Epitope mapping of human thyrotropin

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Abstract. Epitope mapping of hTSH was carried out using 19 monoclonal antibodies prepared with hTSH or its β-subunit as antigen. The affinity constants of the monoclonal antibodies ranged from $9.6 \times 10^7$ to $5.7 \times 10^9$ M⁻¹l/mol for hTSH. The binding activities of monoclonal antibodies were maintained or in some cases rather enhanced after removal of the sugar moeity of the subunits of hTSH, and completely diminished after reduction of intramolecular S-S bonds in the subunits of hTSH. Ten monoclonal antibodies recognized the epitopes on hTSH (αβ subunit combined form) and on free α-subunit form. Eight other antibodies recognized the epitopes on free or combined form of β-subunit, all of which did not recognize any other human glycoprotein hormones. The monoclonal antibodies directed against the α-subunit could bind also other human glycoprotein hormones to a varying extent. On the basis of results from competitive binding studies, the antibodies directed against α-subunit and those against β-subunit were each classified into five subgroups recognizing different antigenic determinants. The remaining one antibody recognized an epitope expressed only by hTSH and not by the free subunits. In addition, a positive cooperativity on the binding of hTSH was observed between monoclonal antibodies directed towards a particular epitope on the α-subunit and those against an epitope on the β-subunit. From these data, two-dimensional map of epitopes on hTSH was constructed. The epitopes on each subunit were found to form a cluster with complicated overlapping, suggesting a highly conformational structure.

Human thyrotropin, and the other glycoprotein hormones such as human chorionic gonadotropin, human luteinizing hormone and follicle stimulating hormone consist of two dissimilar, α- and β-subunits (Pierce 1971; Pierce & Parsons 1981). These subunits are post-translationally assembled to form the natural αβ dimer molecule that is ready for secretion and hormonal action (Ingham et al. 1976). The α-subunit is encoded by a common gene (Fiddes & Goodman 1981) and carries similar antigenic determinants for these four hormones, whereas the β-subunits possess hormone-specific epitopes and give the functional specificity to the respective hormones (Reichert et al. 1970).

Currently, several studies have been reported on the production and characterization of monoclonal antibodies directed towards hTSH (Soos & Siddle 1982; Soos et al. 1984; Benkirane et al. 1987), hLH (Soos & Siddle 1983), hFSH (Hojo & Ryan 1985) and hCG (Schwarz et al. 1986; Thotakura & Bahl 1987; Ozturk et al. 1987). On the basis of epitope analyses using these antibodies, two- or three-dimensional mappings of epitopes were proposed for hLH (Soos & Siddle 1983), hCG (Schwarz et al. 1986) and very recently for hTSH (Benkirane et al. 1987). Such an epitope analysis seems to be useful for construction of available immunoasays for each hormone (Soos et al. 1984; Ozturk et al. 1987; Thotakura & Bahl 1985), and perhaps as a specific tool to probe the molecular structure of such hormones. Furthermore, a study on the functional role of epitope site may give important information to elucidate the mechanism of hormone action on the receptor (Holder et al. 1987; Hill et al. 1987).

To approach these problems, however, much
more information on the epitopes of these glyco- 
protein hormones seems to be needed, in addition 
to several previous analyses.

In this paper, we describe epitope mapping of 
hTSH based on a competition binding study with a 
variety of monoclonal antibodies, and discuss the 
properties of each epitope and the availability of 
the epitope map for a clinical approach.

Materials and Methods

Reagents
A clone of monoclonal antibody to hTSH was obtained 
from Pharmacia Fine Chemical Co, Uppsala, Sweden. 
Highly purified hTSH, hCG, hLH and hFSH were 
obtained from Calbiochem-Behring Co, La Jolla, CA 
or Kabi Vitrum AB, Stockholm, Sweden. Purified α and β-
subunits of hTSH were from UCB-Bioproducts S.A., 
Brussels, Belgium. RPMI culture medium, fetal calf 
serum and complete Freund’s adjuvant were from Difco 
laboratories, Detroit, MI. Bovine serum albumin (BSA) 
(Fraction V), neuraminidase, glucose oxidase and 
lactoperoxidase were from Sigma Chemical Co, St. Louis, 
MO. Endo-β-N-acetylgalactosaminidase H (Endo H) 
and Endo F were from Seikagaku Kogyo Co, Ltd, Tokyo, 
Japan, and Behringer Mannheim GmbH, FRG, respect-
tively. Antisera to class, subclass and type of mouse 
immunoglobulins were from Miles Laboratories, Inc, Elk-
hart, IN. Silicone rubber string (3 mm in diameter) was 
from Sanko Plastic Co, Osaka, Japan. All other chemicals 
used were in the highest available state of purity from 
Wako Pure Chemical Industries, Osaka.

Preparation of monoclonal antibody
Mouse monoclonal antibodies to hTSH were prepared by 
standard techniques (Kennett et al. 1980). Female 
BALB/c mice (3–4 weeks old) were immunized ip twice 
with 10 μg of hTSH (or β-subunit of hTSH) in complete 
Freund’s adjuvant at an interval of 2 weeks between injec-
tions. Booster dose of 20 μg of hTSH (or β-subunit) in 0.1 
mol/l NaCl was given more than 1 month later. The mice 
were killed 3–4 days after the booster injection and their 
spleens were collected. About 2 × 108 spleen cells were 
fused with about 2 × 105 myeloma cells (p3-x63Ag8-UI) in 
the presence of 45% polyethyleneglycol. The fused cells 
were washed once, suspended in RPMI containing 10% 
fetal calf serum, and plated in a 24-well culture plate. A 
solution of hypoxanthin-aminopterin-thidimidine (HAT) 
were added to select hybrid cells. The medium was re-
placed by fresh medium 2 days later and 10–14 days later 
each well was screened to detect antibody-producing 
cells, as follows.

Anti-hTSH antibody in the hybridoma culture me-
dium was detected by its binding to 125I-labelled hTSH 
and 125I-labelled hTSH subunits. 125I-labelled hormone 
(approximately 20 000 cpm) in 100 μl of phosphate-buf-

ered saline (pH 7.4) containing 0.1% BSA was incubated 
with 100μl of culture medium for 1 h at 37°C and then 
with 50 μl of goat anti-mouse IgG for an additional 30 
min. After further incubation with 200μl of a suspension 
of Staphylococcus aureus dead cells (Absorb G, a com-
mercial kit from The Chemo-Sero-Therapeutic Research 
Institute) for 30 min, the assay tube was centrifuged and 
the radioactivity of the precipitate was counted in a 
gamma counter. Iodination of proteins with 125I was per-
fomed according to the method of Tower et al. (1977) 
using glucose oxidase and lactoperoxidase.

Antibody-producing hybrids were subcloned by limit-
ing dilution in the presence of feeder cells (mouse thy-
mococyte monolayer cells). Positive clones were expanded 
and injected into pristane-primed mice for the produc-
tion of ascites antibodies.

Purification of monoclonal antibody
The IgG fraction from mouse ascites was prepared by 
precipitation with 18% Na2SO4 followed by DEAE-cellu-
lose chromatography. The column was developed with a 
linear gradient of 0–0.3 mol/l NaCl in 10 mol/l phos-
phate buffer, pH 8.2. The main peak eluted was em-
ployed as the IgG fraction of monoclonal antibody. The 
monoclonality of the antibodies was confirmed by 
isolectric focusing by the same method as described pre-
viously (Endo et al. 1984).

Characterization of monoclonal antibody
The immunoglobulin class, subclass and type of mo-
noclonal antibodies were determined by immunoprecipita-
tion in agarose gel using antisera specific for mouse im-
munoglobulin class and subclass (γ1, γ2a, γ2b and μ) and 
type (α and β).

The affinity constants of monoclonal antibodies for 
hTSH were determined by Scatchard plot analysis, as de-
scribed previously (Endo et al. 1984).

The specificity of monoclonal antibodies was deter-
mined by measuring their binding ability for 125I-labelled 
α- and β-subunits of hTSH as described above. Their 
binding abilities for 125I-labelled glycoprotein hormones 
were also tested by incubating monoclonal antibodies with 
125I-LH, 125I-hFSH, 125I-hCG instead of 125I-hTSH 
and bound fraction was immunoprecipitated by adding 
anti-mouse IgG antiserum and then Absorb G as de-
scribed above. Especially, the binding activities of α-sub-
unit-specific antibodies for glycoprotein hormones were 
measured at their various concentrations, where the abso-
olute amount of each antibody was adjusted in the 
range of the linearity of the binding response.

For all monoclonal antibodies, the cross-reactivity with 
125I-labelled bovine TSH was estimated by the same 
above method.

Furthermore, monoclonal antibodies were checked for 
their binding activities with deglycosylated or reduced 
subunits of hTSH. Deglycosylated α- and β-subunits were 
prepared by treatment of 125I-subunits (1 to 2 μCi) with
neuraminidase (20 µg), Endo H (0.01 unit) or Endo F (1.2 unit) in a final volume of 300 µl of buffers. The buffers used were 0.1 mol/l sodium acetate (pH 5.0) for the two former enzymes and 0.1 mol/l sodium phosphate (pH 7.2) for the latter. After digestions at 37°C overnight, deglycosylated subunits were passed through a Sephadex G-100 column equilibrated with 10 mmol/l sodium phosphate buffer (pH 7.0) containing 0.1 mol/l NaCl, 0.1% BSA and 0.1% NaN₃ (buffer A). Reduction of ¹²⁵I-subunits (1 to 2 µCi) with 5% β-mercaptoethanol was carried out in 300 µl of 1 mol/l Tris-HCl (pH 8.5) containing 8 mol/l urea at room temperature for 30 min. After treatment, 20 mg of iodoacetate in 50 µl of 0.1 N NaOH was added and further incubated for 15 min. Finally, reduced subunits were passed through the Sephadex G-25 column equilibrated with buffer A. The binding activities of monoclonal antibodies with deglycosylated and reduced subunits were then determined as described above.

**Competitive binding study**

The topological relationship between the binding sites of two different monoclonal antibodies was investigated by a competition study, in which the ability of a soluble antibody to inhibit the binding of ¹²⁵I-labelled hormone to another antibody on a solid phase was tested. Immunoadsorbents coated with monoclonal antibodies (solid-phase antibody) were prepared in the same way described previously (Endo et al. 1979). Briefly, silicone rubber were cut into rods 4 mm in length, and incubated with 30 µg/ml of the IgG fraction of monoclonal antibody for 2 days at 4°C in 0.25 mol/l phosphate buffer, pH 7.5. The adsorbents were then washed with buffer A and stored in the same buffer until use.

An excess amount of soluble monoclonal antibody (1 µg of IgG fraction per tube) or blank medium (buffer A) were pre-incubated with about 40 pg of ¹²⁵I-hTSH (or ¹²⁵I-hTSH subunits) (approximately 50 000 cpm) in 300 µl of buffer A. After 3 h at room temperature, a piece of silicone rod (solid-phase antibody) was added. After further incubation at room temperature overnight, radioactivity in the washed silicone rod was determined. The percent binding of ¹²⁵I-hTSH (or ¹²⁵I-subunits) by solid-phase

**Table 1.**

Summary of properties of monoclonal antibodies.

<table>
<thead>
<tr>
<th>Antibody 1</th>
<th>Affinity (1/nmol)</th>
<th>Specificity</th>
<th>Class subclass</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 7</td>
<td>1.3</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 10</td>
<td>0.096</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 11</td>
<td>0.84</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 12</td>
<td>2.1</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 13</td>
<td>0.13</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 14</td>
<td>0.46</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
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<tr>
<td>A 18</td>
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<td>γ1</td>
<td>✧</td>
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<tr>
<td>A 8</td>
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<td>γ1</td>
<td>✧</td>
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<tr>
<td>A 15</td>
<td>0.48</td>
<td>hTSH, α, hGon</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>A 19</td>
<td>4.1</td>
<td>hTSH, α, hGon</td>
<td>ni</td>
<td></td>
</tr>
<tr>
<td>B 1</td>
<td>nd</td>
<td>β</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>B 2</td>
<td>0.98</td>
<td>hTSH, β</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>B 4</td>
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<td>hTSH, β, bTSH</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>B 5</td>
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<td>hTSH, β, bTSH</td>
<td>γ1</td>
<td>✧</td>
</tr>
<tr>
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<td>γ1</td>
<td>✧</td>
</tr>
<tr>
<td>B 12</td>
<td>0.44</td>
<td>hTSH, β</td>
<td>γ2b</td>
<td>✧</td>
</tr>
<tr>
<td>C 1</td>
<td>nd</td>
<td>hTSH</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

1 Antibodies were prepared in the present study except C1 (Pharmacia), using whole hTSH as antigen except for B1 (hTSH β-subunit).
2 Affinity constants for hTSH calculated from Scatchard plot analysis.
3 Determined by measuring the binding ability for ¹²⁵I-labelled hTSH, its subunits, human gonadotropins (hGon) and bovine TSH (bTSH) as described in Materials and Methods.
4 Determined by immunoprecipitation with specific antiserum. nd: not done; ni: not identified.
antibody was calculated by taking the radioactivity in the absence of soluble antibody as 100%. This study suggests that if two antibodies, one soluble, the other solid phase, compete on the binding of hTSH and the value of percent binding of 125I-hTSH to solid phase antibody is near zero, the antibodies are considered to recognize the same epitope on hTSH molecule. Conversely if the percent binding to solid phase antibody is near 100, different epitopes are being recognized by the antibodies. Results were considered to be full competition, partial competition, no competition and enhancement, when the percent bindings obtained were below 30, 30–70, 70–120% and above 120%, respectively. When the result was not reciprocal, the lower value of the two percent bindings was employed, considering the difference in affinity constants of the antibodies used.

Results

Overall outcome of cell fusion experiments
A total 33 clones of anti-hTSH antibody-producing hybridoma were obtained from 21 independent fusions, each using the spleen cells from a different mouse. Eighteen clones out of these monoclonal antibodies were selected for the following studies, since they had antibody titres high enough to obtain reliable results.

Characterization of monoclonal antibodies
As shown in Table 1, all 18 antibodies established in the present study were composed of N type of light chain, and 16 of these antibodies were of γ1 subclass of heavy chain. An antibody named B12 was of γ2b, but a remaining antibody named A19 was not clearly identified as to its class and subclass.

The affinity constants of these antibodies ranged from $9.6 \times 10^7$ to $5.7 \times 10^9$ mol/l for hTSH. These values were one to three orders lower than those for antisera from the same immunized mice (mean, $7.7 \times 10^{10}$ mol/l).

Antibody specificity
Ten monoclonal antibodies were directed against the α-subunit of hTSH and the remaining 8 against the β-subunit (Table 1). All these antibodies except B1 could also bind whole hTSH (α:β heterodimer). Antibody B1 was directed against free β-subunit, which had been prepared by using spleen cells from a mouse immunized with β-subunit of hTSH. In contrast, a monoclonal antibody obtained commercially, named C1, could bind whole hTSH but not its free subunits. In addition, two antibodies, B4 and B5, cross-reacted with 125I-labelled bovine TSH, but the others did not significantly. All antibodies against β-subunits did not react with other human glycoprotein hormones.

Antibodies directed against α-subunit could also bind with other human glycoprotein hormones, although the binding activities were different for hormones among the antibodies examined (Fig. 1). On the basis of the difference in the binding acti-
Binding activities of monoclonal antibodies with hTSH subunits treated with neuraminidase, Endo H and Endo F. The binding activities after the treatment (—) was expressed by taken the activities before the treatments (—) as 100%.

Table 2.

<table>
<thead>
<tr>
<th>Solid-phase antibody</th>
<th>hTSH</th>
<th>Percent binding of ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Subunit*</td>
</tr>
<tr>
<td>A 7</td>
<td>15.1</td>
<td>34.1</td>
</tr>
<tr>
<td>A 8</td>
<td>9.5</td>
<td>7.0</td>
</tr>
<tr>
<td>A10</td>
<td>6.3</td>
<td>4.1</td>
</tr>
<tr>
<td>A11</td>
<td>9.0</td>
<td>30.2</td>
</tr>
<tr>
<td>A12</td>
<td>8.0</td>
<td>28.6</td>
</tr>
<tr>
<td>A13</td>
<td>3.5</td>
<td>4.3</td>
</tr>
<tr>
<td>A14</td>
<td>3.6</td>
<td>2.7</td>
</tr>
<tr>
<td>A15</td>
<td>2.5</td>
<td>10.2</td>
</tr>
<tr>
<td>A18</td>
<td>11.3</td>
<td>8.8</td>
</tr>
<tr>
<td>A19</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>B 1</td>
<td>0.0</td>
<td>1.2</td>
</tr>
<tr>
<td>B 2</td>
<td>7.2</td>
<td>33.7</td>
</tr>
<tr>
<td>B 4</td>
<td>26.0</td>
<td>42.7</td>
</tr>
<tr>
<td>B 5</td>
<td>23.3</td>
<td>45.3</td>
</tr>
<tr>
<td>B 6</td>
<td>6.1</td>
<td>21.4</td>
</tr>
<tr>
<td>B 8</td>
<td>8.2</td>
<td>12.8</td>
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<td>B10</td>
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<td>8.2</td>
</tr>
<tr>
<td>B12</td>
<td>2.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* 125I-α-subunit and 125I-β-subunit (before and after reduction) were used for the antibodies directed against α-subunit and for those directed against β-subunit as ligands, respectively. nd: not done.
observed that antibody B1 competed partially with the first and second groups for the binding of β-subunit.

Similarly, as shown in Fig. 4, 10 antibodies directed towards α-subunit were classified into five subgroups, based on a competition study with ¹²⁵I-hTSH. The first group (antibodies A7, A11 and A12) was found to compete with the fifth group (antibody A19) and partially with the third (antibody A8), but not with the second (antibody A15). The second group competed with the fifth group but not with the other groups. Closely similar results were obtained from a similar study with ¹²⁵I-α-subunit instead of ¹²⁵I-hTSH (data not shown).

Interestingly, in a competitive binding study using antibodies A8 or A14 and A8 as either solid-phase or soluble-phase antibody, the binding of ¹²⁵I-hTSH to solid-phase antibody was enhanced to about 300%, compared with that in the absence of soluble antibody.

In the following experiments, epitope recognized by antibody C1 was analysed by a competition study, in which the binding of ¹²⁵I-hTSH to the solid-phase antibodies was tested in the presence of antibody C1. As shown in Fig. 5, the presence of antibody C1 inhibited the binding of hTSH to the

antibodies directed towards β-subunit of hTSH were found to fall into five distinct subgroups. The first group (antibodies B4 and B5) competed with the second group (antibodies B8, B10 and perhaps B2) on the binding of ¹²⁵I-hTSH, but not with antibodies such as B6 and B1. The third group (antibody B6) was found to compete with the second group, but not with the first group. The fourth group (antibody B12) competed partially with most of other antibody groups except for B1. The fifth group (antibody B1) did not bind hTSH and so did not compete with any other group on the binding of whole hTSH.

Closely similar results were obtained from another competitive binding study using ¹²⁵I-β-subunit instead of ¹²⁵I-hTSH as ligand (Fig. 3B). It was
Effect of soluble antibody on the binding of hTSH to solid-phase antibody in combination of α-, β-subunit reactive antibodies and antibody C1. The symbols used are the same as in Figs. 3 and 4, and the additional symbol, ♢, represents a percent binding above 300%.

Two-dimensional epitope map of hTSH. The circles represent the epitopes expressed on both whole hTSH and free subunits of hTSH (---), whole hTSH but not free subunit (---), and free subunit but not whole hTSH (-----), respectively. The thickness of the lines expresses the magnitude of affinity constants of monoclonal antibodies which recognize the respective epitopes. A shaded area represents an epitope common to hTSH and hTSH. The arrows in the map represent the positive cooperativity on hTSH-binding and the direction on the arrow represents the effect from soluble-phase to solid-phase antibody.

Fig. 5.

Fig. 6.

Discussion

On the basis of the results of the present competitive binding studies, five different epitopes on each subunit (α- or β-subunit) were identified as shown in Figs. 3 and 4. Fig. 6 shows a two-dimensional epitope map of hTSH constructed according to these results and to additional subsequent observations: 1) monoclonal antibody C1 recognized a bridge region between each epitope on both subunits, and 2) the enhancement of binding of hTSH to solid-phase antibody was found in the coexistence of a particular antibody as a soluble phase. As regards the latter observation, it is likely that such enhancement is observed more frequently when both phase antibodies are bound to different epitopes which are near each other but do not overlap. Although the cooperativity was tested between two antibodies, such positive cooperativity may be involved in three or more antibodies. It is found that the epitopes on each α- and β-subunit form a cluster with complicated overlapping. An antibody subgroup with higher affinity constants recognized an epitope in the centre of the epitope map of each subunit (epitope α-5 on α-subunit and β-2 on β-subunit), suggesting its high immunogenicity. In the classification of epitopes on the α-subunit, the same conclusion was obtained from two independent experiments: a study on the binding ability of the antibodies with four glycoprotein...
hormones (Fig. 1) and a competitive binding study (Fig. 4). This confirms the reliability of the present classification of the epitopes on α-subunit. Compared with previous reports (Soos & Siddle 1982; Soos et al. 1984; Benkirane et al. 1987), the numbers of epitopes found in the present study were similar for β-subunit but more for the α-subunit. No more than two epitopes on α-subunit of TSH have been found in previous reports.

An epitope on β-subunit, β-1, was expressed also on bovine TSH. This may indicate that this epitope is common to both species. Importance of this epitope was also demonstrated by its participation in forming the bridge region between both subunits. It is likely that this region is involved in subunit association to form the functional α:β heterodimer.

In our present experiment, it was found that deglycosylation of hTSH subunits had little effect on its binding to monoclonal antibodies. This suggests that all monoclonal antibodies examined could not recognize either the sugar moiety or the portion related to sugar in the hTSH subunits molecule. In contrast, the remarkable effect of reduction of S-S bonds in hTSH subunits on its binding to monoclonal antibodies suggests that the antibodies tested recognize the conformational sites on hTSH subunits, and that molecular conformation constructed by forming S-S bonds is indispensable to development of antigenicity of hTSH subunits and perhaps hTSH itself. Until now, it has been thought that several factors, such as accessibility, hydrophilicity and mobility of protein segments, are concerned in the antigenicity and immunogenicity of proteins (Berzofsky 1985). The hydrophilicity of a particular region along the amino acid sequence of hTSH could be calculated according to the method of Hopp & Woods (1981). From this analysis, it was found that both α- and β-subunits each have four or five hydrophilic regions which formed with oligomeric peptide (data not shown, see Benkirane 1987). A single antigenic determinant on hTSH seems to be constructed through an assembly of some such hydrophilic segments but not by a single segment, since all epitopes detected in the present study were found to be highly conformational.

It is likely that differences in the immunoreactivities of the four glycoprotein hormones with monoclonal antibodies directed towards α-subunit originate from the differences in conformational changes of the α-subunit, which may be caused through the combination with different β-subunits and/or through posttranslational attachment of different sugar components to α-subunit (Nilsson et al. 1986). The present epitope analysis may provide a useful tool to elucidate the detailed change(s) of hTSH structure. The structural alteration of the TSH molecule seems to be induced frequently according to physiological or pathological states, which are suggested by many previous observations on its microheterogeneity (Yora et al. 1979; Pekonen et al. 1981; Takai & Rapoport 1981) and its abnormality (Faglia et al. 1979; Joshi & Weintraub 1983; Beck-Peccoz et al. 1985, 1986).

References


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