Structure-Function Studies of the Human Thyrotropin Receptor

INHIBITION OF BINDING OF LABELED THYROTROPIN (TSH) BY SYNTHETIC HUMAN TSH RECEPTOR PEPTIDES*

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We have probed the hormone binding regions of the entire putative extracellular domain of the human thyrotropin (TSH) receptor (hTSHR) using synthetic peptides. A series of 26 overlapping peptides comprising the complete sequence of the extracellular domain of hTSHr was synthesized. Each peptide (20 amino acid residues each) was tested for its ability to interact with TSH, as evidenced by inhibition of binding of labeled hormone to native, membrane bound TSH receptors. Four of the 26 peptides interacted with labeled TSH and inhibited its binding to thyroid membranes. The most potent of these peptides was 256–275, which inhibited 125I-h-thyrotropin binding with an IC₅₀ of 31.7 ± 1.3 μM. The remaining peptides were 16–35 (351 ± 9.4 μM), 106–125 (282 ± 20.5 μM), and 226–245 (951 ± 245 μM). An additional peptide, 286–305, showed minimal activity, and the remaining 21 peptides showed no activity. Peptides 256–275, 106–125, and 16–35 also inhibited binding of 125I-human chorionic gonadotropin to ovarian membrane receptors, suggesting that those regions of the receptor are involved in binding of a common glycoprotein hormone structure such as the α-subunit. In contrast, peptides 226–245 and 286–305 did not inhibit human chorionic gonadotropin binding, suggesting that these two regions are involved in hormone-specific activity. Of interest is the finding that the latter two peptides are from regions of TSHr that are largely dis-homologous to the lutropin receptor, whereas the former three, with the exception of 16–35, are from regions that are largely homologous between the two receptors.

The data suggest that multiple, discontinuous regions of the extracellular domain of hTSHr are involved in the binding of the hormone. Furthermore, the binding regions are localized to TSHr-specific sequences as well as to regions that are highly homologous to LHr. This suggests that homologous regions of the two receptors are likely to perform similar functions in the interaction with their specific hormone, suggesting that those regions may be involved in binding of the glycoprotein hormone common α-subunit.

Thyrotropin (TSH) exerts its effects on the thyroid cell by binding to a specific receptor found on the cellular surface. The receptor (TSHr) is one member of a family of glycoprotein hormone receptors that also includes the follitropin receptor (FSHr) and the lutropin receptor (LHr). Through molecular cloning the amino acid sequence of each of these receptors has recently been determined (1–3), making possible detailed studies aimed at determining the nature of the interaction of the hormones with their specific receptor. Such studies of TSHr are especially important because of the central role of TSHr in the most common form of hyperthyroidism, Graves' disease. This disorder is characterized by the presence of TSHr autoantibodies that mimic the thyroid-stimulating action of TSH on the thyroid cell (4).

The glycoprotein hormone receptors constitute a family of highly related proteins that are responsible for specific interaction of the pituitary and chorionic hormones (TSH, LH, CG, and FSH) with their target glands. LH and CG share a common receptor. TSH and FSH have distinct receptors unto themselves (5). The receptor proteins share a common structure that has been deduced from their amino acid sequences. Each has a large extracellular domain beginning at the amino terminus that is approximately 350 residues in length for LH/CG receptor and 400 residues for TSHr and FSHr. Each receptor next has seven hydrophobic regions that represent putative transmembrane-spanning α-helical segments. Finally, each receptor ends with a small intracellular tail of approximately 50 residues at the carboxyl terminus (6). Between the seven transmembrane domains lie three small intracellular and three small extracellular loops (EC1, EC2, and EC3) (6). Compared to other G-protein-linked receptors (such as the β-adrenergic receptor), the glycoprotein hormone receptors have a much larger extracellular domain that appears necessary for binding of these complex hormones. It is therefore the extracellular domain toward which structure-function studies have been directed.

Among the approaches currently available for pursuing such structure-function investigations, two techniques have recently enjoyed favor. The first of these methodologies is site-directed mutagenesis. Several reports have appeared demonstrating the utility of the site-directed mutagenesis approach for the study of TSHr (7–11). However, experiments involving loss of binding or biologic activity of mutants in an expression system may be difficult to interpret because of varying levels of protein expression or the uncertain effects of the mutation.

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on protein folding. Furthermore, construction of chimeras consisting of both TSHr and LHr sequences usually involves substitution of large portions of the protein, making identification of specific residues of importance among the large number surveyed difficult.

The second approach for study of structure-function relationships involves the use of small synthetic peptides replicating the sequence of the protein of interest as probes for regions of interaction of the protein with its specific ligand or receptor. These synthetic peptide strategies offer several advantages over the site-directed mutagenesis approach in the study of structure-function relationships of proteins, as well as several disadvantages. With the synthetic peptide approach, one works with uniformly pure and well-characterized substrates, and large amounts can be available. Because of this it is possible to study low affinity interactions. One need not be concerned about expression of the protein with synthetic peptides. Furthermore, because the peptides used are small, one gains information about small segments of the protein of interest rather than about large regions or domains.

The synthetic peptide strategy has been used successfully in our laboratories to map receptor binding sites on the hormone subunits of LH/hCG (12-14) and TSH (15, 16), as recently reviewed by Keutmann (17).

In the current studies we have examined the entire extracellular domain of the human TSH receptor (hTSHr-ECD) as represented by a series of 26 overlapping peptides plus three additional peptides representing the three putative extracellular loops that lie between the transmembrane domains. The data suggest that multiple regions of the TSHr-ECD are involved in hormone binding and that these regions represent both sequences that are unique to TSHr as well as those that are highly homologous to LHr. The data agree well with the limited information previously available regarding the hormone binding domain of TSHr and extend the preexisting structure-function information regarding the receptor.

**MATERIALS AND METHODS**

**Peptides**—As shown in Fig. 1, the peptides consisted of an overlapping series of 26 fragments of the extracellular domain of the human TSH receptor (hTSHr-ECD), the sequences of which were based on that published by Libert et al. (1). Each peptide was 20 amino acids in length (except for the last of the series, 376-394, which was 19 residues), and each overlapped both its neighboring peptides by 5 amino acids. In this manner the entire extracellular domain was represented. In addition to the overlapping series of hTSHr-ECD, three supplementary peptides representing the small extracellular loops that lie between the putative transmembrane domains were also synthesized. These latter peptides are named EC1, EC2, and EC3.

![Sequence of synthetic hTSHr peptides. Underlined regions represent residues of overlap with neighboring peptides. The sequence of hTSHr was taken from Libert et al. (1). EC1, EC2, and EC3 (residues 453-473, 540-559, and 629-639, respectively) refer to the three small extracellular loops that lie between the putative transmembrane domains 2-3, 4-5, and 6-7, respectively. Note that EC3 was synthesized with the addition of Tyr at the carboxyl terminus for future radiolabeling purposes.](image-url)
Inhibition of TSH Binding by TSHr Peptides

**TABLE I**

<table>
<thead>
<tr>
<th>hTSHr peptide</th>
<th>pTSH RRA</th>
<th>hTSH RRA</th>
<th>hCG RRA</th>
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<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>IC₅₀ (µM)</td>
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<tr>
<td>1-16</td>
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<td>16-35</td>
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<td>106-125</td>
<td>283 ± 20.5</td>
<td>332 ± 64.8</td>
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<td>226-245</td>
<td>951 ± 245</td>
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<td>256-275</td>
<td>31.7 ± 1.29</td>
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<td>286-305</td>
<td>1925 ± 264</td>
<td>1145 ± 119</td>
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FIG. 3. Inhibition of 125I-hCG binding to human thyroid membranes by synthetic hTSHr peptides. Peptide numbers refer to those listed in Fig. 1. Data shown represent mean results of 3-6 separate assays. Nonspecific binding was determined by addition of excess unlabeled hCG (100 milliunits/ml). The remaining 24 hTSHr peptides were tested similarly to those shown in the figure, and no activity was found.

**FIG. 4.** Inhibition of 125I-hCG binding to ovarian membranes by synthetic hTSHr peptides. Peptide numbers refer to those listed in Fig. 1. Data shown represent mean results of 3-6 separate assays. Nonspecific binding was determined by addition of excess unlabeled hCG. Not included in this figure are the results of assays of peptides 226-245 and 286-305, both of which showed a B/B₀ of greater than 90% at concentrations up to 1000 µM.

Gradient to 80% acetonitrile in 0.1% trifluoroacetic acid. A single homogeneous peak of each peptide was collected and lyophilized. Following HPLC purification, the structure of each peptide was confirmed by amino acid composition analysis and mass spectroscopy. For the composition determination, the peptides were hydrolyzed with 6 N HCl at 155 °C for 1 h and examined with a Beckman 6300

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respectively (residues 453-473, 540-559, and 629-639). Note that the numbering scheme used for the peptides does not include the signal peptide sequence.

*Peptide Synthesis*—The peptides were synthesized with an ABI 431A automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) using Fmoc chemistry and 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink resin (Ref. 18), purchased from Advanced ChemTech, Louisville, KY). Completed peptides were cleaved from their resin with 95% trifluoroacetic acid (Pierce Chemical Co.) containing 5% scavenger solution (anisole/ethanedithiol/ethylmethylsulfide, 3:1:1 ratio) for 1.5 h at 20 °C, and then precipitated in ice-cold methyl tert-butyl ether, centrifuged, and dried under vacuum.

Each peptide was purified to homogeneity by reversed-phase HPLC in 0.1% trifluoroacetic acid on a Vydac C4 column developed with a gradient to 80% acetonitrile in 0.1% trifluoroacetic acid. A single homogeneous peak of each peptide was collected and lyophilized. Following HPLC purification, the structure of each peptide was confirmed by amino acid composition analysis and mass spectroscopy. For the composition determination, the peptides were hydrolyzed with 6 N HCl at 155 °C for 1 h and examined with a Beckman 6300.
Inhibition of TSH Binding by TSHr Peptides

Four of the 26 overlapping peptides from hTSHr-ECD inhibited binding of labeled TSH to its receptor. The peptide 256–275 was most potent in both the human and the porcine membrane assays, demonstrating an IC50 of 44.7 ± 34.2 and 31.7 ± 1.3 μM, respectively. Peptides 16–35 and 106–125 were less active but similar to each other, with IC50 values as shown in Table I. Of note, peptide 16–35 was relatively more potent in the human RRA than it was in the porcine assay. Peptide 226–245 and 286–295 showed shallow but significant activity, with the former being somewhat more potent than the latter in both assay systems. The 21 peptides representing the remainder of hTSHr-ECD showed no displacement of TSH binding whatever, demonstrating the specificity of the five active peptides.

The three small extracellular loops that lie between the putative transmembrane domains were also synthesized and tested in the RRA. As shown in Table I, none of these peptides inhibited binding of labeled TSH to either human or porcine membranes when tested at concentrations up to 1000 μM.

hCG RRA—Fig. 4 shows the activity of the hTSHr in the hCG RRA. Several of the peptides showed ability to inhibit binding of labeled hCG to receptor: 16–35 (EC50 = 132 ± 13.7 μM), 256–275 (49.2 ± 14.8 μM), and 106–125 (31.2 ± 4.8 μM). However, two of the peptides that were noted to have activity in the TSH RRA (226–245 and 286–305) were not active in the hCG assay when tested up to 1000 μM. Of note is the finding that peptide 106–125 is considerably more potent in the hCG RRA than it was in either of the TSH assay systems.

**DISCUSSION**

Employing a comprehensive peptide strategy, we have identified four regions of the human thyrotropin receptor extracellular domain that interact with TSH in a radio-receptor assay. These regions were 16–35, 106–125, 226–245, and 256–275. Of these 256–275 was most potent. A fifth region (286–305) showed minimal but detectable activity as well, but because the activity was indeed minimal, its importance is in question.

Several recent publications have reported synthetic peptide studies of TSHr (25–29). These previous experiments have studied only selected portions of the TSHr-ECD, chosen because secondary structure prediction analysis of the sequence indicated antigenic potential or possible turn structure. Some regions were studied because they represented some regions of the TSHr-ECD in its entirety. Therefore, we elected to study the TSHr-ECD in its entirety.

In the current studies, we examined the entire hTSHr-ECD using a series of overlapping peptides. The results suggest that at least four or five regions of the ECD are involved in hormone binding. Two of the peptide sequences represent regions that are largely or entirely unique to TSHr when compared to LHR and FSHr. 16–35 and 286–305 (see Fig. 6).
Inhibition of TSH Binding by TSHr Peptides

These sequences represent insertions into TSHr that have no homolog in LHr when compared by homology searching. The cross-reactivity studies, however, suggest that only one of these two regions is involved in hormone specificity, because the peptide 16–35 inhibited binding of both TSH and hCG to their respective receptors, whereas 286–305 was active only in the peptide 16–35. Hormone binding activity of this region of the receptor has been shown previously by both site-directed mutagenesis (8) and the synthetic peptide approach (25, 29). The 8-residue sequence is entirely TSHr-specific and appears as an insertion when compared to the sequence of LHr. FSHr does contain the peptide 16–35 further supports the importance of this region of the receptor in hormone binding and demonstrates agreement of our results with those of other laboratories.

A study similar to the current experiments but using the LH/CG receptor was recently reported (30). The findings were similar to those we report here with TSHr and are compared to the results of the current studies in Fig. 6. Several synthetic peptides from various regions of the LH receptor interacted with the hormone. Active regions of LHr were placed in regions of the receptor almost identical to those we report here with TSHr and are therefore considered insertions. Shaded boxes indicate residues within active synthetic peptides from the current study. Hashed boxes indicate residues from active regions of LHr as recently reported by Roche et al. (30).

Comparison of the two receptor sequences for homology comparison of the ECD of TSHr and LHr was performed using the program BESTFIT from the University of Wisconsin Genetics Computer Group package of sequence analysis software running on a DEC VAX 8600 (31). A vertical line (|) between residues indicates identity between the two sequences; a colon (: ) indicates highly similar residues; a period (.) indicates similar residues; no symbol represents dissimilar residues. Gaps in the sequence of LHr define the regions of TSHr that have no homolog within LHr and are therefore considered insertions.
Inhibition of TSH Binding by TSHr Peptides

35, 226–235, and 286–305) would, therefore, contain regions that bind to β-TSH. However, it is possible that residues from both the α- and β-subunits interact with a common segment or region of TSHr.

Our studies of the cross-reactivity of the hTSHr peptides in the hCG RRA are a means of directly examining the issue of common glycoprotein hormone binding versus receptor-specific binding. As predicted by the above homology examination, we found that peptides 106–125 and 256–275 showed binding activity in both the TSH and hCG RRA, suggesting that they are involved in binding of a common hormone structure such as the α-subunit. The data also demonstrate that peptides 226–235 and 286–305 showed binding only in the TSH RRA, suggesting that those regions are involved in hormone-specific binding, as again predicted by the homology examination above. Peptide 16–35 was active in both assay systems, suggesting that this region is important in interaction of both TSH and hCG with the respective receptor even though it contains the S-residue insertion in TSHr noted above. Of interest, the LHR peptide representing the same region of that receptor showed a similar ability to inhibit binding of hCG to its receptor, supporting the common activity of this region in hormone interaction.

REFERENCES