Synthetic Analogs of the Carboxyl-Terminus of β-Thyrotropin: The Importance of Basic Amino Acids in Receptor Binding Activity†

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ABSTRACT: Previously, using a synthetic peptide strategy, we determined that four distinct regions of human β-thyrotropin (βTSH) were responsible for interaction of TSH with the TSH receptor. The most potent of these four regions was the carboxyl-terminus of the subunit, represented by the peptide sequence β101–112, which inhibited binding of radiolabeled βTSH to receptor in radioreceptor assay with an IC_{50} of approximately 100 μM. In the current studies, we systematically substituted the native amino acids in region β101–112 with alanine, and we have determined which residues within this span are important to the binding activity of TSH to its receptor. Substitution of Lys^{101}, Asn^{103}, Tyr^{104}, Cys^{105}, Lys^{107}, and Lys^{110} with alanine each caused a significant fall in activity as compared to the native sequence, whereas substitution at the remaining positions had little or no effect. Because three of these residues are positively charged at physiologic pH, we hypothesized that this charge may be important to the binding activity of the sequence. We modified the charge characteristics of the region by synthesizing two series of analogs in which the residues identified in the alanine substitution studies were substituted with Arg, D-Lys, and D-Arg at each position. In addition, a series of analogs containing basic residues, either added to or substituted for nonbasic residues in the sequence β101–112, was synthesized. Substitution of Arg, D-Lys, and D-Arg for Lys^{101}, Lys^{107}, and Lys^{110} had little effect on activity; however, inclusion of additional basic residues in the β101–112 sequence significantly enhanced the inhibitory activity of the region. Substitution of Ala, Ser, Lys or D-Lys for Cys^{105} resulted in marked reduction in activity. In order to determine if the activity of the region was sequence specific or, rather, due to the amino acid composition of the region, we reversed and scrambled the amino acid residues in an additional series of peptides. The peptide with reversed sequence as well as four peptides with scrambled sequence possessed equal activity to the native peptide, suggesting that the amino acid composition and the net charge of the region is more important than its specific sequence. We concluded that a net positive charge of region 101–112 of βTSH is an important factor in the inhibitory activity of peptides representing this portion of the hormone. Enhancement of the charge, by addition of basic residues, may increase the potency of interaction of TSH with its receptor. However, positive charge is not all important, as removal of Cys^{105} results in marked loss in activity even though the resulting peptide may have overall significantly greater charge.

Thyrotropin (TSH) is a member of the glycoprotein hormone family that also includes lutropin (LH), follitropin (FSH), and choriogonadotropin (CG). These proteins are heterodimers consisting of an α-subunit, which is identical in all four hormones within a species, and a hormone-specific β-subunit. Because the α-subunit is shared, the β-subunit must confer receptor and tissue specificity upon the intact molecule either as an intrinsic property of β or by inducing a unique conformation upon α. Prior work has shown, however, that both subunits interact with the receptor (Pierce & Parsons, 1981; Ryan et al., 1988). In agreement with this hypothesis, we have previously demonstrated, using a synthetic peptide strategy, that multiple regions from both the α- (Morris et al., 1988a,b) and β- (Morris et al., 1990) subunits of human TSH have binding activity with the TSH receptor.

One potential utility of structure–function information of TSH is the application of that knowledge in the production of synthetic peptide analogs and antagonists of the hormone. In order to make high potency synthetic peptide analogs, however, it will be necessary to be as efficient as possible at selecting sequences within the hormone that are involved in receptor interaction. Because the regions of activity we previously noted were fairly broad (i.e., 15–20 residues each), we have begun a series of studies aimed at determining which specific residues within these regions are critical to the observed activity.

In the glycoprotein hormone α-subunit, the region of highest receptor binding activity lies between residues 26 and 46. By utilizing peptide analogs, we demonstrated that within this sequence were two smaller regions of binding activity, α31–35 and α42–45 (Leinung et al., 1991). In βTSH, we previously identified and reported four distinct regions of activity. The two most active regions were the sequences β101–112 (the -COOH terminus of βTSH) and β31–52 [the "intercysteine loop" region of LH and hCG as described by Keutmann et al. (1988, 1987)]. In the current series of experiments, we focus on the carboxyl-terminus of βTSH and apply a similar substitution and synthetic analog strategy to the study of its structure and function relationships.

MATERIALS AND METHODS

Peptide Synthesis. Synthetic peptide analogs comprising batches 1–3 were synthesized by the manual "tea bag" method of peptide synthesis (Houghten, 1985; Houghten et al., 1986;

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Table I: Schedule for "Tea Bag" Manual Solid-Phase Peptide Synthesis[a]

step	procedure	time
1	deprotection, 20% piperidine in DMF, perform twice	15 min
2	wash with DMF, perform three times	10 min
3	wash with NMP, perform twice	10 min
4	wash with NMP	30 min
5	couple with 30 mM Fmoc-amino acid-OPfp, 30 mM HOBt, in NMP	16 h
6	wash with NMP, perform twice	10 min
7	wash with DMF, perform three times	10 min

[a] Abbreviations used are as follows: NMP, 1-methyl-2-pyrrolidinone; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; OPfp, pentafluorophenyl ester; HOBt, 1-hydroxybenzotriazole.

		101				105					110		112
1	Native	Lys	Thr	Asn	Tyr	Cys	Thr	Lys	Pro	Gln	Lys	Ser	Tyr
2	Ala^{101}	Ala	-	-	-	-	-	-	-	-	-	-	-
3	Ala^{102}	-	Ala	-	-	-	-	-	-	-	-	-	-
4	Ala^{103}	-	-	Ala	-	-	-	-	-	-	-	-	-
5	Ala^{104}	-	-	-	Ala	-	-	-	-	-	-	-	-
6	Ala^{105}	-	-	-	-	Ala	-	-	-	-	-	-	-
7	Ala^{106}	-	-	-	-	-	Ala	-	-	-	-	-	-
8	Ala^{107}	-	-	-	-	-	-	Ala	-	-	-	-	-
9	Ala^{108}	-	-	-	-	-	-	-	Ala	-	-	-	-
10	Ala^{109}	-	-	-	-	-	-	-	-	Ala	-	-	-
11	Ala^{110}	-	-	-	-	-	-	-	-	-	Ala	-	-
12	Ala^{111}	-	-	-	-	-	-	-	-	-	-	Ala	-
13	Ala^{112}	-	-	-	-	-	-	-	-	-	-	-	Ala

FIGURE 1: Synthetic strategy for alanine substitution analogs (batch 1). Peptide 1 contains the native βTSH-101–112 sequence. Hyphens indicate identity of the residue at that position with peptide 1.

		101				105				110		112 (113)
1	Native	Lys	Thr	Asn	Tyr	Cys	Thr	Lys	Pro	Gln	Lys	Ser	Tyr
2	Arg^{101}	Arg	-	-	-	-	-	-	-	-	-	-	-
3	Arg^{107}	-	-	-	-	-	-	Arg	-	-	-	-	-
4	Arg^{110}	-	-	-	-	-	-	-	-	-	Arg	-	-
5	Lys^{113}	-	-	-	-	-	-	-	-	-	-	-	- Lys
6	Lys^{103}	-	-	Lys	-	-	-	-	-	-	-	-	-
7	D-Lys^{101}	D-Lys	-	-	-	-	-	-	-	-	-	-	-
8	D-Lys^{107}	-	-	-	-	-	-	D-Lys	-	-	-	-	-
9	D-Lys^{110}	-	-	-	-	-	-	-	-	-	D-Lys	-	-
10	Thr^{101},Lys^{102}	Thr	Lys	-	-	-	-	-	-	-	-	-	-
11	Lys^{106},Thr^{107}	-	-	-	-	-	Lys	Thr	-	-	-	-	-
12	Ser^{110},Lys^{111}	-	-	-	-	-	-	-	-	-	Ser	Lys	-
13	Ser^{105}	-	-	-	-	Ser	-	-	-	-	-	-	-
14	Phe^{104}	-	-	-	Phe	-	-	-	-	-	-	-	-
15	101-110	-	-	-	-	-	-	-	-	-	-	xx	xx
16	D-Arg^{107}	-	-	-	-	-	-	D-Arg	-	-	-	-	-
17	D-Arg^{110}	-	-	-	-	-	-	-	-	-	D-Arg	-	-
18	D-Arg^{101}	D-Arg	-	-	-	-	-	-	-	-	-	-	-

FIGURE 2: Synthetic strategy for batch 2 analogs. Peptide 1 contains the native β101–112 sequence. Hyphens indicate identity of the residue at that position with the native sequence. Substitutions are noted by amino acid abbreviations in three letter code at the given positions.

Reed et al., 1991; Stewart & Young, 1984). Each tea bag was loaded with 0.05 mmol of 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink) resin (Rink, 1987). The synthetic schedule is shown in Table I. The sequences of the three batches of βTSH-101–112 peptides made by this technique are shown in Figures 1–3. Batch 1 consisted of 13 peptides, the first containing the native βTSH-101–112 sequence and the remaining 12 containing the native sequence except for substitution of a single residue with alanine, as demonstrated in Figure 1. Batches 2 and 3 consisted of a total of 34 peptide analogs of the β101–112 sequence as shown in Figures 2 and 3, respectively.

Peptide analogs of batch 4 were synthesized on an automated multiple peptide synthesizer (Advanced Chemtech ACT-350, Louisville, KY) using Fmoc chemistry and software provided by the manufacturer. The sequences and synthetic stratey for this batch of analogs are shown in Figure 4.

Completed peptides were cleaved from the resin with 95% TFA (Pierce, Rockford, IL) and 5% scavenger solution

		101				105					110		112	115					
1	Native	Lys	Thr	Asn	Tyr	Cys	Thr	Lys	Pro	Gln	Lys	Ser	Tyr						
2	101-118	-	-	-	-	-	-	-	-	-	-	-	-	Leu	Val	Gly	Phe	Ser	Val
3	D-Lys^{101,107,110}	D-Lys	-	-	-	-	-	D-Lys	-	-	D-Lys	-	-						
4	D-Lys^{101,107,110,113}	D-Lys	-	-	-	-	-	D-Lys	-	-	D-Lys	-	-	D-Lys					
5	Lys^{105}	-	-	-	-	Lys	-	-	-	-	-	-	-						
6	D-Lys^{105}	-	-	-	-	D-Lys	-	-	-	-	-	-	-						
7	D-Lys^{101,107,110},Gly^{109}	D-Lys	-	-	-	-	-	D-Lys	-	Gly	D-Lys	-	-						
8	Lys^{103},Lys^{113}	-	-	Lys	-	-	-	-	-	-	-	-	-	Lys					
9	D-Lys^{103},D-Lys^{113}	-	-	D-Lys	-	-	-	-	-	-	-	-	-	D-Lys					
10	D-Lys^{113}	-	-	-	-	-	-	-	-	-	-	-	-	D-Lys					
11	D-Lys^{103}	-	-	D-Lys	-	-	-	-	-	-	-	-	-						
12	Glu^{109}	-	-	-	-	-	-	-	-	Glu	-	-	-						
13	Gly^{109},D-Lys^{113}	-	-	-	-	-	-	-	-	Gly	-	-	-	D-Lys					
14	Met^{110}	-	-	-	-	-	-	-	-	-	Met	-	-						
15	Orn^{113}	-	-	-	-	-	-	-	-	-	-	-	-	Orn					
16	Orn^{103}	-	-	Orn	-	-	-	-	-	-	-	-	-						
17	Orn^{110}	-	-	-	-	-	-	-	-	-	Orn	-	-						
18	Orn^{103,113}	-	-	Orn	-	-	-	-	-	-	-	-	-	Orn					

FIGURE 3: Synthetic strategy for batch 3 peptides. Analog sequences are noted as described in legends for Figures 1 and 2.

		101				105					110	112	
1	Native	Lys	Thr	Asn	Tyr	Cys	Thr	Lys	Pro	Gln	Lys	Ser	Tyr
2	Ala^{102,3,4,6,8,9,11,12}	-	Ala	Ala	Ala	-	Ala	-	Ala	Ala	-	Ala	Ala
3	Arg^{102,3,6,8,9,11,12}	-	Ala	Ala	-	-	Ala	-	Ala	Ala	-	Ala	Ala
4	Reverse	Tyr	Ser	Lys	Gln	Pro	Lys	Thr	Cys	Tyr	Asn	Thr	Lys
5	Shuffle 1	Lys	Pro	Asn	Lys	Ser	Tyr	Cys	Tyr	Thr	Gln	Thr	Lys
6	Shuffle 2	Gln	Pro	Ser	Lys	Lys	Thr	Tyr	Cys	Lys	Thr	Tyr	Asn
7	Shuffle 3	Lys	Pro	Thr	Gln	Tyr	Asn	Lys	Ser	Thr	Tyr	Lys	Cys
8	Shuffle 4	Lys	Lys	Asn	Lys	Pro	Tyr	Cys	Thr	Gln	Thr	Ser	Tyr

FIGURE 4: Synthetic strategy for batch 4 peptides. Analog sequences are noted as described in legends for Figures 1 and 2.

(anisole/ethanedithiol/ethylmethylsulfide, 3:1:1 ratio) for 1.5 h at 20 °C, then precipitated in ice-cold methyl *tert*-butyl ether, centrifuged, and dried under vacuum.

All peptides were purified by reversed-phase HPLC using a VYDAC C8 column (2.2 cm × 25 cm, VYDAC, Hesperia, CA) in 0.1% TFA/water (buffer A) and a gradient of from 5% to 80% acetonitrile in 0.1% TFA/water (buffer B). A single homogenous peak of each peptide from the HPLC column was collected and lyophilized. In order to confirm their structure, each peptide was subjected to acid hydrolysis with 6 N HCl at 155 °C for 1 h followed by amino acid analysis using a Beckman 6300 automated amino acid analyzer. If discrepancies in composition were suspected, the peptides were analyzed by a Bio-Ion mass spectrometer (Applied Biosystems) and by gas-phase microsequencing on an ABI 470A protein sequencer. In all cases, the synthetic peptide structures agreed with the expected amino acid sequences.

Synthesis resins, amino acid derivatives, HOBt, and tea bag mesh for peptide synthesis were obtained from Advanced Chemtech, Louisville, KY. All solvents for peptide synthesis and HPLC were obtained from EM Science, Gibbstown, NJ. Piperidine and trifluoroacetic acid were purchased from Aldrich, Milwaukee, WI.

TSH Radioreceptor Assays (RRA). The RRA for TSH using crude porcine thyroid membranes and [^{125}I]bTSH has been described previously (Morris et al., 1988b; Takahashi et al., 1978). Within each of the three batches of peptides, the native peptide β101–112 was resynthesized (peptide number 1 in each batch) and used as a control for all assays utilizing peptides from that batch. Thus, the activity of each peptide was compared to that of the native peptide from the synthetic batch in which it was made.

Statistical Analysis. Dose–response curves for each synthetic peptide analog were obtained from three or four data points performed in duplicate for each assay, and an IC_{50} was calculated. The results from three to five separate assays were averaged. Results were expressed as percent activity of each peptide as compared to its appropriate native control. Statistical significance was tested by ANOVA comparing the IC_{50} values of each peptide to those of its native control.

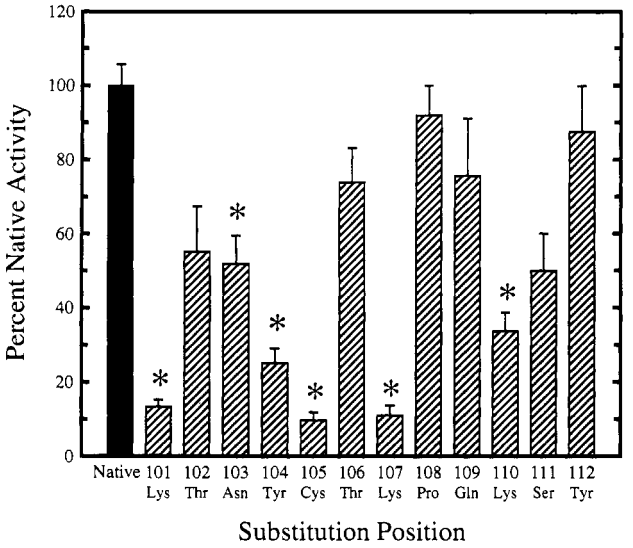

FIGURE 5: Activity of alanine substitution analogs (batch 1) in the TSH RRA. Results of alanine substitution peptides in TSH receptor binding assays shown as their percent activity compared to the native sequence (Native) β101–112. Data shown represent the mean ± SE of 5 separate radioreceptor assays. Sequences of the peptide analogs are shown in Figure 1. An asterisk (*), denotes a confidence interval of $p < 0.05$ when compared to the activity of the native peptide (Native) as determined by ANOVA. The IC_{50} of the native control peptide was 174 ± 11.0 μM.

RESULTS

Alanine Substitution Analogs, Batch 1. This "nested set" of peptides consisted of 13 members, one containing the native β101–112 sequence and 12 others in which each individual residue was substituted systematically with alanine as shown in Figure 1. Figure 5 shows the activity of these peptides in the TSH RRA. Substitution of several specific residues with alanine resulted in a significant reduction of activity. Most significant were residues Lys^{101}, Cys^{105}, and Lys^{107} in which substitution by alanine resulted in reductions of activity to 13.3 ± 1.9, 9.6 ± 2.2, and 10.9 ± 2.7% of native β101–112 respectively. Substitution of Tyr^{104} and Lys^{110} also reduced the activity of these peptides but to a lesser degree (25.1 ± 3.9 and 33.6 ± 5.1%, respectively). Finally, substitution of Asn^{103} with alanine caused a fall to 51.8 ± 7.7% of native ($p < 0.05$). Thus, five residues within the 12 amino acid span of β101–112 are important to the activity of the native sequence, with a sixth residue (Asn^{103}) also playing a role, although its activity is of somewhat lesser importance. The remaining residues allowed substitution with alanine without significantly effecting activity.

101–112 Analogs, Batch 2. Because three of the important residues noted above have basic side chains, we hypothesized that the positive charge, imparted by lysine residues in this region of βTSH, is important in receptor binding. We further hypothesized that increasing the positive charge of this region might enhance binding activity. The analogs in batch 2 were devised to investigate these hypotheses. As shown in Figure 2, the next series analogs consisted of 17 peptides plus the native sequence, which was resynthesized simultaneously for use as a reference control. In order to test our hypotheses, we added basic amino acids to the native sequence (peptides 5 and 6), made substitutions for the existing Lys residues with Arg, D-Lys, or D-Arg, shifted the lysine position by one residue, or substituted some native residues with Phe (for Tyr^{104}) or Ser (for Cys^{105}).

Table II shows the activity of the analogs in batch 2 as compared to that of native β101–112. Substitutions of Arg for Lys (peptides 2–4), D-Lys for Lys (peptides 7–9), and D-Arg for Lys (peptides 16–18) had no effect on activity. In peptides 10–12, we shifted the position of the three Lys residues by one amino acid. This also failed to change the activity. However, inclusion of a fourth basic residue in the sequence at positions 113 or 103 (peptides 5 and 6) increased the activity by approximately 2.5-fold. This result supports our hypothesis of the importance of the positive charge in the activity of β101–112 and, further, suggests that the activity may be enhanced by increasing the charge of the region.

Table II: Activity of Batch 2 Analogs[a]

peptide	% activity	SE
(1) native	100.0	10.50
(2) Arg^{101}	128.1	16.96
(3) Arg^{107}	128.2	17.08
(4) Arg^{110}	108.3	18.01
(5) Lys^{113}	205.6	25.25*
(6) Lys^{103}	252.2	56.63*
(7) D-Lys^{101}	117.7	14.11
(8) D-Lys^{107}	83.4	14.41
(9) D-Lys^{110}	71.2	13.93
(10) Thr^{101},Lys^{102}	82.5	20.03
(11) Lys^{106},Thr^{107}	84.8	15.14
(12) Ser^{110},Lys^{111}	98.8	15.62
(13) Ser^{105}	27.4	2.54*
(14) Phe^{104}	82.8	13.12
(15) 101–110	182.7	21.20*
(16) D-Arg^{107}	126.1	18.04
(17) D-Arg^{110}	104.5	13.95
(18) D-Arg^{101}	160.3	31.70

[a] Activity is expressed as percent of peptide number 1. Data shown represent means and SE of 4–5 separate assays. (*) $p < 0.05$ versus peptide 1 as determined by ANOVA. The IC_{50} value for the native control peptide was 182 ± 77.6 μM.

The importance of Cys^{105} was tested again in analog batch 2 by substituting it with Ser (peptide 13) as shown in Table II. This substitution resulted in a 72.7 ± 2.5% reduction in activity, agreeing with the results of the above experiment regarding the importance of this residue. Finally, peptide 14 of batch 2 demonstrated that the aromatic amino acid Phe can be freely substituted for Tyr^{104} without loss of activity.

101–112 Analogs, Batch 3. This set of peptides was designed to further test the influence of charged residues on the activity of the β101–112 sequence as well as to investigate the influence of some of the "experiments of nature" manifest as interspecies heterogeneity within this region. The sequences are shown in Figure 3, and the activity of the analogs in TSH RRA is shown in Table III.

In peptides 15 and 16 we included the basic amino acid ornithine (Orn) at positions 113 and 103, respectively. As observed in the peptide analogs of batch 2, the introduction of this fourth basic residue into the sequence dramatically increased the activity of these two peptides. Substitution of Orn for Lys^{110} (peptide 17) resulted in no change in activity. However, the addition of a fourth positive charge by substitution of Lys or D-Lys for Cys^{105} resulted in a 90.8 ± 1.8 and 87.5 ± 1.1% *reduction* in activity. Thus, it appears that although a fourth positive charge is capable of increasing the activity of the sequence, this capability is present only in specific positions and/or in the presence of other critical residues. This result also agrees with the Ala substitution data noted above in batches 1 and 2, in that Cys^{105} appears critical for the activity of the peptide.

In several of the peptides of this batch, we introduced both a fourth and a fifth basic residue into the sequence (peptides 8, 9, and 18). Lys or D-Lys at these positions resulted in an approximately 5-fold increase in activity over the native

Table III: Activity of Batch 3 Analogs[a]

peptide	% activity	SE
(1) native	100.0	29.21
(2) 101–118	160.6	48.70
(3) D-Lys^{101,107,110}	57.6	10.77*
(4) D-Lys^{101,107,110,113}	189.4	51.29*
(5) Lys^{105}	12.0	5.16*
(6) D-Lys^{105}	13.3	3.70*
(7) D-Lys^{101,107,110},Gly^{109}	56.9	14.05*
(8) Lys^{103},Lys^{113}	476.4	110.10*
(9) D-Lys^{103},D-Lys^{113}	519.2	129.37*
(10) D-Lys^{113}	185.6	44.75
(11) D-Lys^{103}	232.6	63.92
(12) Glu^{109}	27.9	5.69*
(13) Gly^{109},D-Lys^{113}	206.3	38.58
(14) Met^{110}	101.5	78.93*
(15) Orn^{113}	286.7	64.41*
(16) Orn^{103}	340.7	70.86*
(17) Orn^{110}	118.7	28.43
(18) Orn^{103,113}	753.7	172.78*

[a] Activity is expressed as percent of peptide number 1. Data shown represent means and SE of 4–5 separate assays. (*) $p < 0.05$ versus peptide 1 as determined by ANOVA. The IC_{50} value of the native control peptide was 108 ● 24.4 μM.

Table IV: Activity of Batch 4 Analogs[a]

peptide	% activity	SE
(1) native	101.5	8.77
(2) Ala$^{102-4,6,8,9,11,12}$	63.7	4.64*
(3) Ala^{102,3,6,8,9,11,12}	84.3	7.09
(4) reverse	140.4	7.80*
(5) shuffle 1	138.8	13.67
(6) shuffle 2	151.7	29.57
(7) shuffle 3	126.6	18.23
(8) shuffle 4	132.2	10.79

[a] Activity is expressed as percent of peptide number 1. Data shown represent means and SE of 4–5 separate assays. (*) $p < 0.05$ versus peptide 1 as determined by ANOVA. The IC_{50} value for the native control peptide was 138 ± 11.0 μM.

sequence, and addition of Orn at the same two positions caused a (7.3 ± 0.6)-fold increase in activity (Table III, peptide 18).

The mRNA sequence for human βTSH includes code for six additional residues (Figure 3, peptide 2) that are not found in the protein when purified and sequenced from pituitary extracts (Carr et al., 1987; Hayashizaki et al., 1985; Maurer et al., 1984; Wondisford et al., 1988). We tested the possible importance of these residues in hormone binding (Table III) and found that inclusion of this sequence in the peptide β101–112 did not significantly alter activity. In another experiment of nature involving this region, residue Gln^{109} is altered to Glu in the porcine βTSH subunit (Maghuin-Rogister et al., 1976). The peptide incorporating this variant (peptide 12) had significantly lower activity than the human sequence (27.8 ± 2.2%). This finding adds further support to the hypothesis regarding the importance of positive charge within this region because substituting the acidic residue Glu for the neutral Gln would decrease the positive charge of the resulting peptide.

101–112 Analogs, Batch 4. This series of peptides was designed to confirm the findings from the batch 1 analogs as well as to study the sequence specificity (as opposed to composition specificity) of the activity found in region 101–112 of βTSH. As shown in Figure 4, two peptides were synthesized containing only the residues noted in batch 1 to be essential to the activity of this region, and all the remaining amino acids were replaced with alanine. The results are summarized in Table IV. While a minor reduction in the activity of peptide 2 was noted, peptide 3 (which differed from peptide 2 by the addition of Tyr^{104}) was statistically as active as the native peptide. These findings suggest that the residues noted to be important for receptor interaction in the batch 1 experiments are essentially the only residues necessary to reproduce the native receptor binding activity of region β101–112, as the remaining amino acids can be freely substituted with alanine.

Peptides 4–8 were designed to determine if the activity of β101–112 is sequence specific or related only to the composition of the hormone region, i.e., composition specific. Peptide 4 is the native β101–112 sequence in reverse. Peptides 5–8 are randomly scrambled sequences that contain the same amino acid composition as the native peptide [sequences were scrambled with the program SHUFFLE of the Genetics Computer Group package of sequence analysis software (Devereux et al., 1984)]. As shown in Table IV, each of these peptides possessed activity statistically indistinguishable from the native sequence (peptides 4 and 6 actually had slightly enhanced activities).

DISCUSSION

Using a synthetic analog strategy we have determined that six residues within the carboxyl-terminal region of human βTSH are important in the interaction of the hormone with the TSH receptor. These include Lys^{101}, Asn^{103}, Tyr^{104}, Cys^{105}, Lys^{107}, and Lys^{110}. The three Lys residues and Cys^{105} appear to be of greatest importance, whereas the remaining two residues are somewhat less critical. Of note are the characteristics of the important residues. Three of the five most important amino acids are positively charged (Lys^{101}, Lys^{107}, and Lys^{110}), another is hydrophobic and aromatic (Tyr^{104}), and the last is a cysteine residue (Cys^{105}) involved in disulfide cross-linking [probably to Cys^{19} (Pierce & Parsons, 1981; Ryan et al., 1988)] in the native β-subunit.

Electrostatic interactions are known to be important in TSH–TSHr interactions (Furmaniak & Rees Smith, 1990; Hashim et al., 1986). The pK_a of the TSHr has previously been measured around pH 5 and that of native TSH from 6.5 to 8.7 with receptor-active fractions generally possessing higher isoelectric points (Powell-Jones et al., 1982; Rees Smith, 1971). Thus, the finding that positively charged residues in this sequence are important in the binding activity suggests that the β101–112 region may be involved in this type of interaction. Further support of this hypothesis is given in the current studies by analogs from the last three synthetic experiments. Enhancement of the positive charge of the molecule by addition of a fourth or fifth basic amino acid at positions 103 and 113 increased the activity of the peptide.

It appears, however, that charge is not the only factor providing the activity of this region of βTSH. Addition of a fourth positively charged residue at position 105 did not increase activity. Indeed, substitution of either Lys or D-Lys for Cys^{105} resulted in significant *losses* of activity as compared to the native sequence (Table III, peptides 5 and 6). The isoelectric point of peptides 5 and 6 calculate at 10.38, which is considerably higher than the 9.8 calculated for native βTSH-101–112. This suggests that the positive charge, while important in activity, is not itself sufficient, and speaks for other specific requirements regarding the amino acid composition of the region and against an all important effect of charge.

Further evidence of specificity is seen with substitution of arginine, which at any position, did *not* result in enhancement of activity, whereas ornithine (Orn) substitution did increase activity to a degree similar to that seen with Lys (Table III, peptides 15, 16, and 18). This suggests that the longer and

more complex side chain of Arg may sterically inhibit interaction of the peptide with the receptor, even though it possesses a positive charge at physiologic pH, whereas the simpler and shorter side chains of Lys and Orn allow such interaction.

The results of the experiments with the batch 4 analogs are particularly interesting (Table IV). The data show that the sequence of β101–112 can be reversed or completely randomized without significantly impairing the activity of the peptides. This finding suggests that it is the composition of this region of the hormone (and especially the positive charge within this region) that is responsible for the interaction with the TSH receptor rather than the specific sequence of amino acids. The presence of the positive charge imparted by lysine residues appears critical as does the presence of cysteine, although their relative position within this span is elective. However, as described in the above paragraph, arginine cannot impart the same enhancement of activity as lysine, and ornithine is perhaps superior to lysine in this respect. This suggests that the nature of the charged species, in addition to its presence, is also important.

Cys^{105}, as noted in all three synthetic experiments above, appears critical to the activity of the peptide. This residue, which is absolutely conserved throughout all known species of pituitary glycoprotein hormones, is believed to be cross-linked by disulfide bonding to Cys^{19} of hTSHβ (Ryan et al., 1988). The function of this residue in receptor binding and the reason for its importance are not clear. Reichert et al. (Boniface & Reichert, 1990; Santa-Coloma et al., 1991) have suggested that βFSH possesses thioredoxin-like activity, and through this activity may be involved with disulfide rearrangement of the hormone and receptor, or between the two. Thus, it is conceivable, but not proven, that covalent binding of the hormone–receptor complex could occur through their Cys residues (Santa-Coloma et al., 1991). Our data do not directly address this issue, but the findings do support this type of interaction for Cys^{105}. Along this line, we previously reported that the synthetic peptide βTSH-11–25, which includes Cys^{19}, also contained receptor binding activity (Morris et al., 1990). This finding suggests that the disulfide pair Cys^{19}–Cys^{105} may form a portion of a receptor binding "face" of βTSH. Further studies are required to clarify this question.

The mRNA for human βTSH includes coding sequences for an additional six residues after Tyr^{112} (Carr et al., 1987; Hayashizaki et al., 1985; Maurer et al., 1984; Wondisford et al., 1988), however, the protein as extracted and sequenced from pituitaries does not include these amino acids (Pierce & Parsons, 1981; Weintraub et al., 1985). It has been suggested that some portion of the circulating and biologically active form of the hormone may contain these residues and that they may be important in the activity of the hormone (Wondisford et al., 1988). Inclusion of the residues into the synthetic peptide β101–112 does not significantly effect the activity (Table III, peptide 2), suggesting that the sequence is superfluous to hormone binding. This data does not exclude importance of the residues in hormone bioprocessing, secretion, or activation of receptor; but it does suggest that they are not required for optimum binding of the hormone to its receptor.

In conclusion, our data suggest that both the presence of a positive charge created by multiple lysine residues and the presence of a cysteine residue near the carboxyl-terminus of human βTSH are important in the interaction of that region of the hormone with the TSH receptor. Further, the requirement appears to be largely compositional in nature rather than sequence specific. The findings confirm the importance of charge–charge interactions in the binding of TSH to the TSH receptor and suggest that alterations of that charge may serve to enhance or reduce the activity of future synthetic analogs of human TSH.

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