Inhibition of Thyrotropin Binding to Receptor by Synthetic Human Thyrotropin β Peptides*

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In order to study the structure and function relationships of the thyrotropin (TSH)-specific β-subunit, we produced 11 synthetic overlapping peptides containing the entire 112-amino acid sequence of human β-TSH and tested them for activity in TSH radioreceptor assay using both human and porcine thyroid membranes. Synthetic peptides representing four regions of the β-subunit demonstrated the ability to inhibit binding of 125I-bovine TSH to crude thyroid membranes. The peptide representing the -COOH terminus of the subunit (β101-112) possessed highest binding activity, inhibiting binding of labeled TSH with an EC50 of 80 μM. The remaining active peptides were: β71-85 (104 μM), β81-45 (186 μM), β41-55 (242 μM), and β1-15 (331 μM). Specificity of the binding activity was shown by the inability of the peptides representing the remainder of the subunit to inhibit binding of labeled TSH at concentrations two peptides by 5 residues as shown in Fig. 2. Each peptide consisted of 15 amino acid residues and overlapped its adjacent two peptides by 5 residues as shown in Fig. 1.

The synthetic peptide strategy was employed in order to reduce fragmentation of active regions. Each peptide consisted of 15 amino acid residues and overlapped its adjacent two peptides by 5 residues as shown in Fig. 2.

The peptides were synthesized by standard solid phase techniques (6, 7) using an automated Applied Biosystems 430A peptide synthesizer on p-methylbenzhydrylamine (copolymerstyrene) resin. Each residue after the first 5 was subjected to two coupling cycles as were all Arg, Asn, and Gin residues to ensure completion of the coupling reaction. Completed peptides were removed from the resin by hydrolysis with liquid hydrogen fluoride at 0 °C for 1.25 h and then purified by column chromatography (Sephadex G-25, Pharmacia LKB Biotechnology Inc.) in 1 M acetic acid and/or reversed phase HPLC. Analytical HPLC using a 220 x 2.1-mm C-18 column (Brownlee Labs, Santa Clara, CA) showed a single major peak for each peptide.

Thyrotropin (TSH) exerts its effects by interaction with specific surface receptors found on the plasma membrane of thyroid follicular cells. Although the amino acid sequence of TSH and the structurally similar glycoprotein hormones follicitropin, lutropin (LH), and choriotropin have been known for several years (1), description of the three-dimensional structure of the hormones and the specific hormone regions responsible for receptor interaction and exertion of biologic activity remains limited.

The glycoprotein hormones are heterodimers consisting of a common α-subunit, the sequence of which is identical between the four hormones, and a β-subunit, the sequence of which is hormone specific. Previously, using a comprehensive synthetic peptide approach, we identified two hormone sites within the common α-subunit (α26-46 and α81-92) that interact with TSH receptors on human and rat thyroid membranes (2) and with the LH-hCG receptor on rat ovarian membranes (3). These synthetic peptides proved to be inhibitors of TSH bioactivity, and the former (α26-46) was shown to inhibit the bioactivity of TSH receptor autoantibodies (4) from patients with Graves' hyperthyroidism. However, the affinity of these synthetic peptides for receptor was quite low (10- to 10- M) suggesting that multiple hormone sites were involved in receptor interaction.

Because the α-subunit is shared between all the glycoprotein hormones, the β-subunit must, in some as yet unknown fashion, confer hormone-receptor specificity (1, 5). We herein describe studies in which we have extended the synthetic peptide approach to β-TSH. The studies support the hypothesis of interaction of both α-TSH and β-TSH with the TSH receptor. Further, they suggest that the interaction of this glycoprotein hormone with its receptor is multifaceted, with the overall affinity of hormone-receptor interaction resulting from the simultaneous effort of several hormone binding sites.

MATERIALS AND METHODS

Peptide Synthesis—Eleven synthetic peptides were assembled representing the entire 112-amino acid sequence of the β-subunit of human TSH. As shown in Fig. 1, an overlapping strategy was employed in order to reduce fragmentation of active regions. Each peptide consisted of 15 amino acid residues and overlapped its adjacent two peptides by 5 residues as shown in Fig. 2.

The peptides were synthesized by standard solid phase techniques (6, 7) using an automated Applied Biosystems 430A peptide synthesizer on p-methylbenzhydrylamine (copolymerstyrene) resin. Each residue after the first 5 was subjected to two coupling cycles as were all Arg, Asn, and Gin residues to ensure completion of the coupling reaction. Completed peptides were removed from the resin by hydrolysis with liquid hydrogen fluoride at 0 °C for 1.25 h and then purified by column chromatography (Sephadex G-25, Pharmacia LKB Biotechnology Inc.) in 1 M acetic acid and/or reversed phase HPLC. Analytical HPLC using a 220 x 2.1-mm C-18 column (Brownlee Labs, Santa Clara, CA) showed a single major peak for each peptide.

Amino acid composition of the peptides was confirmed by subjecting each to acid hydrolysis followed by precolumn derivatization with phenylisothiocyanate as described by Hennrikson and Meredith (8) and as modified as noted in the Waters Pico-Tag system. The derivatized samples were analyzed by HPLC utilizing the Waters Pico-Tag column, Beckman 114 pumps, and a Beckman 160 fixed wavelength detector. The data were collected and integrated using an IBM PS/2 model 50Z with the Beckman System Gold chromatography software. The phenylisothiocyanate and amino acid standards were purchased from Pierce Chemical Co. All solvents were HPLC grade. If discrepancies were suspected after composition analysis, the peptide sequence was confirmed by gas-phase microsequencing using
an Applied Biosystems 470A protein sequenator employing the OC8PTH programs provided by the manufacturer.

Membrane Preparation—Thyroid tissue from patients with Graves' disease was obtained at surgery and promptly frozen and maintained at -70 °C. Crude membrane preparations were prepared from the frozen tissue as previously described (6). Briefly, the tissue was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), and the 2000 × g fraction was collected by centrifugation, washed, and resuspended in 40 mM Tris, 200 mM sucrose, pH 7.4. Membrane preparations were maintained at -70 °C until assay.

Frozen porcine thyroid glands were purchased (Pel-Freeze Biologicals, Rogers, AK) and prepared using the same procedures as for the human glands.

TSH Radioreceptor Assay (RRA) utilized for the studies has been well described previously (2, 9). Briefly, for either human or porcine thyroid RRA the incubation mixture consisted of the following: 10 ng eq of crude thyroid membrane preparation (weight, 0.125 ng of [125I]iodo-bTSH (approximately 20,000 cpm), 0.2% Triton X-100, and the noted amounts of synthetic peptides or unlabeled bTSH in 0.5 ml of 40 mM Tris, 0.1% bovine serum albumin, pH 7.4. Bound label was separated from free after 2 h of incubation at 25 °C by addition of 1 ml of 30% polyethylene glycol (J. T. Baker Chemical Co.) 6000 in 1 M NaCl and centrifugation. The resulting pellet was counted in a y counter. Nonspecific binding was determined by the addition of 1 ml of 30% polyethylene glycol (J. T. Baker Chemical Co.) 6000 in 1 M NaCl and centrifugation as above.

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EGF Radioreceptor Assay—The EGF RRA was a modification of that described by Humphries et al. (10). Purified murine EGF (a gift of Dr. N.-S. Jiang, Mayo Clinic) was radioiodinated by a chloramine-T technique to a specific activity of 65 &i/fig. The incubation mixture was determined by the addition of 0.5 pg of unlabeled EGF. Bound label was separated from free by precipitation with polyethylene glycol and centrifugation as above.

RESULTS

Human Thyroid RRA—Fig. 3 and Table I show the results of the human thyroid radioreceptor assays. Four regions of the human bTSH subunit were represented by five active peptides. The highest activity was found in the peptide representing the COOH terminus of the subunit bTSH-112 which inhibited [125I]iodo-bTSH binding with an EC50 of 80 ± 9 &g.

Two peptides representing the region of the subunit between residues 71 and 95 were also active, bTSH-81-95 with an EC50 of 104 ± 15 &g and bTSH-91-95 with an EC50 of 1198 ± 139 &g. Because these two peptides overlap each other and because both show activity, the active region might be assumed to involve the region of overlap (residues 81-85) and, perhaps, extend somewhat toward the amino end of the region in order to explain the higher activity of the more proximal peptide bTSH-81-95.

Activity was also observed in the two peptides bTSH-104-112 and bTSH-81-95. Cysteine residues at 31 and 52 of bTSH correspond to those at 38 and 57 of bLH and hCG (1) so that this region corresponds to the "intercysteine loop" region of bLH and bCG described by Kruttman et al. (11). Synthetic peptides representing the sequence of the region possessing binding.

**TABLE I**

Inhibition of TSH binding to thyroid membrane homogenates by synthetic βTSH peptides

Values represent mean ± S.E. of at least three separate assays. EC50 represents the concentration of peptide or hormone needed to inhibit binding of [125I]iodo-bTSH to the respective membrane fraction by 50%.

| Peptide                        | Human EC50 μM | Porcine EC50 μM |
|--------------------------------|--------------|-----------------|
| 1-11                          | μM           |                 |
| 1-15                           | 931 ± 45     | 1159 ± 401      |
| 1-25                           | >1000        | 186 ± 28        |
| 1-35                           | >1000        | >1000           |
| 1-45                           | 186 ± 23     | 200 ± 26        |
| 1-55                           | 242 ± 58     | 216 ± 88        |
| 1-65                           | >1000        | >1000           |
| 1-75                           | >1000        | >1000           |
| 1-85                           | 104 ± 15     | 137 + 14        |
| 1-95                           | 1196 ± 139   | >1000           |
| 1-10                           | >1000        | >1000           |
| 1-112                          | >1000        | >1000           |
| 1-112                          | 80 ± 9       | 106 ± 8.7       |

bTSH (pm) 540 ± 0.04 114 ± 0.01
βTSH Peptides Inhibit TSH Binding

activity in the hCG-LH radioreceptor assay and stimulated testoster- one production in Leydig cell bioassay. The activity of this region in the TSH RRA suggests that the intercysteine loop region may be important for receptor interaction of all the glycoprotein hormones. However, the βTSH sequences were significantly less potent in inhibition of TSH binding (186 ± 25 and 242 ± 59 μM for βTSH1-45 and βTSH41-55, respectively) than was the βLH sequence in the hCG-LH RRA (20.3 μM) (11) suggesting that this region may be less important for TSH binding than it is for the gonadotropins.

Finally, activity was noted in the peptide representing the amino terminus of βTSH, β1-15 which inhibited binding of the label with an EC₅₀ of 331 ± 45 μM.

The peptides representing the remainder of the βTSH sequence possessed no ability to inhibit hormone-receptor interaction, indicating that the effect of the active peptides is sequence specific.

In order to determine if the inhibition of binding of labeled TSH might be mediated by dissociation of the subunits of the hormone by the peptide, we performed preincubation experiments in which the active peptides were incubated for 1 h with labeled TSH prior to the addition of the membrane preparation. No increase or enhancement of activity was noted in these experiments, suggesting that the peptides did not interact directly with the label. In additional experiments, labeled TSH was incubated with the two most active peptides (βTSH110-112 and βTSH71-85) under the same conditions as the RRA but without thyroid membranes. The mixture was then examined by Sephadex G-100 (Pharmacia LKB Biotechnology Inc.) chromatography. The 1.6 × 50-cm column was pre-equilibrated with the RRA incubation buffer and calibrated with 125I-labeled TSH and with 125I-Iodo-thyroglobulin. There was no appearance of free α- or β-subunit in these experiments, suggesting that no dissociation of labeled hormone occurred. Thus, it is quite unlikely that the binding inhibition seen was secondary to peptide-induced dissociation of subunits of the radiolabel.

Porcine Thyroid RRA—Fig. 4 shows the activity of the synthetic βTSH peptides in the TSH RRA using porcine thyroid membranes. Four regions of activity similar to those found in the human thyroid RRA were found. Again βTSH110-112 and βTSH71-85 were most potent showing EC₅₀ values of 106 ± 8.7 and 137 ± 14 μM, respectively. The intercysteine loop region was represented by the peptides βTSH31-45 and βTSH41-55 (EC₅₀ of 200 ± 36 and 216 ± 18 μM, respectively). The amino-terminal peptide β1-15 was also active with an EC₅₀ of 1158 ± 431 μM.

The peptide βTSH1-25 showed differential activity in the two TSH RRA preparations. Although it was inactive in the human membrane assay, it showed significant inhibitory activity in the porcine assay with an EC₅₀ of 186 ± 28 μM. This may represent a region of βTSH that is recognized by the porcine TSH receptor but is not recognized by the human receptor. This may also, at least in part, explain the higher binding activity of unlabeled TSH in the porcine RRA versus the human membrane assay (Table I).

As noted in Fig. 4, the remaining peptides had no activity, again demonstrating the sequence specificity. The Ec₅₀ of unlabeled EGF in this assay (252 pm) is quite similar to that of unlabeled TSH in the TSH RRA using the same porcine membranes (114 pm). As shown in the figure, none of the active synthetic βTSH peptides significantly inhibited binding of [125I]iodo-EGF to its thyroid membrane receptor.

DISCUSSION

Current evidence for TSH (13-15) as well as LH-hCG (16) suggests that both the α- and β-subunits interact with the receptor. However, direct studies of the binding activity of the individual subunits have been difficult to perform due to problems related to the apparent very low affinity binding of the isolated subunits and to the detection of this activity in preparations of uncertain purity (6). Thus, in order to study the interaction of βTSH with the TSH receptor we have extended the comprehensive synthetic peptide approach we previously used for the study of the glycoprotein α-subunit (2, 3) and, thereby, prepared and tested overlapping peptides representing the entire sequence of human βTSH.

Four regions of activity within βTSH are represented by synthetic peptides. The most potent activity was noted in the peptide representing the -COOH terminus of the subunit, βTSH110-112. Activity of this region of βTSH has been proposed previously by Cheng et al. (17) using enzymatic modification. Carboxypeptidase treatment of isolated βTSH resulted in reduced receptor binding activity of intact hormone when the modified β-subunit was recombined with α. The authors suggested that the residues Ser¹¹³ and Tyr¹¹⁶ were responsible for the activity. Our synthetic peptide data strongly support the importance of this region. Further, because the synthetic peptide βTSH110-112 had no activity, the active residues would be

![Fig. 4. Porcine thyroid membrane TSH radioreceptor assay](http://www.jbc.org/)

**Fig. 4.** Porcine thyroid membrane TSH radioreceptor assay. See legend for Fig. 3. Assay conditions were identical to those of the human assay except that 10 mg eq of porcine thyroid membranes were utilized as the receptor source.

![Fig. 5. Porcine thyroid membrane epidermal growth factor radioreceptor assay](http://www.jbc.org/)

**Fig. 5.** Porcine thyroid membrane epidermal growth factor radioreceptor assay. 40 mg eq of crude porcine thyroid membranes was incubated with 0.5 ng of [125I]EGF and increasing concentrations of unlabeled EGF or synthetic βTSH peptides. Nonspecific binding was determined by the addition of 0.5 μg of unlabeled EGF. As shown, none of the active βTSH peptides inhibited binding of [125I]EGF to its thyroid membrane receptor.
expected to lie after residue 105, in agreement with the carboxypeptidase findings.

The region between cysteine residues at positions 31 and 52 of \( \beta \)TSH corresponds to the region termed the "intercysteine loop," believed to be a looped region in the native hormone that is formed by disulfide bonding between the cysteines (11). The intercysteine loop region of \( \beta \)LH and \( \beta \)CG (\( \beta \)LH38-57) has been shown, by a similar synthetic peptide approach, to possess binding activity in \( \beta \)LH-hCG radioreceptor assay using ovarian membrane preparations (11). The synthetic peptide \( \beta \)LH38-57 also caused stimulation of testosterone production in the Leydig cell bioassay, further indicating its role in lutropin-receptor interaction. These data support our finding of activity in the peptides representing the intercysteine loop region of \( \beta \)TSH. However, the activity noted in the two peptides \( \beta \)31-45 and \( \beta \)41-55 was significantly lower than that noted for the \( \beta \)LH peptides in the previous studies, suggesting that the region, although still possessing binding activity for TSH, may not be as important in the overall activity of the intact hormone as it appears to be for \( \beta \)LH-hCG.

Activity of the amino terminus of \( \beta \)TSH has not been previously reported. However, chemical modification studies have suggested activity for the amino terminus of \( \beta \)LH (1); thus activity in this region of \( \beta \)TSH should not be surprising. The amino terminus of \( \beta \)TSH has not proved susceptible to similar chemical modification; thus the synthetic peptide approach has demonstrated activity in this region where more prototypic studies failed.

The peptide \( \beta \)71-85 was second only to the \(-\text{COOH-} \)terminal peptide in its potency in the TSH RRA. Activity in this region has not been previously alleged for \( \beta \)TSH, as the region is relatively devoid of residues susceptible to chemical modification, and those residues which might be susceptible have not been proven to be so in the whole hormone (1, 18). This further confirms the utility of the synthetic peptide approach for study of ligand-receptor interaction.

Several authors have noted higher TSH binding activity of porcine thyroid membranes as compared with human (10, 19). The current studies have shown that the region 11-25 of the \( \beta \)TSH subunit has significantly greater activity when tested with porcine receptor than with human receptor, suggesting that the binding activity of this region may be responsible, at least in part, for the higher activity of the porcine receptor. In addition, reduced activity of \( \beta \)1-15 in the porcine assay suggests that the binding site of TSH in this region for the porcine receptor is shifted toward the \(-\text{COOH} \) end of 11-25 versus the site recognized by the human receptor. The significance of these findings is increased when one notes the similarity of the activity of the remaining peptides between the two assays (Table I). Additional studies of this region are needed to characterize further which residues are responsible for this apparent species-dependent activity.

As we previously noted with the synthetic \( \alpha \)-subunit peptides (2, 3), the affinity of interaction of the synthetic \( \beta \)-subunit peptides with the receptor is quite low when compared with that of the native hormone (10-8-10-7 M versus 10-30-10-30 M).

If one assumes that four regions of the \( \beta \)-subunit and two regions of the \( \alpha \)-subunit participate in receptor binding, the affinity of each interaction must be rather low; otherwise the overall affinity of the hormone for receptor would greatly exceed that observed. Some negative cooperativity may therefore occur if all these sites interact with the receptor simultaneously. Thus, the noted activity of the peptides suggests that the interaction of TSH with its receptor is multifaceted, not only involving each subunit but also multiple sites within each subunit, the final affinity thereby resulting from a simultaneous cooperative contribution of a combination of these sites.

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