Analysis of the Interaction of Thyrotropin Receptor with the Ligands by Using Its Synthetic Peptides

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IT IS WIDELY believed that thyrotropin receptor (TSH-R) autoantibody plays a central role in the pathogenesis of Graves’ disease [1]. Clinically, these antibodies have been detected by several different methods. They are measured by their ability to bind the thyroid membrane (TSH-binding inhibitor immunoglobulin (TBI) activity), by their ability to stimulate thyrocytes (thyroid-stimulating antibody (TSAb) activity) or by their inhibitory effect on the TSH-induced cAMP increase in thyroid cells (thyroid stimulation blocking antibody (TSBAb)) [1].

However, these different assay procedures for detecting TSH-R antibody (TRAb) have revealed that there exists an imperfect correlation between TBI and TSAb [1]. Furthermore, there is a report of Graves’ disease whose IgG shows both TSAb and TSBAb activities [2]. These discrepancies have been explained by the heterogeneity of the antibody and it has been actively debated whether their epitopes are the same or not. Since some IgGs stimulate thyrocytes and others inhibit the function [3], determination of the sites on TSH-R that interact with TSH or TRAb would be helpful in understanding the pathogenesis of Graves’ disease. But such assay procedures with thyroid membrane or thyrocytes seem to have a limitation in clarifying the issues and details remain unknown.

Recently, TSH-R cDNAs of various species have been cloned and their deduced amino acid sequence has been reported [4–8]. And the data opened the way to a new approach to the determination of the domains responsible for the signal transduction of TSH or TRAb.

One of the approaches is the use of the deleted TSH-R or TSH-luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor chimera, and much effort has been made to clarify the TSH binding site(s) or TRAb binding domain(s) on TSH-R [9].

Another new approach is the application of the synthetic peptides of TSH-R and the evaluation of their antibody activities. And many studies have also been made to clarify the issues. We review here the analysis of the structure and function of TSH-R and the studies of TRAb binding site(s) on TSH-R with TSH-R peptides. The problems of these studies and their application will also be discussed.

Search for TSH or TSAb binding site(s) on TSH-R using TSH-R peptides

The first report of dog TSH-R cDNA has revealed that the receptor belongs to the G-protein coupled receptor family [4]. Although the majority of the receptor family have a small extracellular domain, TSH-R as well as LH/CG receptor have a large extracellular domain (398 amino acids) and the primary structure of the extracellular segment of TSH-R was very similar to that of LH/CG receptors [10]. However, comparison of the amino acid sequence of the TSH and LH/CG receptors shows that the TSH-R has two unique insertions that are not present in the LH/CG receptor: an 8-amino acids tract (No. 19–26) near the N-terminus and an approximately 50-amino acid tract (No. 298–347) near the transmembrane segment (Table 1). Therefore, initially, most atten-
tion was focused on these TSH-R unique insertions as the candidates for TSH or TRAb binding sites on TSH-R.

Indeed, Murakami and Mori reported that peptide # 12-36, which contains a TSH-R unique insertion, binds strongly to Graves' IgGs. They synthesized seven peptides corresponding to various segments of human TSH-R. Tyrosine moiety was added to each of these peptides at the N-terminus for labeling with 125I and the binding activity of Graves' IgG to the labeled peptides was determined. They found that only one peptide (# 12-36) possessed statistically significant binding to Graves' IgGs, showing the importance of the region for IgGs binding to the receptor [11].

Subsequently, Piraphatdist et al. observed that peptide P-194 (# 103-111) slightly absorbed TBII activity of IgG from Graves' patients (Table 1). They found that only one peptide (# 12-36) possessed statistically significant binding to Graves' IgGs, showing the importance of the region for IgGs binding to the receptor [11].

In these three reports, peptides investigated by them did not overlap each other, so that a detailed comparison of their data is difficult. However, when compared to subsequent studies concerned with the search for the TSH-binding domain on TSH-R using synthetic peptide, there exists a discrepancy to be explained.

Atassi et al., in turn, labeled ligands such as TSH, LH or FSH with 125I, and then examined the ligand binding to the synthetic human TSH-R peptide [14]. They observed that peptide #12-30, and #324-344 significantly bind to TSH but not to LH or FSH (Table 1).

Table 1. Interaction of TSH-R peptides with Graves' IgG or TSH

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Graves' IgG binding</th>
<th>TBII activity</th>
<th>TSAb activity</th>
<th>TSH binding</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Murakami et al.</td>
<td>12-36</td>
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<td>Piraphatdist et al.</td>
<td>103-111</td>
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<td>Mori et al.</td>
<td>333-343</td>
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<tr>
<td>Attasi et al.</td>
<td>12-30</td>
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<td>324-344</td>
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<td>Ohmori et al.</td>
<td>16-31</td>
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caused an increase in their TSAb activity. They interpreted this as being due to the binding of the peptide to IgG which has an inhibitory effect on TSAb measurements [12].

However, Mori et al. identified the sequence from #333-343 (P-195) on human TSH-R as a TSAb binding site (Table 1). Preincubation of 5 Graves' IgGs with the peptide resulted in dose-dependent reductions in TSAb activity. The binding activity of the radiolabeled peptide to IgGs was correlated with their TSAb activity, indicating that this region contains some TSAb binding sites [13].
Ohmori et al. also found that preincubation of the peptide (#16–31) with TSH inhibited cAMP formation in FRTL-5 cells, but the peptide (#379–398) had no effect on it (Table 1). They also failed to absorb TSAb activity of Graves’ IgGs with the peptide #16–31, and suggested that there exist multiple immunogenic sites for TSAb [15].

The peptide employed in the latter two reports accidentally contained the amino acid sequence of the human TSH-R studied in the former. For instance, #324–344 peptide synthesized by Atassi et al. [14] contained the sequence of the P-195. Although #324–344 peptide significantly binds TSH, P-195 did not bind TSH [13]. These results suggest that modification of the peptide by adding tyrosine residues or a few additional sequences largely affects their binding to TSH or TSAb.

Biological activity of antibody to TSH-R peptide

Production of the antibody to the TSH-R peptides and determination of their biological activities are another approach to the studies on the effects of IgG binding to the receptor and also on the receptor function itself.

We firstly developed the antibody to the synthetic peptides corresponding to various segments of the human TSH-R in chicken, and measured their TBII, TSAb and TSBAb activities. We found that the antibodies to the peptide #353–378 and the peptide #322–339 had both TSBAb and TBII activities, and the antibody to the peptide #630–642 corresponding to the third extracellular loop in the membrane spanning region possessed only TSBAb activity [16].

In order to investigate the functional activity of the antibody to the N-terminal TSH-R specific insertion, the peptide #10–38 (N peptide) was immunized in rabbits, and their biological activities were also examined [17]. Some of these experimentally produced antibodies successfully mimic the action of TSH on TSH-R, namely TSAb activity, but, by immunizing the same peptide, others were positive for TSBAb activity. However, none of these showed TBII activity. The results indicated that there exists TSAb (189%) or TSBAb (66%) without TBII activity, and supported the theory of the importance of the N-terminal region for the epitope of Graves’ TRAb.

To study whether these TSAb activities are specific for antibodies to the N-terminal region, the antibodies to two other regions, one corresponding to the mid region (C-peptide, #153–183) and the other to the TSH-R unique insertion near the transmembrane domain (P-peptide, #322–352), of the extracellular domain were further produced in rabbits, and their activities were also measured. Antibody to the C-peptide, which does not contain TSH-R specific sequence, surprisingly showed very strong TSAb activity (4127% and 2548%), almost comparable to Graves’ TSAb [18]. In addition, one of the antibodies to the P-peptide also had potent TSAb activity (3468%). However, even in this experiment, none of the antibodies had TBII activity. Since these experiments were carried out independently, direct comparison of their TSAb activity seems to be meaningless. But these results suggest that the domains responsible for Graves’ TSAb are not necessarily site-specific, and that TBII positive Graves’ IgG may recognize the secondary or tertiary structure of the receptor.

This type of study may serve to analyze the functional domains of TSH-R, but, to interpret the data obtained from the study of the biological activity of the antibodies to the peptides, it seems to be important that, if the antibody to some specific segment had TSAb activity, it does not mean that the region is the epitope for Graves’ TSAb.

Molecular cloning of TSH-R has revealed that various species of TSH-R are homologous to each other, and clinically, Graves’ TSAb activity has been measured with porcine or rat thyroid cells. Thus, if TSH-R peptides were immunized in rabbits and the antibody has TSAb activity, it is likely that the antibody stimulates the thyroid glands of the immunized rabbits.

To test this possibility, Ohmori et al. produced an antibody to the unique N-terminal region of human TSH-R (N-peptide, #10–38) in 8 rabbits and measured their TSAb activity and serum T3 and T4. Their results showed that the post-immune sera had higher mean T3 and T4 than did the pre-immune sera [19]. Although histological examination has revealed that there exist no destructive or inflammatory changes in the thyroid glands of the animals, the possibility remains that a hyperthyroid state was induced as a result of thyroiditis. Further studies such as thyroidal iodine uptake in the immunized rabbits will there-
fore be needed to confirm the hypothesis that the hyperthyroid state was due to TSAb. However, this is the first report of experimentally induced hyperthyroidism in animals, and may open the way to establish a new treatment trial for Graves' disease.

By immunizing rabbits with a peptide (#12–30), Sakata et al. obtained similar results that their antibody possessed TSAb activity and that potency of TSAb correlated with serum T₃ and T₄ values. They also noted that the antibodies to the peptide (#324–344) have TSBAb activity and their titers also correlated with thyroid hormone levels [20]. Although the antibody to each peptide was raised in only one rabbit, the data showed that the immunization of rabbits with TSH-R peptide produces a change in the serum hormone concentration.

Since TSH-R has a much larger extracellular domain than that of α- or β-adrenergic receptor [21], it is natural to presume that the functional region for TSH or TRAb binding site(s) is located in this extracellular component. And indeed all the above data indicate that part of the extracellular domain is involved in TSH or TRAb binding. But the extracellular loops of the membrane spanning region of the α- or β-adrenergic receptor form the entire ligand-binding pocket, and catecholamines are capable of binding even to receptors lacking the extracellular domains [22]. TSH-R has three putative extracellular loops in the membrane spanning domain [4] and the structure is very similar to that of the catecholamine receptor. If so, what is the role of the extracellular loops of TSH-R? The study of the biological activity of TSH-R peptide-antibody provided a clue to this.

Endo et al. developed rabbit antibodies to the peptides corresponding to the first (E₁, #459–478), second (E₂, #542–561) and third (E₃, #630–633) extracellular loops of the human TSH-R, and measured their TSH-R antibody activities. They found that anti-E₂ and anti-E₃ antibodies possessed strong TSAb activity, and their values were over 1000%. The results suggested that although the major concerns as to the ligand binding sites were focused on the extracellular domains, the extracellular loop regions also might play a role in ligand binding and their signal transduction [23]. In addition, the data also suggest that the regions might be one of the candidates for the epitope to TSAb in patients' with Graves' disease. In fact, Nagy et al. found that the binding activity of Graves' IgG was higher than that of the normal control IgG to the peptide corresponding to the second extracellular loop [24].

Application of TSH-R peptide and their antibodies to the study of autoimmune thyroid disease

At present, TBII, TSAb and TSBAb activity is measured by radioreceptor assay or with porcine thyroid membrane in a bioassay with cultured thyroid cells. If a specific segment is responsible for the binding of Graves' TRAb to the receptor, it is ideal to detect them by ELISA in diagnosing autoimmune thyroid disease. Recently Nagy et al. [24] and Ikeda et al. [25] have tried to establish an ELISA system for detecting Graves' IgG by coating the synthetic peptides on ELISA plate.

Nagy et al. synthesized 27 peptides, and carried out systematic screening of IgG interaction with them. As a result, they obtained positive results with peptide #331–350 and peptide EC-2 (2nd extracellular loop).

Ikeda et al. also established a system with N peptide (#12–30), C peptide (#153–183), P₁ peptide (#379–398) and P₃ peptide (#340–352). They found that all of the peptides investigated were recognized by sera from patients with Graves' disease, and the individual sera recognized different numbers and combinations of these peptides. But, as pointed out by Nagy et al., there were many similarities between the binding pattern of Graves' IgG and the normal control. Therefore, for interpretation and comparison of the data, it seems to be important to keep in mind that each synthetic peptide possesses its own physicochemical properties which differ from the whole TSH-R molecule.

Apart from the search for TRAb binding sites on TSH-R, another most useful application of the peptide or its antibody is the analysis of the structure of TSH-R by specific antibody or the search for TSH-R-immunoreactivity with it.

With the antibody to the peptide corresponding to the N-terminus of human TSH-R, Murakami et al. searched for certain components of TSH-R in human peripheral blood and identified a TSH-R-immunoreactive substance. Its molecular weight is about 60 KDa and the amount of it was significant-
ly greater in Graves' plasma than those in normal subjects or in Hashimoto's thyroiditis [26]. With the antibody to the N-terminal peptide, Endo et al. clarified the subunit structure of the TSH-R [27]. Before this study, the model most widely used for TSH-R was a heterodimer linked by disulfide bonds [1]. However, their antibody clearly showed by Western blot analysis that TSH-R is composed of a single subunit with a molecular weight of 104 kDa.

Recently, with the same antibody, they also reported that TSH-R, though it has been thought to be a thyroid-specific protein, exists even in the rat retro-orbital tissues and suggested that TSH-R might be the antigen in the pathogenesis of exophthalmos in Graves' disease [28].

At present, as to the binding site(s) for TSH or TRAb on TSH-R, the results obtained from the experiments with synthetic peptides of TSH-R do not always coincide with each other. However, this is not the case in the studies with TSH-R peptides. When the issue was analyzed with deleted TSH-R or TSH-LH/CG chimera receptor, there were also contradictory results. With deleted human TSH-R, Wadsworth et al. reported that TSAb from patients with Graves' disease loses its response to the receptor lacking N-terminal unique segment (#19–44) but fully retained its response to the receptor lacking an amino acid segment #298–347 [29]. With TSH/LH receptor chimera, Nagayama et al. [30] have revealed that amino acids #152–241 contribute to TSAb binding to the receptor. Kosugi et al. have used deleted rat TSH-R in identifying threonine (#21) as a TSAb binding site and tyrosine (#366) or sequence #276–287 as a TSBAb interacting site [31].

All these discrepancies might be due to the following reasons: 1) there are few data on the tertiary structure of TSH-R and there is little conformational information about it. It therefore remains unclear whether the synthetic peptide employed retained its original structure or not. What is the effect of the modification of the peptide by other residues on its structure? Deletion or substitution of the receptor segment might largely change its conformation and might result in complete loss of its receptor function. Under these conditions, is it possible to estimate the TSH or TRAb binding site on TSH-R? 2) the sera from the patients with Graves' disease were limited in number and their characterization seems to be insufficient. It is evident that Graves' IgGs are heterogeneous and there exist different types of TSAb [32]. What type of sera did the study employ in identifying the epitope? If the epitope(s) for TRAb differs from case to case, it is inevitable that the presumed epitopes are different in each case.

In spite of these limitations in identifying the epitope(s) to TRAb or the functional domain in TSH binding on TSH-R, the studies with TSH-R peptide have provided new information about the role of the extracellular domain, extracellular loops and the subunit structure of TSH-R. In addition, they also revealed new information about the location of TSH-R and TSH-R immunoreactivity.

In the near future, the tertiary structure of TSH-R should be clarified, and the TSH-R peptide which possesses the original structure might be synthesized. With such peptides, it is desirable to help to clarify the pathogenesis of autoimmune thyroid disease.

References

luteinizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for thyrotropin receptor function. Proc Natl Acad Sci USA 88: 902–905.
