 molecules from the medium, it did not extract any TSHR autoantibody-neutralizing activity from the same medium (Fig. 6B). These data suggested the existence of two forms of native TSHR-289, with mAb 3BD10 recognizing a dominant component lacking biological activity.

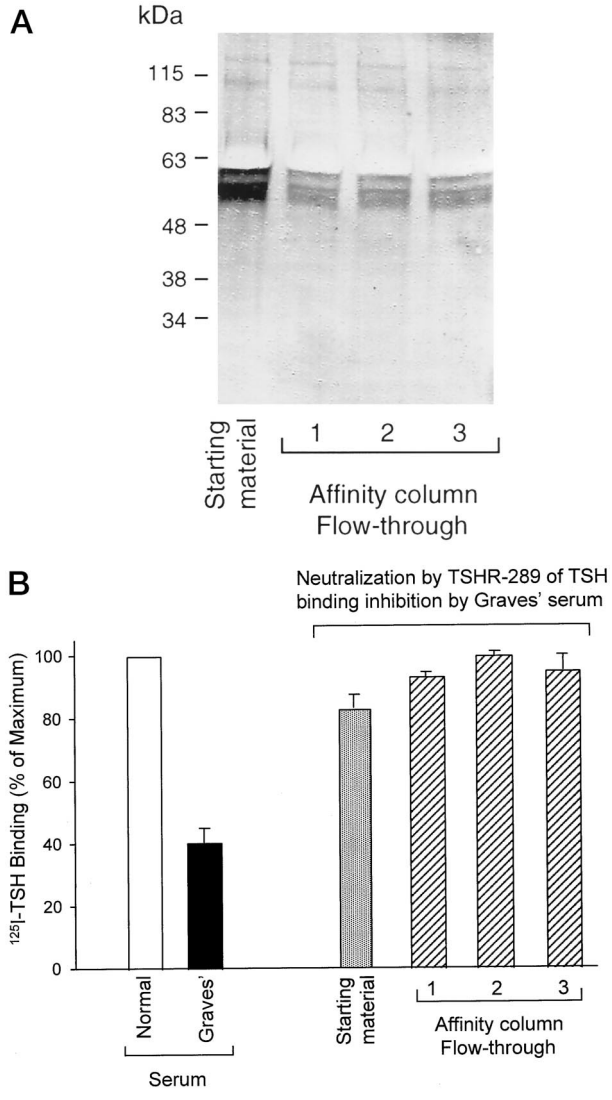

FIG. 6. A, Inability of the 3BD10-Sepharose affinity column to extract all TSHR-289 in conditioned medium. A small volume (50 mL) of conditioned medium from TSHR-289 CHO cell cultures was applied three times to a 3BD10 affinity column with a very large capacity. The total amount of TSHR-289 applied was less than 50 μg, and the column (5 mL; 10 mg 3BD10 IgG) is capable of capturing milligram quantities of antigen. The starting material and each of three successive flow-throughs (20 μL; diluted 1:5; affinity column flow-throughs 1, 2, and 3) were electrophoresed under reducing condition (SDS-10% polyacrylamide gel). Samples transferred to membranes were probed with mAb A10 (18), which recognizes denatured TSHR. Dilution of samples before electrophoresis was necessary to reduce the compression artifact caused by the high albumin content of the medium (size similar to that of TSHR-289). Even with this dilution, artifactual banding of TSHR-289 is still evident (compare with Fig. 3). B, Inability of the 3BD10-Sepharose affinity column to extract TSHR-289 capable of neutralizing TSHR autoantibodies in serum. A Graves' serum (*black bar*), unlike serum from a normal individual (*clear bar*), inhibits [^{125}I]TSH binding to solubilized porcine thyroid membranes. Conditioned medium from cultures of CHO cells secreting TSHR-289 (starting material; *speckled bar*) neutralizes the TBI activity of the autoantibodies in Graves' serum. Three applications of this medium over the mAb 3BD10-Sepharose column (affinity column flow-throughs 1, 2, and 3; *hatched bars*) do not extract the neutralizing activity. Compare this unreduced bioactivity with the extraction of TSHR-289 protein from the same samples (A). *Bars* indicate the mean ± range of duplicate incubations.

Stability of TSHR-289 at tissue culture temperatures

There existed, therefore, a perplexing situation that mAb 3BD10 preferentially recognized TSHR-289 under native conditions but was unable to bind to biologically active TSHR-289. One possible explanation for this phenomenon was the existence of two forms of native TSHR-289, with mAb 3BD10 recognizing a dominant component lacking biological activity. The basis for such a difference could be the reduced stability of TSHR-289 in medium at tissue culture temperature (37 C) despite its stability at ambient temperature. Moderate TSHR-289 instability at tissue culture temperature was indeed found. Thus, incubation of harvested conditioned medium for 7 h at 37 C reduced the ability of the material to neutralize TSHR autoantibodies in serum (Fig. 7). No loss of activity was seen when the medium was maintained at 21 or 28 C for the same time period.

Immunodepletion of TSHR autoantibody-neutralizing activity in conditioned medium

Another (more disturbing) possible explanation for the inability of mAb 3BD10 to immunopurify material with TSHR autoantibody-neutralizing activity was that this activity in conditioned medium was not inherent to the TSHR-

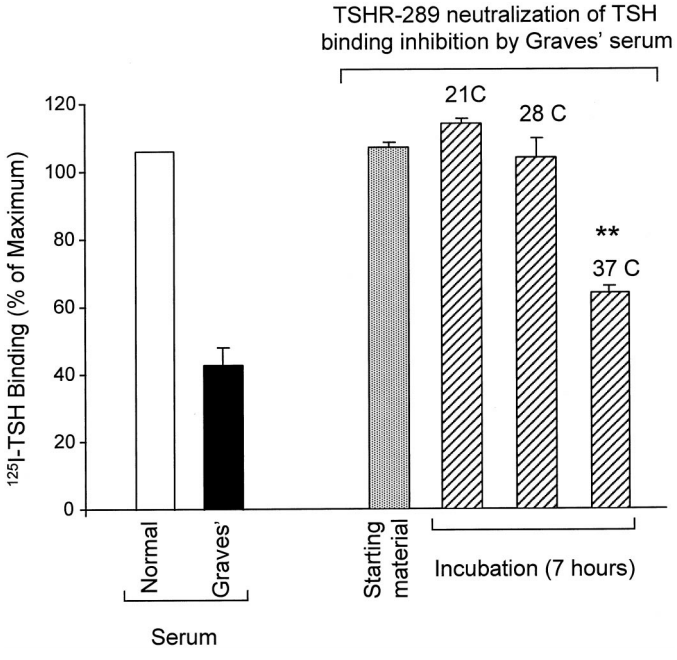

FIG. 7. Instability of TSHR-289 bioactivity under tissue culture conditions. Conditioned medium harvested from cultures of CHO cells secreting TSHR-289 was stored in aliquots at −80 C. When used immediately after thawing, TBI activity in Graves' serum (*black bar*) is completely neutralized (starting material; *speckled bar*). The lack of TSH binding inhibition by normal serum is indicated by the *clear bar*. Incubation of thawed conditioned medium for 7 h at room temperature (21 C) or at 28 C before the assay has no effect on its ability to neutralize the TSHR autoantibodies in Graves' serum. Incubation at tissue culture temperature (37 C) reduces this activity (*hatched bar*) relative to that of the starting material (**, $P < 0.005$, by Student's t test). Note, to obtain significant amounts of TSHR-289 protein, medium is harvested from cell cultures every 2–3 days. *Bars* indicate the mean ± range of duplicate incubations. The data shown are representative of three experiments

289 molecule. We, therefore, attempted to immunodeplete TBI-neutralizing activity in conditioned medium using a mAb to the six histidine residues at the C-terminus of TSHR-289. Such an antibody is unlikely to be influenced by subtle conformational changes in the autoantibody-binding site. Indeed, almost all TBI-neutralizing activity was removed by addition to the medium of the antihistidine mAb followed by passage over a protein G column (Fig. 8). Consistent with the previous data, mAb 3BD10 was largely ineffective.

Discussion

The TSHR has been a remarkably difficult molecule to study. Despite much effort, the protein is less stable than the closely related LH/CG receptor and has never been purified from thyroid tissue in significant quantities. Even after its molecular cloning and expression in cultured mammalian cells, scraping of cells from the tissue culture dish results in the loss of most TSH-binding activity, a phenomenon not prevented by proteolytic inhibitors (20). Contributing to this difficulty is the unique cleavage of the TSHR into two disulfide bond-linked subunits (A and B) (21), which appears to occur at two sites with the release of a putative C peptide (22–24). The present study indicates that even when the TSHR ectodomain has been engineered to convert it into a secreted form that retains recognition by patients' autoantibodies (10), the protein is unstable, especially when purified to homogeneity.

On the other hand, our data provide valuable new insight into this remarkable molecule that will contribute to the long term goal of understanding its interaction with TSH and disease-causing autoantibodies. It is apparent that two forms of native TSHR-289 exist, only one of these being recognized by TSHR autoantibodies. In a reciprocal manner, mouse mAb 3BD10 only recognizes TSHR-289 that does not interact with autoantibodies and *vice versa*. In our view, the most reasonable explanation for this phenomenon is that 3BD10 has a conformational epitope that is cryptic on the "super" native molecule but is revealed by subtle unfolding, imperceptible other than by loss of autoantibody binding. Further denaturation of TSHR-289 (reduction and alkylation) leads to loss of the 3BD10 epitope.

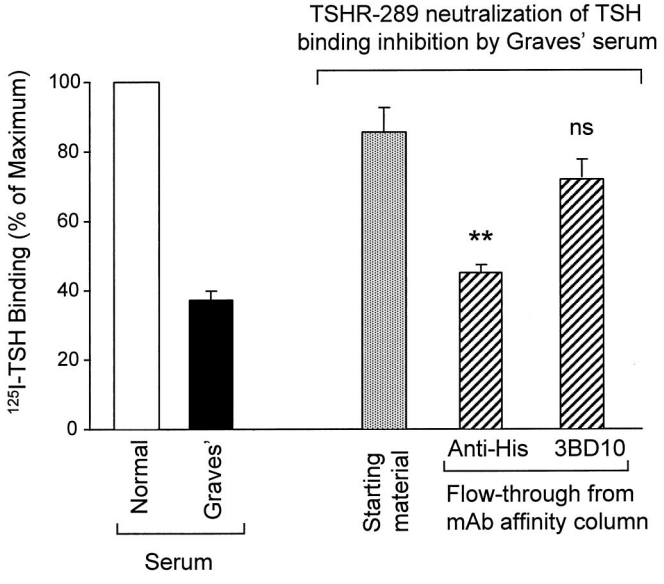

FIG. 8. Immunodepletion of TSHR autoantibody-neutralizing activity in conditioned medium with an antibody to the C-terminus of TSHR-289. TBI activity in Graves' serum (*black bar*) is completely neutralized by conditioned medium from CHO cells secreting TSHR-289 (starting material; *speckled bar*). Mouse mAb 3BD10 to the N-terminus of the molecule or Penta-His mAb to the six histidine residues at the C-terminus of the molecule was added to the same conditioned medium. After 16 h at 4 C, the mixtures were passed over a protein G column, and the flow-through was tested for TSHR autoantibody-neutralizing activity (*hatched bars*). All samples were assayed simultaneously. *Bars* indicate the mean ± range of duplicate incubations. **, $P < 0.01$, by Student's t test. ns, Not significant. The data shown are representative of two experiments.

These observations carry a number of important implications. First, the data help to resolve the still controversial concept that TSHR autoantibodies recognize exquisitely conformational epitopes. The discontinuous nature of these epitopes was revealed by studies using chimeric TSH-LH/CG receptor molecules (25). On the other hand, studies too numerous to describe have used TSHR-based synthetic peptides and prokaryotic fragments to report a myriad of linear epitopes apparently recognized by patients' autoantibodies (reviewed in Ref. 1). Parenthetically, the evidence most commonly cited for the conformational nature of TSHR autoantibody epitopes is the inability of Graves' patients' sera to detect TSHR cDNA fragments expressed in a prokaryotic library (26). However, this conclusion depends on recognition by Graves' sera of the TSH holoreceptor in such libraries, a finding that does not occur (26) (Nagayama, Y., and B. Rapoport, unpublished data).

A second intriguing conclusion from our study is that TSHR autoantibody concentrations in patients' sera are even lower than previously estimated, a phenomenon of pathophysiological relevance. TSHR autoantibodies in the majority of Graves' patients cannot be detected by indirect immunofluorescence (27) or flow cytometry (14) on TSHR-expressing mammalian cells (thyroidal or nonthyroidal). In contrast, thyroid peroxidase (TPO) autoantibodies in autoimmune Hashimoto's thyroiditis, with titers typically 50-fold higher, are easy to detect by the same approach (14). The availability of partially purified ectodomain variant TSHR-261 permitted quantitative neutralization studies of TSHR autoantibodies in patients' sera (10). By this means, relatively few sera were estimated to have TSHR autoantibodies in the microgram per mL range (10), whereas TPO autoantibody levels can attain 1 mg/mL (28). The present study with TSHR-289 indicates that only approximately 25% of the antigen molecules in conditioned medium contain the TSH binding-neutralizing activity. Therefore, TSHR autoantibody levels in patients' sera are clearly in the nanogram per mL range. The extremely low level of TSHR autoantibody in serum is consistent with the hypothesis (29) that these antibodies arise at a very early stage of the autoimmune process. Support for this concept is provided by restricted κ or λ light chain usage (30–32) and relative restriction to the IgG1 subclass (33) of TSHR autoantibodies.

The epitope for mouse mAb 3BD10 as well as those for the other two mAb raised against TSHR-289 are also of interest. Previously, using the same cDNA fragment library approach for mouse mAb that bind to denatured TPO, we could readily

narrow the cognate region to 15 amino acids (15). Likewise, a mAb (A9) that recognizes the TSHR after reduction has an epitope of only 14 amino acids (18). In contrast, despite analyzing 16 clones with TSHR fragments recognized by 3BD10, the linear epitope of 3BD10 could not be narrowed to less than 27 residues (amino acids 25–51). Because a typical antibody makes contact with 15–22 amino acid residues (34), the large size of the 3BD10 epitope is consistent with our data demonstrating its conformational nature and suggests that it may even be discontinuous. Support for this concept is that 15 of the 16 clones analyzed contained the cluster of 4 cysteine residues (Cys^{24}, Cys^{29}, Cys^{31}, and Cys^{41}) at the extreme N-terminus of the ectodomain (after signal peptide deletion). Modeling of the structurally rigid, leucine-rich repeats in the TSHR ectodomain (35) suggests that these four cysteines are spatially distant from the other 7 cysteines in the 397-amino acid residue ectodomain. Disulfide bonds are therefore likely to occur between the four N-terminal cysteines. Such bonding in a relatively small segment could, in turn, create a highly structured, possibly discontinuous, epitope. It is possible that folding variability in this region could result in the reciprocally exclusive recognition of mAb 3BD10 and Graves' TSHR autoantibodies. Indeed, chimeric receptor and other mutagenesis studies have implicated residues Ser^{25}-Glu^{30} (36) and Thr^{40} (37) as being a part of the TSHR autoantibody-binding site. Mutation of Cys^{41} also eliminates TSH binding (38), although whether this receptor trafficks to the cell surface is unknown.

In conclusion, studies involving a TSHR ectodomain variant truncated at residue 289 indicate the exquisite conformational requirements of TSHR autoantibodies. Even under native conditions, only a minority of molecules in highly potent TSHR-289 preparations neutralize patients' autoantibodies. Therefore, Graves' disease is caused by even lower concentrations of autoantibodies than previously thought. Finally, reciprocally exclusive binding to TSHR-289 by human autoantibodies and a mouse mAb with a defined epitope provides strong complementary evidence to the results of mutagenesis studies that the extreme N-terminus of the TSHR is important for autoantibody recognition.

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