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# A SYNTHETIC OLIGOPEPTIDE DERIVED FROM HUMAN THYROTROPIN RECEPTOR SEQUENCE BINDS TO GRAVES' IMMUNOGLOBULIN AND INHIBITS THYROID STIMULATING ANTIBODY ACTIVITY BUT LACKS INTERACTIONS WITH TSH*

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An 11-residue oligopeptide, P-195, was synthesized to match human thyrotropin (TSH) receptor structure from No. 333 to 343 of amino acid sequence. Preincubation of 5 Graves' IgGs with P-195 up to 10 $\mu$g resulted in dose-dependent reductions of thyroid stimulating antibody (TSAb) activity. [$^{125}$I] labeled P-195 was found to bind Graves' IgG. The bound radioactivity correlated significantly with their TSAb activity (N=25, r=0.587, p<0.01). A peptide having a completely reverse sequence as P-195 did not show such biological activity. The peptide did not affect TSH and thyrotropin binding inhibitor immunoglobulin (TBII) on their receptor binding nor biological activities. P-195 was concluded to have a part of TSAb binding sites. 

Graves' disease has been postulated to be an autoimmune thyroid disorder involving an autoantibody against TSH receptor, thyroid stimulating antibody (TSAb) (1-3). The causative role of the antibody has been confirmed clinically in cases of neonatal transient thyrotoxicosis induced by placental passage of maternal TSAb into the fetus (4).

Cloning and sequencing of TSH receptor cDNAs of dog, human and rat have been achieved recently (5-9). Through transfection of the cDNA into eukaryotic cells, binding of and cAMP production by TSH and TSAb as well as inhibition of TSH binding by thyrotropin binding inhibitor immunoglobulin (TBII), another TSH receptor antibody, can readily be observed (5-10). Several recent

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reports using site-directed mutagenesis or synthetic oligopeptides indicated some regions of the TSH receptor to be responsible for biological and/or immunological specificity (10-14). Irrespective of numerous efforts, however, the TSAb binding site has not been identified clearly.

The present study investigated the binding site of TSAb on the TSH receptor using a synthetic oligopeptide composed of a section of human TSH receptor amino acid sequence, and the peptide was found to bind and inhibit TSAb. The antigenic site of TSAb is discussed in relation with TSH receptor structure.

## Materials and Methods

1) Preparation of P-195

An 11-residue oligopeptide, P-195, was synthesized by Ishihara Industrial Co. Ltd. (Kusatsu, Shiga, Japan) to match No.333 to 343 amino acids of human TSH receptor sequence reported by Libert et al.(7). This part was one of 5 regions of whole TSH receptor sequence initially chosen to be potentially antigenic according to both acrophilicity plot and hydrophilicity plot by Kihou et al. (Japanese patent pending, unpublished observations). The sequence was H-Tyr-Val-Phe-Phe-Glu-Glu-Gln-Glu-Asp-Glu-Ile-OH. The synthetic procedures were carried out on phenyl acetamidomethyl resin (0.5mmol/g) by solid phase techniques using an Applied Biosystems Model 430A peptide synthesizer (15).

P-195 is white powder which is insoluble in water but easily soluble in a minimal amount of 0.01N NaOH. The peptide was kept at -80℃ until use, and aliquots were dissolved immediately before the usage. Radioiodination using [$^{125}$I] sodium iodide of the peptide was performed by Chloramine-T method giving specific activity of 90 $\mu$Ci/$\mu$g. Another peptide, P-237, having a completely reverse sequence of P-195 was also synthesized to see the non-specific effects of amino acid compositions of P-195.

2) Binding of P-195 to serum protein, especially to Graves' IgG

Binding of [$^{125}$I] P-195 to serum protein from one each of normal subject and Graves' patient was studied. To 50 $\mu$l of 1 to 5 diluted serum samples and phosphate buffer containing 1% bovine serum albumin, pH 7.4, 2.2 x $10^4$ cpm of [$^{125}$I] P-195 was added. After incubation for 2 hr at 4℃, the incubates were applied on a GW-3000 column of high performance liquid chromatography (HPLC) system (Waters Ltd., Milford, MA) and radioactivity of fractions was measured by a gamma counter. [$^{125}$I] P-195 binding to serum immunoglobulin (IgG) from 20 Graves' patients and 5 normal subjects was also studied. After incubation, a 50$\mu$l of 10% Staphylococcal Protein A (Pansorbin, Nakarai Chemical Co. Kyoto) was added and mixed well. Bound radioactivities were separated by a centrifuge after 30 min at room temperature and counted.

3) TSAb measurement

TSAb activity in IgG fractions was assayed using rat thyroid cells, FRTL-5, and under low salt-hypotonic incubation conditions as reported previously (16). In the case of peptide application, 0, 1, 2 and 10 or 50 $\mu$g of P-195 were added onto 1 mg of IgG fractions from 5 Graves' patients or normal IgG containing 0, 20 and 1000 $\mu$U of bTSH, and incubated for 2hr at 4℃. After incubation the solutions were added to FRTL-5 cells at 37 ℃. Produced cAMP contents in the cultured media after 3 hr incubation at 37℃ were measured by RIA kit (Yamasa Co., Choshi, Japan). Effects of upto 50 $\mu$g of P-237 on TSAb and bTSH activities were similarly studied.

4) Binding of P-195 to TSH and its effect on TBII activity

Using TBII assay system, effects of P-195 on [$^{125}$I] bTSH binding to porcine thyroid membrane and also on TBII activity were studied. The assay procedures were slightly modified from those detailed previously (14,17). A 50 $\mu$l aliquot of 0, 0.1, 1 and 10 $\mu$g of P-195 in the assay buffer were preincubated with [$^{125}$I] bTSH and 50 $\mu$l of low TSH serum from a patient with painless thyroiditis or a TBII positive serum for 2hr at 4℃. Then 50 $\mu$l of TSH receptor preparation was added and incubated further for 90 min at 37℃.

Receptor bound radioactivities were separated by polyethylene glycol precipitation and counted.

Binding to hTSH was studied using hTSH immunoradiometric assay system (TSH RIA Bead II kit, Dainabot Lab., Tokyo). The peptide solutions of 0, 2, 10 and 50 $\mu$g were added to the standard hTSH preparations containing 0, 0.6 and 4.0 $\mu$U/ml and normal serum with 2.3 $\mu$U/ml of TSH. After incubation for 2 hr at 4℃, the TSH concentration of the samples was measured using 2 kinds of anti-hTSH monoclonal antibodies as described previously (14,18).

## RESULTS

1. Effects of P-195 on TSH and TBII

Fig. 1 shows effects of P-195 on [$^{125}$I] bTSH binding to the porcine thyroid membrane preparation. Levels upto 10 $\mu$g of the peptide did not affect the bTSH binding to the receptor. In addition, no effect on TBII activity was

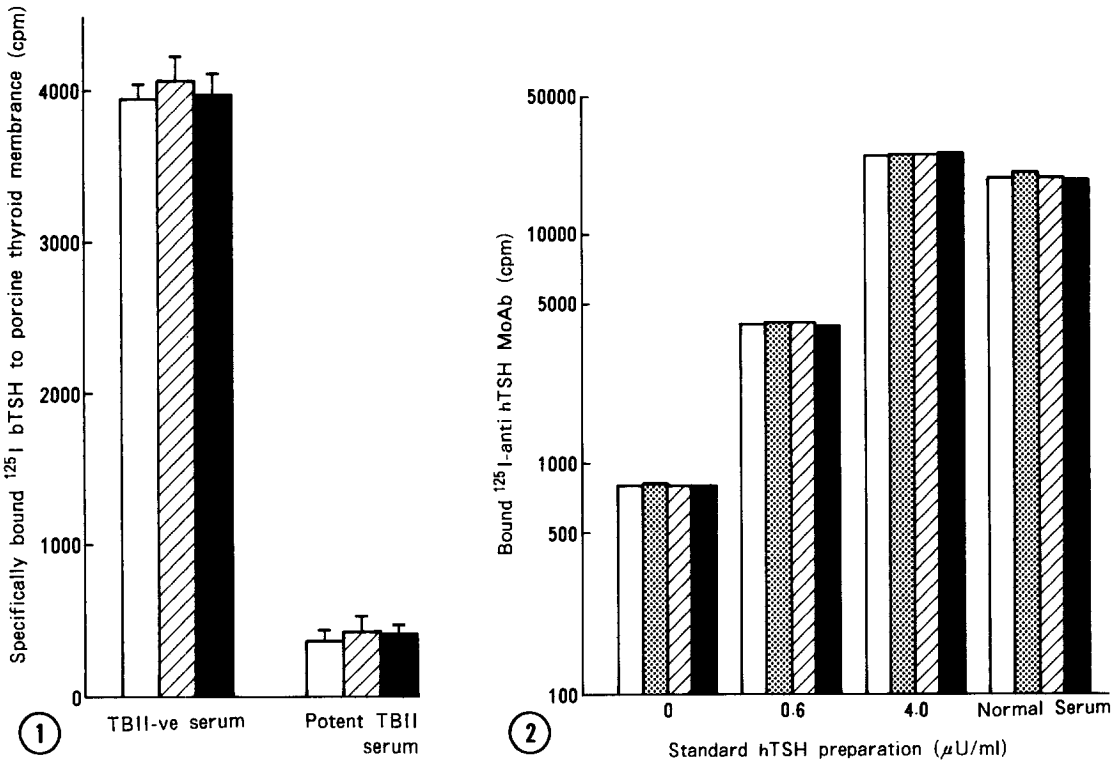


Fig. 1. Effects of P-195 on [$^{125}$I] bTSH binding to the porcine thyroid membrane and on TBII activity.

TBII negative serum was obtained from a patient with painless thyroiditis and did not contain any detectable TSH, while TBII serum obtained from a patient with hypothyroidism had activity of 99%. Open, striated and closed columns represent 0, 1 and 10 $\mu$g of P-195, respectively. Error bars show 1 S.D. of triplicated experiments, and no significant difference by the amount of added peptide was observed.

Fig. 2. Effects of P-195 on hTSH recovery on a specific immunoradiometric assay applying 2 anti-hTSH monoclonal antibodies.

Columns indicate different amounts of added peptide as described in Fig. 1. and dotted column for 0.1 $\mu$g peptide. Error bars were not presented but the highest coefficient of variance among tripricated observations was 5.9%, and no significant difference was observed.

seen. The effects of upto 50 $\mu$g of the peptide on 25 known TBII positive sera were also found to be negative (data not shown).

Preincubation of hTSH preparations with the peptide did not alter the amounts of hTSH which were detected by 2 kinds of anti-hTSH monoclonal antibodies (Fig. 2). Further, when hTSH preparations were incubated with [$^{125}$I] P-195 and then trapped by anti-hTSH monoclonal antibody conjugated beads, radioactivities bound to the beads remained almost negligible and unchanged irrespective of the concentrations of hTSH applied (data not shown). From these results, P-195 was concluded not to bind TSH at all and not to interfere with TBII activity either.

2. Binding of P-195 to Graves' IgG

Fig. 3 shows radioactivity distribution patterns obtained by HPLC of [$^{125}$I] P-195 incubated with BSA containing buffer, normal serum and Graves' serum. In the cases of buffer and normal serum, only one big radioavtivity peak of low molecular weight was observed. On the other hand, the incubate with Graves' serum revealed another small but discrete radioactivity peak of high molecular weight indicating [$^{125}$I] P-195 binding to some serum component(s) of Graves' patient. Fig. 4 demonstrates relationship between bound [$^{125}$I] P-195 radioactivities to 25 serum IgG fractions and their TSAb activities. Though the bound radioactivities were rather low, they show a significant correlation with individual TSAb activities ($r=0.587$, $p<0.01$).

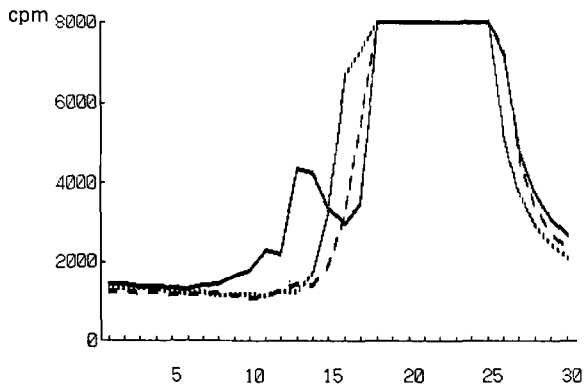


Fig. 3. Radioactivity distribution patterns of HPLC fractions obtained after incubation of [$^{125}$I] P-195 with buffer, normal serum and Graves' serum. Dotted, broken and solid lines indicate radioactivity counts of [$^{125}$I] P-195 incubates with buffer, normal serum and Graves' serum, respectively.

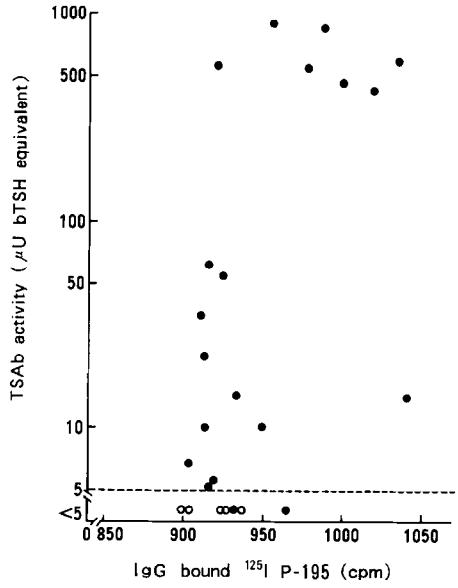


Fig. 4. Relationship between IgG bound [$^{125}$I] P-195 and TSAb activity in 20 patients with Graves' disease and 5 normal subjects.
Closed circles indicate Graves' patients and open circles are of normal subjects. Dotted line indicates the least detactable range of the assay.

3. Effects of P-195 on TSAb

Fig. 5a shows effects of P-195 on TSAb activities of 5 Graves' IgGs. In 3 of 5 IgGs studied, TSAb activity was inhibited by the addition of P-195 dose-dependently and the remainder also showed significant inhibitions by the addition of 10 $\mu$g of the peptide. On the other hand, upto 50 $\mu$g of P-195 did not reveal any significant inhibitory effects on cAMP production by 2 doses of bTSH or normal IgGs (Fig. 5b). When P-237 was used instead of P-195, any noticeable effects on TSAb activity by upto 50 $\mu$g of the peptide were not observed (data not shown). These results indicate that P-195 contains a binding site to Graves' IgG, presumablly to TSAb, and the peptide binding results in a subsequent decrease in cAMP production on FRTL-5 cells by TSAb stimulation.

## DISCUSSION

The present study demonstrated that TSAb binds to the amino acid sequence No. 333 to 343 of the proposed hTSH receptor structure (7), and the thyroid stimulating activity of the antibody is effectively inhibited by preincubation with a synthesized oligopeptide of this sequence.

As mentioned, cloning and sequencing of dog, human and rat TSH receptor cDNA have been achieved, and high homology among them have also been reported (5-9). Transfection studies using cDNA revealed: 1)[$^{125}$I] bTSH binds specifically to the transfected cells as well as thyroid cells, 2) bTSH and

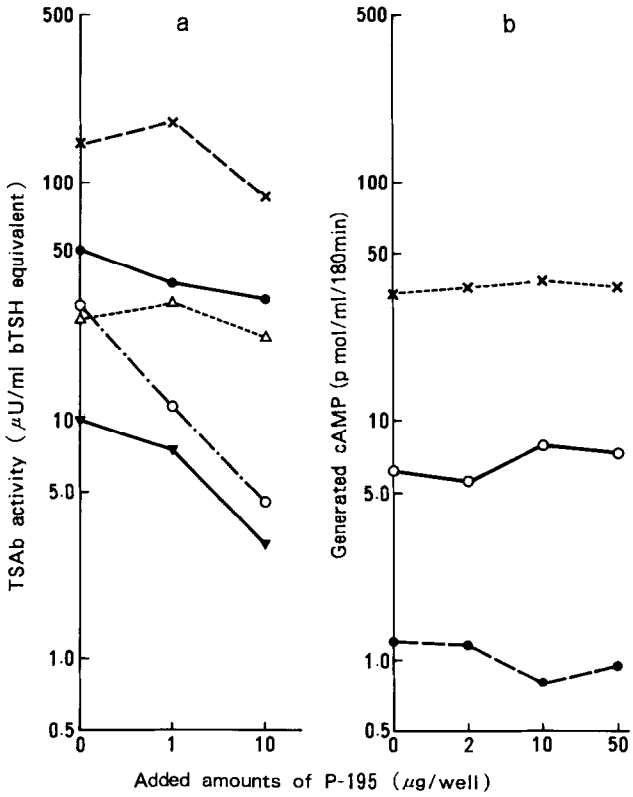


Fig. 5. Effects of P-195 on cAMP production by Graves' IgG and bTSH.
a): Effects on TSAb activity.
As detailed in Materials and Methods, 5 IgGs with various TSAb activity were preincubated with various amounts of P-195 and then measured for TSAb activity. A statistically significant decrease was seen ( paired t test, $p<0.01$ by 10 μg).
b): Effects on bTSH induced- or basal cAMP production.
Peptide effects on cAMP production by 0, 20 and 1000 μU bTSH are shown by closed circles with broken line, open circles with solid line and cross marks with dotted line, respectively. No significant effects were seen.

TSAb-IgG from Graves' patients (10) stimulate the cells to produce cAMP, and 3) TBII-IgGs from hypothyroid patients and Graves' patients inhibit the [$^{125}$I] bTSH binding to the cells. Thus, the proposed sequences of TSH receptors are very likely to be genuine.

Using sophisticated site-directed mutagenesis technique, Wadsworth et al. (10) recently reported that an 8 amino acid sequence from No.38 to 45 has an important role on the binding and thyroid stimulation of TSH and TSAb. Further, Murakami and Mori (13) indicated an immunogenic region of the hTSH receptor using a synthetic peptide around this 8 amino acid sequence. However, recent reports rather denied the significance of this region (11,12), and we also found no effects of a synthetic peptide containing this 8 amino acid sequence on TSH binding and TSAb activities (unpublished observations). Thus, TSAb binding site on the TSH receptor has not been identified clearly.

The presented P-195 is located in the TSH receptor-specific insertion amino acid sequence which is not present in the LH/CG receptor (19). Production of a unique stimulatory receptor autoantibody in patients with only Graves' disease may allow us to understand if certain antigenic sites are localized within this insertion sequence. We have chosen 11 residue sequences that contain high acrophilicity and hydrophilicity potentials. This partial sequence was identical among dog, human and rat TSH receptors (5-9).

Preincubation of Graves' IgGs with the peptide resulted in an apparent decrease of their TSAb activity. Later we studied 20 other Graves' IgGs for their TSAb after incubation with the peptide. Again we observed reductions of almost all TSAb activity. It was necessary to use 50 $\mu$g peptide to achieve similar effects, possibly due to fragility of the peptide during storage (data not shown). Such inhibitory activity to TSAb was observed only by P-195 among 32 peptides so far synthesized. As for possible non-specific effects of amino acid compositions of P-195, another peptide with a completely reverse sequence of P-195 was studied similarly and no biological effects were observed at all. In addition to the inhibitory effect of the peptide on TSAb activity, we observed a significant correlation between TSAb activity and [$^{125}$I] P-195 binding to the serum IgG fractions. However, the binding of [$^{125}$I] P-195 was rather low compared with biological effect of unlabeled material. The reason is not clear at present. As will be detailed elsewhere (Inoue et al, in preparation), we have succeeded in producing monoclonal TSAb-IgM antibodies from peripheral blood of Graves' patients by B-cell cloning procedures. Interestingly, while the TSAb-IgM did not inhibit the binding of [$^{125}$I] bTSH to FRTL-5 cells, the binding of [$^{125}$I] TSAb-IgM was inhibited effectively by unlabeled TSAb-IgM, TSAb-IgG and also by P-195, but not by bTSH or potent TBII-IgG. The antigenicity of P-195 sequence agaist TSAb should better be understood by immunoabsorption study, however, we have not been able to achieve this by P-195 or various P-195 conjugates. The sequence may be too small to hold the antigenic site free after conjugation. As for the effects of the peptide on TSH, the amount of receptor binding of [$^{125}$I] bTSH and 2 antigenic epitopes of hTSH did not change, and any significant binding of [$^{125}$I] P-195 with hTSH was not observed. Further, pretreatment of Graves' IgG with the peptide did not result in any noticeable decrease in TBII activity.

From these results we conclude that the P-195 amino acid sequence is identical to the sequence of at least a part of the antigenic site of TSAb but not of the binding site of TSH or TBII.

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