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Immunoglobulins from Graves' Disease Patients Interact with Different Sites on TSH Receptor/LH-CG Receptor Chimeras than either TSH or Immunoglobulins from Idiopathic Myxedema Patients

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To examine the identity of binding sites for thyrotropin (TSH) and thyroid stimulating antibodies (TSAbs) associated with Graves' disease, we constructed eight human TSH receptor/rat LH-CG receptor chimeras. Substitution of amino acid residues 8-165 of the TSH receptor with the corresponding LH-CG receptor segment (Mc1+2) results in a chimera which retains high affinity TSH binding and the cAMP response to TSH but loses both the cAMP response to Graves' IgG and Graves' IgG inhibition of TSH binding. Two of three IgGs from idiopathic myxedema patients which contain thyroid stimulation blocking antibodies (TSBAbs) still, however, react with this chimera. Chimeras which substitute residues 90-165 (Mc2) and 261-370 (Mc4) retain the ability to interact with TSH, Graves' IgG, and idiopathic myxedema IgG. The data thus suggest that residues 8-165 contain an epitope specific for TSAbs and that TSH receptor determinants important for the activities of TSAbs and TSH are not identical. Further, binding sites for TSBAbs in idiopathic myxedema may be different from receptor binding sites for both Graves' IgG TSAb as well as TSH and may be different in individual patients.

The thyrotropin (TSH) receptor plays a critical role both in the function and growth of the thyroid gland (1). Graves' disease and idiopathic myxedema are autoimmune thyroid disorders whose expression is associated with antibodies to the TSH receptor (2-8). In Graves' disease, thyroid stimulating antibodies (TSAbs) are competitive agonists of TSH, elevate cAMP levels, and cause hyperthyroidism as well as goiter (2-8). In idiopathic myxedema, thyroid stimulation blocking antibodies (TSBAbs) are TSH antagonists, inhibiting TSH binding or TSH-increased cAMP levels and causing hypothyroidism and thyroid atrophy (2, 3). The different activities of TSAbs and TSBAbs suggest they might interact with different epitopes on the TSH receptor (2-8). Definition of the binding sites on the TSH receptor for TSAb, TSBAb, and TSH would clearly contribute to our understanding of the structure and function of TSH receptor and the

Abbreviations used: TSH, thyrotropin; LH, luteotropin; CG, chorionic gonadotropin; TSAb, thyroid stimulating antibody; TSBAb, thyroid stimulation blocking antibody.

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pathophysiology of Graves' disease and idiopathic myxedema. In one approach to address this question, we constructed eight TSH receptor/LH-CG receptor chimeras which spanned the extracellular domain of these receptors. We hoped some might be integrated into the membrane, conserve the tertiary structure of the TSH receptor, and allow studies differentiating the interactions of TSAb and TSBAbs as well as TSH.

MATERIALS AND METHODS

Human TSH Receptor Cloning - cDNA was prepared using a cDNA synthesis kit (Amersham) and Graves' thyroid poly(A)$^+$RNA, isolated as described (9). A human TSH receptor cDNA fragment was obtained using PCR (10), human thyroid cDNA as template, the following oligonucleotides as primers, GAGGACTTCAGAGTCACCTGCAAGGATATT and GGTCAGGTCAGGGAACATTTTAAGTCC. A random primed cDNA library was constructed using the EcoRI site of lambda gt11 and screened with ^{32}P-labeled human TSH receptor cDNA fragment (9, 11). Isolation and sequencing of positive clones was with standard methods (9, 11). Two positive clones, RM3-3 (2.6 kb) and RM19-3 (3.5 kb), contained the full length coding region for the human TSH receptor and had the sequence reported (12-14).

Construction of TSH Receptor/LH-CG Receptor Chimeras - The extracellular domain of the human TSH receptor was divided into 5 segments with preexisting restriction sites (Fig. 1). A new wild type clone (RC2.43) was constructed by substituting a 2036 bp EcoRI-BglII fragment of RM3-3 with a 1868 bp EcoRI-BglII fragment of RM19-3 to remove a AflII restriction site in the 5'-untranslated region. The new 2.43 kb wild type receptor cDNA was subcloned into pGEM 9Z (Promega) to construct chimeras. Rat ovarian cDNA was synthesized from its poly(A)$^+$RNA using the Amersham cDNA synthesis kit. Rat LH-CG receptor cDNA fragments were amplified by PCR (10). Primers were synthesized based on the published sequence of the rat LH-CG receptor (15) and contained appropriate restriction sites on the 5'end; rat ovarian cDNA or a 2.5 kb rat LH-CG receptor cDNA clone obtained from the rat ovarian cDNA library was used as template. PCR fragments were cut by restriction enzymes to produce cohesive ends and ligated to RC2.43 which had been cut by the same restriction enzymes. NsiI, the isoschizomer for PstI, was used for ligation of segment 1 or Segment 1+2 to the PstI sites of RC2.43 in the case of chimeras, Mc1 or Mc1+2. PCR fragments of rat LH-CG receptor cDNA and junctions of PCR fragments within the RC2.43 human TSH receptor clone were sequenced in full. Only chimeras having the human TSH receptor and rat LH-CG receptor cDNA sequences identical to the published data (12-15) were subcloned into the pSG5 expression vector.

Assays of TSH Binding and cAMP Production - Assays were performed 48 hours after transfection of the receptor chimeras into Cos-7 cells by electroporation as described (16). Before binding assays, cells were washed three times with modified Hanks'-HEPES (20 mM) buffer, pH 7.4, without NaCl (17) but containing 222 mM sucrose (16). Binding incubations were at room temperature for 8 hours in the same modified Hanks'-HEPES buffer containing 0.4% BSA, [^{125}I]TSH (70uCi/ug), and different concentrations of nonradioactive TSH ($1x10^{-11}$ to $1x10^{-7}$M) or IgG from patients with Graves' or idiopathic myxedema (16). The cAMP assays in the transfected cells were performed as described (16-18) with the exception that cells were incubated for two hours at 37° in the different concentrations of TSH or patient's IgG. All assays were performed in duplicate and repeated on three separate occasions. Values are normalized by the DNA content of the cells as described (18).

Materials - Sources of all materials, i.e. purified bovine TSH, [^{125}I]cAMP radioimmunoassay kits, [α-^{32}P]dCTP, and restriction enzymes, were the same as previously described (9, 16). IgGs from patients or normal subjects were purified using DEAE Affi-Gel Blue columns (Bio-Rad Laboratories); sera and thyroid tissue were kindly provided by Drs. Kunihiko Ito and Naoko Momotani (Ito Hospital, Tokyo, Japan) or Dr. W. Valente (U. of MD, Baltimore, MD).

RESULTS

After transient expression in Cos-7 cells, 4 of the 8 chimeric TSH/LH-CG receptors described in Figure 1 exhibited specific TSH binding and a TSH-induced cAMP response (Fig. 2). Mc2, which substitutes residues 90-165 of the TSH receptor, has the same or a slightly better K_d ($2.0x10^{-10}$M) and EC_{50} ($2x10^{-10}$M) for TSH binding and cAMP responsiveness, respectively, than wild type TSH receptor (Fig. 2A and 2B). The same is true for Mc1+2 (K_d=$2.6x10^{-10}$M and EC_{50} $4x10^{-10}$M) which substitutes residues 8-165 (Fig. 2C and 2D). Mc4 (residues 261-370 substituted) and Mc2+4 (residues 90-165 and 261-370 substituted) have a poorer K_d for TSH binding ($4.5x10^{-10}$M and $6.3x10^{-10}$M, respectively) and EC_{50} for cAMP response ($5x10^{-9}$M and $8x10^{-9}$M, respectively (Fig. 2C and 2D). Four chimeras have the same low level of background TSH binding and the same lack of a TSH-induced cAMP response as control antisense constructs of the wild type TSH receptor: Mc3 (Fig. 2A and 2B), Mc2+3 (Fig. 2A and 2B), Mc2+3+4 (Fig. 2C and 2D) and Mc1 (Fig. 2C and 2D).

The four chimeras (Mc2, Mc1+2, Mc4 and Mc2+4) which expressed high affinity TSH binding and could increase cAMP levels in response to TSH were evaluated for their ability to

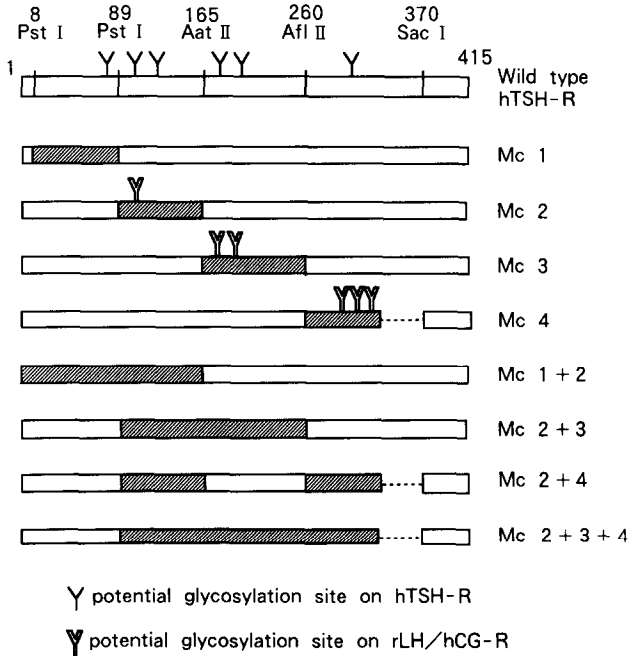

Figure 1. Structure of human TSH receptor/rat LH-CG receptor chimeras. Open bars indicate TSH receptor sequence; hatched bars denote rat LH-CG receptor sequence. The wild type human TSH receptor is diagrammatically represented on top; the restriction sites used for chimera construction are noted as are potential glycosylation sites. Numbers indicate the amino acid residue starting each segment as numbered from the methionine start site. Segment 4 of the LH-CG receptor is shorter than that of the TSH receptor; broken lines are therefore used to depict sequence gaps. Chimera receptors are named according to the segment substituted by LH-CG receptor sequence.

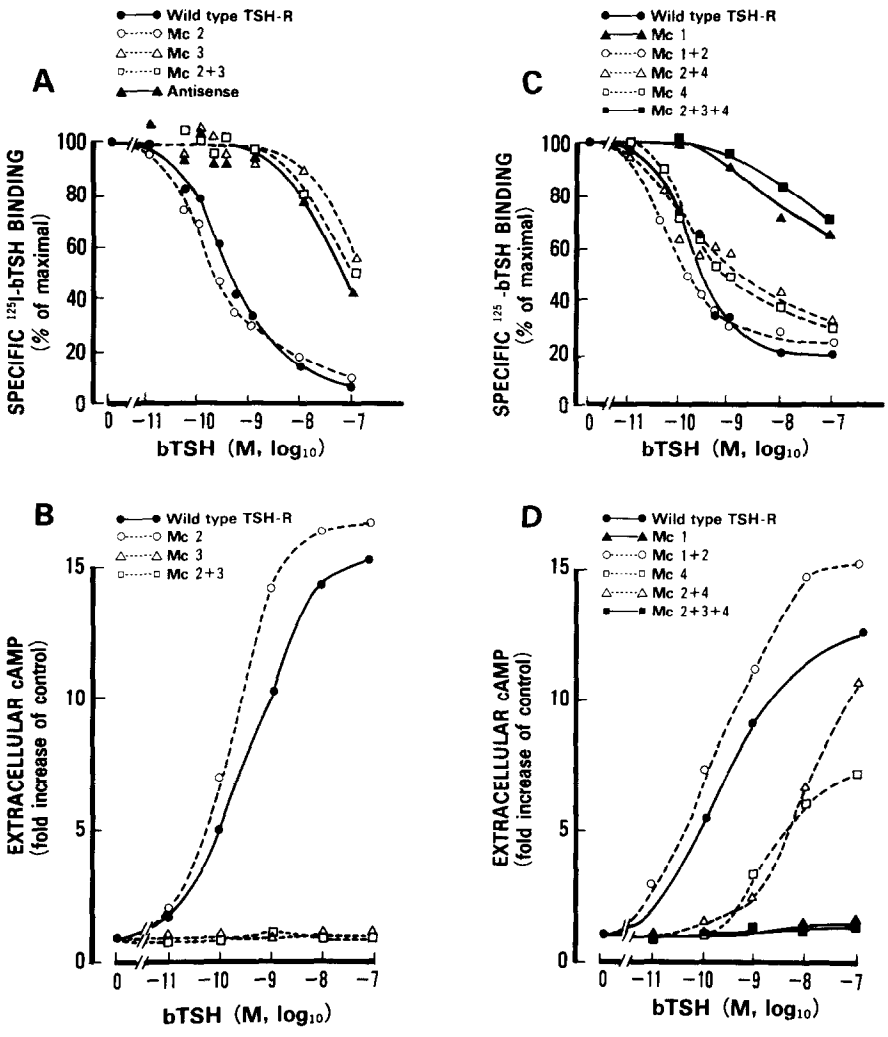

Figure 2. (A) [^{125}I]TSH binding and (B) concentration-dependent, TSH-enhanced cAMP production in Cos-7 cells transfected with wild type human TSH receptor, its antisense counterpart, or the TSH receptor/LH-CG receptor chimeras Mc2, Mc3, and Mc2+3. (C) [^{125}I]TSH binding and (D) concentration-dependent, TSH-enhanced cAMP production in Cos-7 cells transfected with wild type human TSH receptor or the TSH receptor/LH-CG receptor chimeras Mc1, Mc1+2, Mc4, Mc2+4, and Mc2+3+4. Assays were performed as described in Materials and Methods. In (A and C) results are expressed as the percent of [^{125}I]TSH bound in the absence of unlabeled hormone; in (B and D) data are presented as the ratio to control cAMP levels in the absence of TSH. In all panels (A-D) each point is the mean of duplicate dishes and data are representative of three separate experiments.

respond to Graves' IgG preparations (Fig. 3A). IgG from Graves' patients can inhibit TSH binding as well as increase cAMP levels (2-8); the two activities can, however, represent the action of separate antibodies as evidenced in both clinical studies and studies of monoclonal antibodies to the TSH receptor (2-8). To preliminarily evaluate the activity of Graves' IgG with each chimera, we used a pooled IgG preparation from several patients which was known to have

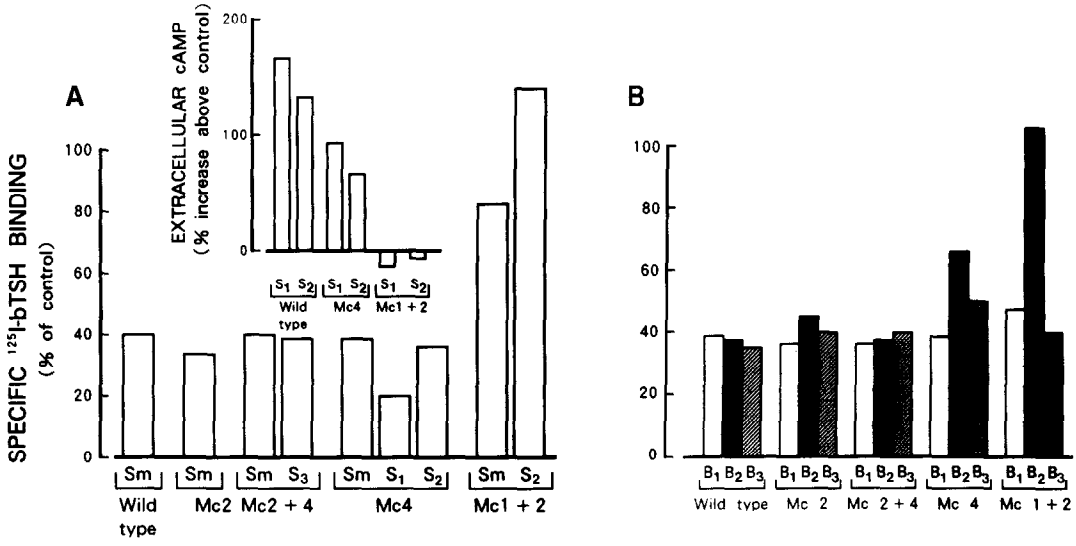

Figure 3. (A) The ability of Graves' IgG (2 mg/ml) to inhibit [^{125}I]TSH binding to Cos-7 cells transfected with wild type human TSH receptor or the TSH receptor/LH-CG receptor chimeras Mc2, Mc2+4, Mc4, or Mc1+2. In the **Inset** the ability of Graves' IgG (1 mg/ml) to increase cAMP levels, i.e. their TSAb activity, is measured in Cos-7 cells transfected with wild type human TSH receptor or the TSH/LH-CG receptor chimeras Mc4 or Mc1+2. Sm indicates pooled IgG from three Graves' patients; S_1, S_2, and S_3 are the three individual IgGs pooled. (B) The ability of three IgGs (B_1, B_2, B_3) from idiopathic myxedema patients to inhibit [^{125}I]TSH binding to Cos-7 cells transfected with wild type human TSH receptor or the TSH receptor/LH-CG receptor chimeras Mc2, Mc2+4, Mc4, or Mc1+2. IgG was present at 2 mg/ml. In both (A) and (B) results are expressed relative to controls tested with the same concentration of normal IgG. Each bar is the mean of duplicate assays and is representative of three separate experiments.

high levels of both activities. The pooled Graves' preparation (Sm in Fig. 3A) inhibited TSH binding to Mc2, Mc4, Mc2+4, and wild type TSH receptor but not to Mc1+2. This result was also true for IgG from the individual Graves' patients (S_1, S_2, S_3 in Fig. 3A) which had high levels of TSH binding inhibiting activity and were included in the pool. This suggested that substitution of segments 1 and 2 of the human TSH receptor with rat LH-CG receptor might retain high affinity TSH binding but did not retain the activity for a Graves' IgG to inhibit TSH binding. The pooled Graves IgG was also unable to increase cAMP levels in Mc1+2 (data not shown). This is, however, illustrated in the **Insert** of Figure 3A using Graves' IgG from two patients (S_1 and S_2) who had high TSAb activity in standard thyroid cell bioassays (17, 18); wild type TSH receptor and Mc4 are used as comparisons. Thus, the Mc1+2 chimera, wherein residues 8-165 of the TSH receptor are replaced by LH-CG receptor sequences, loses determinants essential for Graves' IgG activity in assays measuring both inhibition of TSH binding and TSAb activity in cAMP assays. The full retention of TSH binding and activity by Mc1+2 insures this data reflects the loss of one or more specific determinants and not the inability of the receptor to insert itself into the membrane for whatever reason.

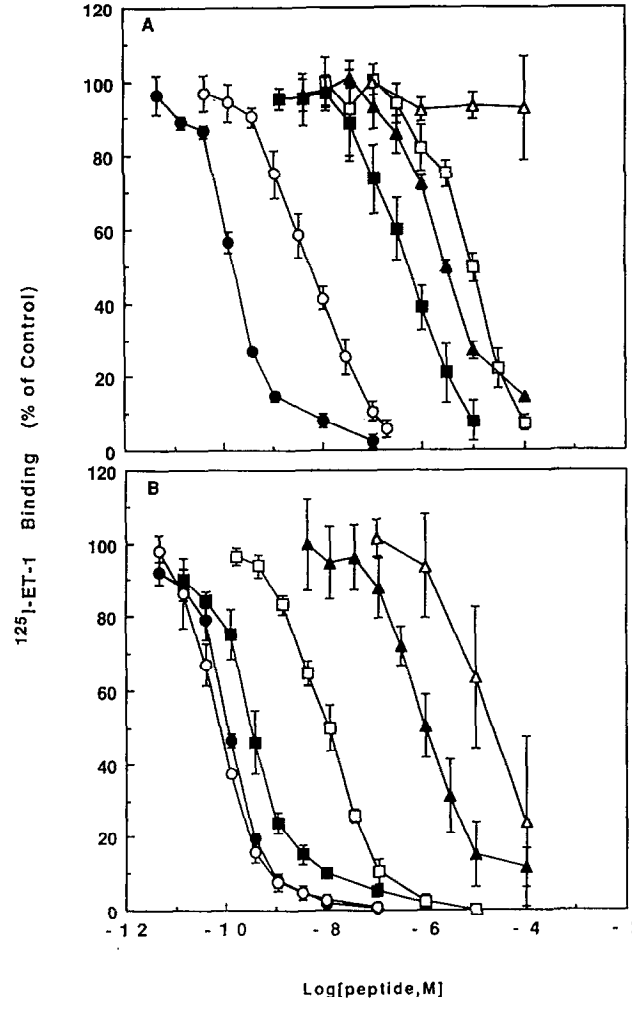

Fig.1. Inhibition of [^{125}I]ET-1 binding to (A) porcine aortic smooth muscle membranes and (B) cerebellar membranes by ET-1(●), ET-3(○), 4AlaET-1(■), N-Ac-4AlaET-1(10-21) (□), 4AlaET-1(11-21)(▲) and 4AlaET-1(6-20)(△). All points represent the average of more than three experiments.

whereas 4AlaET-1(11-21) and 4AlaET-1(6-20) scarcely elicited vasorelaxation at 10μM (Fig.2).

DISCUSSION

The structure-activity relationship of ET-1 analogs for vasoconstriction and binding to ET_A has been discussed in several reports (8,16,17). At the least, the disulfide bridges and the C-terminal Trp^{21} seem to be essential for vasoconstriction and binding to ET_A. However, there is little information about the structure-activity relationship of ET-1 for ET_B binding and function. Hiley *et al.* reported that the linear ET-1 analog 4AlaET-1 was nearly equipotent to ET-1 and ET-3 in inhibiting [^{125}I]ET-1 binding to rat cerebellar homogenate (9). Furthermore, 4AlaET-1 is a weak vasoconstrictor (18), and cerebellar membrane fraction is rich in ET_B (10,12).

will be necessary to determine if epitopes for the two different Graves' IgG activities, inhibition of TSH binding and cAMP elevation, are different, as predicted by monoclonal studies (5-8).

The ability of TSBAb preparations from two idiopathic myxedema patients to inhibit TSH binding to Mc1+2, whereas the Graves' IgG could not, indicates that the two types of antibodies have different epitopes despite the fact both are measured by inhibition of TSH binding. Again this was suggested in studies of monoclonal antibodies to the TSH receptor which showed that antibodies which inhibited TSH binding did not inhibit TSAb activity (5); in contrast, TSBAbs can inhibit TSAb activity (2, 3). The failure of a TSBAb from one idiopathic myxedema patient to inhibit TSH binding to Mc1+2 indicates that the binding site for TSBAb may vary between different patients or that idiopathic myxedema patients may have multiple types of TSH-binding inhibiting antibodies, some of which are the same as in Graves' patients. The data do not exclude the possibility that the two types of inhibiting antibodies recognize different epitopes; nor do they indicate TSBAbs interact with the same sites as TSH. This localization requires specific receptor mutations not simply broad substitutions as in the present study.

In sum, although TSH receptor/LH-CG chimera receptors can define regions of the TSH receptor which can be substituted with no loss of TSH binding or activity, the data are limited in defining specific determinants important for TSH binding activity. The chimeras have, however, unequivocally indicated that determinants important for TSH, TSAb and TSBAb activities are nonidentical and have localized a region within residues 8-165, possibly within residues 8-89, that contains a critical TSAb site.

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