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IDENTIFICATION OF IMMUNOGENIC REGIONS IN HUMAN THYROTROPIN RECEPTOR FOR IMMUNOGLOBULIN G OF PATIENTS WITH GRAVES' DISEASE

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SUMMARY To identify immunogenic regions in human thyrotropin (TSH) receptor for immunoglobulin G (IgG) of patients with Graves' disease, seven different peptides (each consisting of 14-29 residues long) corresponding to segments of the extracellular domain of the receptor were synthesized. Graves' sera and IgG significantly bound to two out of seven peptides (the amino acid sequence of peptide #1, HQEEDFRVTCKDIQRIPSLPPSTQT; that of peptide #5, LRQRKSVNALNSPLHQEYEENLGDSIVGY). The present data indicate the characteristic existence of immunogenic regions in human TSH receptor for IgG of patients with Graves' disease. © 1990 Academic Press, Inc.

Graves' disease is a thyrotoxic disorder ascribed to auto-antibodies raised against thyrotropin (TSH) receptor which play pivotal roles in interaction with TSH receptor and stimulation of thyroid functions (see references in Ref. 1). Recently, human TSH receptor-encoding cDNA was cloned and its amino acid sequence was determined (2,3). This has a distinctive, putative extracellular domain, which is composed of 398 amino acids, connected to a 346 residue carboxy-terminal domain involving seven putative transmembrane segments. In analogy to anti-receptor antibodies in Graves' disease, myasthenia gravis is a disease featured by generating auto-antibodies for acetylcholine receptor and the detailed analysis has been in progress to determine the main immunogenic regions in the extracellular domain of human muscle acetylcholine receptor for patient IgG (4). The successful analytical methodologies include the application of synthetic peptides to the determination of epitopes binding to patient IgG (4). Implementation of this strategy has prompted us to evaluate which specific segments in human TSH receptor may be responsible for the immunogenicity for Graves' IgG. In the present study, seven different peptides existing in segment of the putative extracellular domain of human TSH receptor

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were synthesized and their binding to sera and IgG from patients with Graves' disease were examined.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma Chemical Co. (MO) and Wako Pure Chemical Industries (Osaka, Japan), unless otherwise indicated.

Patients: Sera were collected from eight control subjects (4 males and 4 females) and eight patients with non-treated Graves' disease (4 males and 4 females). Diagnosis of Graves' disease was made on the basis of clinical hyperthyroidism with diffuse goiter and abnormal laboratory findings including increased thyroxin levels, decreased TSH levels, and increased titers of TSH receptor antibody in the peripheral blood (determined by Baxter's TSH receptor antibody kit, R.S.R. Ltd., Cardiff, U.K.).

Peptide synthesis and radioiodination: Seven different segmental peptides in human TSH receptor were synthesized depending on three determinant bases; 1, secure turn segments which are insinuated by Chou-Fasman's protein secondary structure (Genetyx, Genetic Information Processing Software, Software Development Co., Ltd., Tokyo, Japan) and are known as important features of antibody-binding epitopes (4); 2, strong antigenic segments of the amino acid composition which have increased antigenic determinants (5); 3, sequential amino acids with low homology with pig luteinizing hormone-chorionic gonadotropin (LH-CG) receptor (2). These peptides (each consisting 14-29 residues long as shown in Figure 1) were synthesized by Peptide Institute, Osaka, Japan. For radioiodination, a tyrosine moiety was added to each of these peptides at the N-terminal side except for peptide #6. The composition of peptides was determined by an amino acid analyzer (JLC-300, Nihon-Denshi Industrial Co., Tokyo, Japan) after acid hydrolysis. Peptide purity was 88-99%, which was assessed by HPLC using YMC-Pack A-302 column (YMC Co., Kyoto, Japan) with a gradient of 10-60% acetonitrile in 0.1% trifluoroacetic acid (elution speed = 1 ml/min, fractionation = 1 min/tube). Ten μg of each peptide was radioiodinated with 0.5 mCi of ^{125}INa (Amersham International plc., Buckinghamshire, U.K.) by the chloramine-T method. Radioiodinated peptides were separated by Sephadex G-25 and further purified by HPLC using Sep-Pak C_{18} column (Waters Associates, MA) with a gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid (elution speed = 0.37 ml/min, fractionation = 1 min/tube).

Preparation of IgG: Serum was dialyzed against phosphate buffer (0.02 M KH_2PO_4, pH 8.0) overnight and applied to a DEAE column (Bio-Rad Laboratories, Richmond, CA). Eluates containing IgG were concentrated using Amicon's macrosolute concentrator (Division of W.R. Grace & Co., Danvers, MA) and adjusted to 10 mg/ml with phosphate buffer. Concentrations of IgG were determined by the Bradford's method (Bio-Rad Laboratories).

Immunoprecipitation: One hundred μl of radioiodinated peptide ($\sim$20,000 cpm) in phosphate buffered saline (PBS, 0.01 M PO_4, 0.15 M NaCl, pH 7.5) containing 0.25% bovine serum albumin (PBS-BSA), 50 μl of serum or IgG (0.5 mg), and 150 μl PBS containing 0.05 M EDTA were incubated at 4°C for 48 h. A solution of 500 μl of 8 mg/ml bovine γ-globulin and 500 μl of 30% polyethylene glycol (molecular weight = 6,000) was added to the incubation mixture. After centrifugation at 2,000 x g for 30 min, the precipitates were counted for radioactivities. Specific binding was calculated by subtracting radioactivities of precipitates without serum or IgG.

To estimate effects of protein A on immunoprecipitation by patients' sera, one ml of serum was incubated with 250 mg of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at room temperature for 1 h and centrifuged at 1,500 x g for 10 min, and the supernatants were used for immunoprecipitation.

Statistics: Results were expressed as mean ± SE. Statistical difference was calculated by Student's t test or Duncan's multiple range test.

RESULTS

Figure 1 shows the amino acid sequence of the putative extracellular domain in human TSH receptor (2,3) and the localization of synthesized peptides.

Figure 2 shows the representative elution profiles of radioiodinated peptides #1 and #5 on HPLC with Sep-Pak C_{18} column.

Figure 3 depicts the bindings of patient sera to synthesized peptides. In the series of the present study, the identical sera were used to assess the bindings to different peptides. Peptide #1 was significantly precipitated by sera of patients with Graves' disease as compared to those of control subjects (the patient group, 2.96 ± 0.80 vs. the control group, 1.13 ± 0.12% of original count, $P<0.05$). Although no statistical significance was observed in difference between precipitations of peptide #5 by patient and control sera, three out of

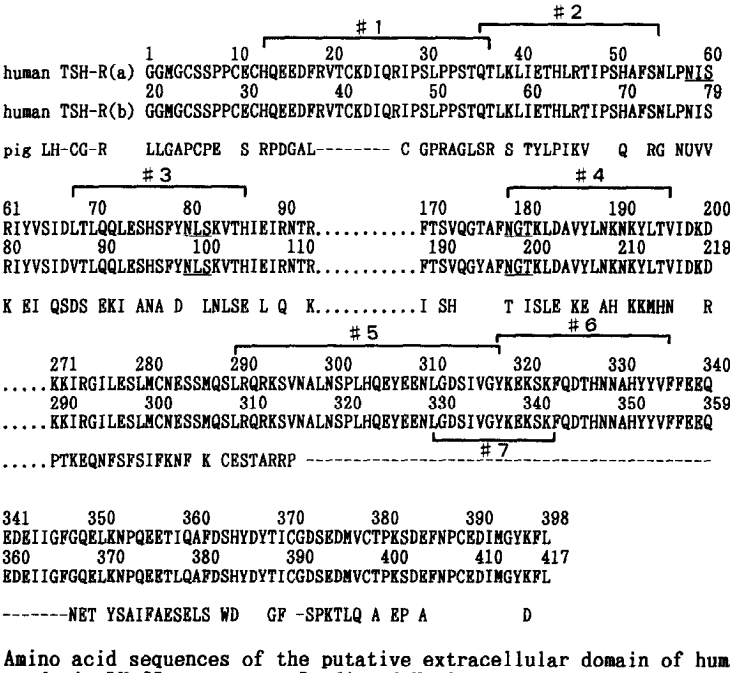

Fig. 1. Amino acid sequences of the putative extracellular domain of human TSH receptor and pig LH-CG receptor. Predicted N-glycosylation sites are underlined. Only non conserved amino acids and gaps (---) are indicated for pig LH-CG receptor. The different sequential numbers of amino acids indicated correspond to those mentioned by two groups of investigators [TSH-R (a) in Ref. 3; TSH-R (b) in Ref. 2]. Localization of synthesized peptides (#1-#7) is indicated.

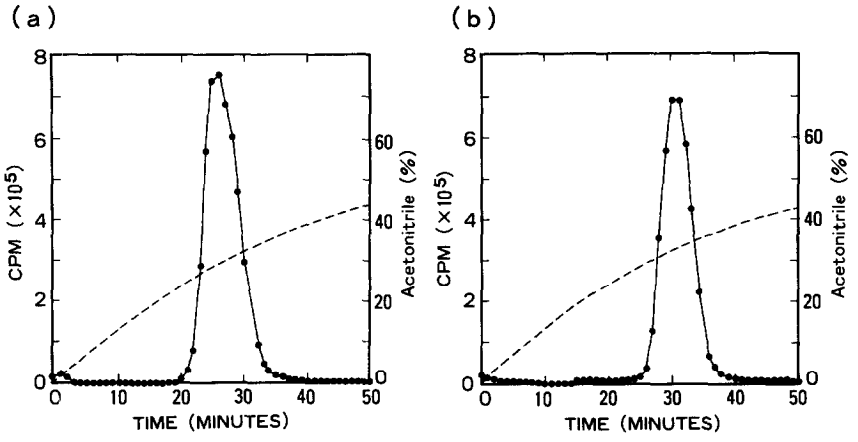

Fig. 2. The representative elution profiles of radioiodinated peptides on HPLC. Radioiodinated peptides were purified by HPLC using Sep-Pak C_{18} column as described in Materials and Methods. Activities of radioiodinated peptides and concentrations of acetonitrile are shown as dotted and dashed lines, respectively. (a), peptide #1; (b), peptide #5.

eight patients' sera tested were observed to significantly bind to peptide #5. The statistical correlation between the binding degrees of patient sera to peptides #1 and #5 was not significant ($r=0.438$, $P>0.05$). When compared to the control sera, sera of Graves' disease did not significantly bind to peptide #2 (the patient group, -3.48 ± 0.10 vs. the control group, -2.85 ± 0.31% of original count), peptide #3 (-1.24 ± 0.12 vs. -1.06 ± 0.10%), peptide #4 (0.59 ± 0.12 vs. 0.59 ± 0.04%), peptide #6 (0.16 ± 0.11 vs. 0.09 ± 0.09%), or peptide #7 (0.75 ± 0.10 vs. 0.88 ± 0.02%).

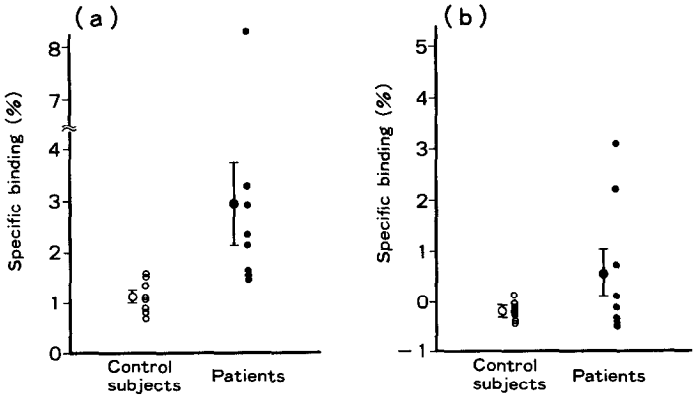

Fig. 3. Precipitation of peptides #1 and #5 by sera of control subjects (N=8) and patients with Graves' disease (N=8). Radioiodinated peptides were incubated with sera, and specific bindings were obtained as described in Materials and Methods. Each point is shown as a mean of duplicate determination. Mean and SE in each group is indicated. (a), peptide #1; (b), peptide #5.

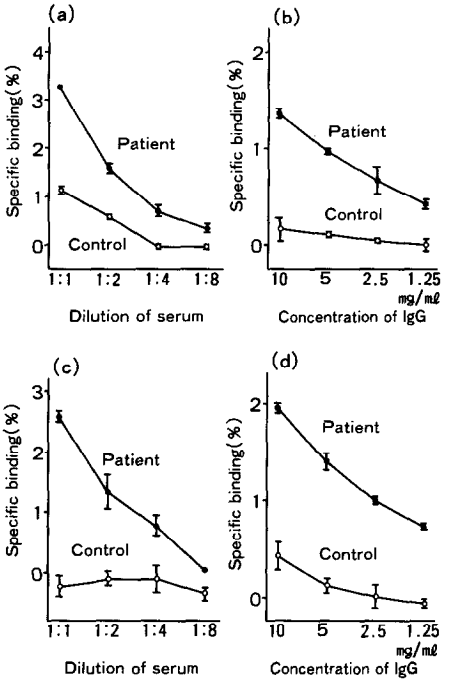

Fig. 4. Dose-response curves of precipitation of peptides #1 and #5 by serum and IgG. Radioiodinated peptides were incubated with sera and IgG, and specific bindings were obtained as described in Materials and Methods. Each point is shown as mean ± SE of triplicate determination. (a), precipitation of peptide #1 by sera; (b), precipitation of peptide #1 by IgG; (c), precipitation of peptide #5 by sera; (d), precipitation of peptide #5 by IgG.

Figure 4 shows the dose-dependent bindings of patient sera and IgG to peptides #1 and #5. To evaluate the prominent natures of binding, sera and IgG showing the second highest binding to peptide #1 and the highest binding to peptide #5 were utilized in this study. The binding efficiencies of the patient sera to peptides #1 and #5 decreased with increasing dilution (Figure 4a and 4c). When patient sera were pretreated with protein A as described in Methods, the binding of sera to peptides significantly decreased [to peptide #1, from 7.53 ± 0.10 (N=3) to 1.86 ± 0.02% (N=3), $P<0.01$; to peptide #5, from 4.01 ± 0.06 (N=3) to 2.08 ± 0.05% of original count (N=3), $P<0.01$]. The data indicate that the IgG component of serum contributed to the binding to the peptides. IgG obtained from Graves' patients apparently bound to peptides #1 and #5 in the dose-dependent manner, as shown in Figure 4b and 4d.

DISCUSSION

The present study demonstrated that sera of Graves' disease significantly bound to the greater extent to peptide #1 than did those of the control

subjects, in contrast to other peptides (#2, 3, 4, 6, and 7) which were not significantly precipitated by patient sera. To peptide #5, three out of eight patient sera showed the distinct binding. Hydrophilic regions have been initially considered to have the potentially antigenic determinants, but it is nowadays identified that not all antigenic regions are hydrophilic and not all hydrophilic regions are antigenic (5). Therefore, the synthesis of segmental peptides examined in the present study was not contingent on the hydrophilic natures (2) of sequential amino acids in human TSH receptor. Alternatively, the segmental peptides located in the extracellular regions of human TSH receptor were synthesized on the basis of the following criteria; the strong turn potential propounded by Chou-Fasman secondary structure (4), the heightened antigenicity of amino acid sequence (5), and the sequential amino acids with low homology with the amino acid sequence of LH-CG receptor that is a member of the family of pituitary glycoprotein hormone receptors.

The high degree of homology (71%) was observed in the amino acid sequence of the transmembrane regions between human TSH receptor and pig LH-CG receptor (2) as previously predicted (6), but the extracellular regions of the former had low homology (only 33%) with those of the latter. Moreover, the extracellular domain of human TSH receptor has unique "gap" arrangements which are not conserved in LH-CG receptor (2,3). Synthesized peptides #1 and #5 which were specifically precipitated by Graves' IgG contained the "gap" segments. Whereas the sequential amino acids of peptides #6 and #7 were also localized in the "gap" segments, these peptides were not significantly precipitated by patient IgG. These results imply that not all portions of "gap" segments contributed to exaggerated immunogenicity for auto-antibodies. With respect to the homology of human TSH receptor with the dog counterpart, Libert et al (3) described similitude of 90.3%. The amino acid compositions of peptides #1 and #5 possessed respectively 88.0 and 75.9% homology with the corresponding portions of the dog TSH receptor. The sequential amino acids of peptides #2, 3, 4, 6 and 7 were observed to have respectively 78.9, 84.2, 88.8, 68.4 and 64.3% similarity with those of the dog TSH receptor, the data indicating that the species difference was not meaningfully related to immunogenic properties to raise

antibodies. It was concluded from the present data that the amino acid sequence of peptide #1 (HQEEDFRVTCKDIQRIPSLPPSTQT) and the portion of peptide # 5 (LRQRKSVNALNSPLHQEYEENLGDSIVGY) were substantially associated with immunogenic regions for Graves' IgG.

It is well recognized that the apparent dissociation between titers of TSH-binding inhibitory antibody and activities of thyroid stimulating antibody occurs in the same IgG of some patients with Graves' disease (1). This phenomenon is interpreted to support the possibility that there may exist heterogeneous forms of the immunogenic region rather than uniformity in human TSH receptor. Because no homology between two peptides #1 and #5 was found, these two sequential portions in the human TSH receptor had considerably different epitopes for antibodies. Therefore, the present results confirm the existence of at least two divergent immunogenic regions in the TSH receptor as important features of antibody-binding epitopes.

Further studies are in progress in our laboratory to evaluate which antibodies raised against segmental peptides may be associated with the stimulatory functions of the thyroid gland that cause Graves' disease.

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