Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9707–9711, November 1991
Biochemistry

Further characterization of the receptor-binding region of the thyroid-stimulating hormone α subunit: A comprehensive synthetic peptide study of the α-subunit 26–46 sequence

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Communicated by J. Edward Rall, August 8, 1991 (received for review April 12, 1991)

ABSTRACT **Previously, using a synthetic peptide strategy, we determined that the region of the common glycoprotein hormone α subunit between residues 26 and 46 is a site of interaction of the hormone with the thyroid membrane-bound receptor for thyroid-stimulating hormone (TSH). We have undertaken to identify further the specific residues within this 21-amino acid span that are critical in hormone receptor binding. We synthesized three nested sets of peptide, two in which we systematically truncated the amino-terminal region of the sequence and another in which we truncated the carboxyl-terminal region, and we synthesized a fourth nested set in which we systematically substituted alanine for the native residues from the region of highest activity. Each peptide was tested in a TSH radioreceptor assay for its ability to inhibit binding of ^{125}I-labeled bovine TSH to porcine thyroid membranes. Removal, either by truncation or alanine substitution, of several specific residues resulted in a significant reduction in the ability of the sequence to interact with receptor; these residues included Cys^{31}, Cys^{32}, Phe^{33}, Arg^{35}, Arg^{42}, Lys^{44}, and Lys^{45}, suggesting that they are crucial for binding activity. Loss of activity also occurred with substitution for Gly^{30} and Ser^{34}, but the reduction was less pronounced. Amino-terminal truncation of the sequence through Arg^{35} (leaving the α-subunit peptide 36–46) resulted in greater than 98% loss of activity of the sequence. We conclude that two distinct receptor binding regions lie within the α-subunit 26–46 sequence. The first lies between residues Gly^{30} and Arg^{35} and includes Cys^{31}, Cys^{32}, and Phe^{33} as important constituents, and the second region lies between residues Arg^{42} and Lys^{45} and includes Lys^{44} as an important residue and Ser^{43} as a less important component.**

The glycoprotein hormones [thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin (CG)] are heterodimers consisting of a common α subunit and a hormone-specific β subunit (1, 2). Both subunits are required for expression of full biologic activity, but the individual contributions of each to receptor binding and bioactivity remain under active investigation. Although some early reports of crystallization of αTSH (3), αLH, and deglycosylated human CG (hCG) (4, 5) have appeared, the three-dimensional structure of these molecules has not been elucidated. Thus, other methods have been used to investigate the structure–function relationships, such as chemical modification (1, 2) and monoclonal antibody mapping (6–10). Because individual subunits interact with the receptor with low affinity, studies on the interaction of isolated subunits with receptors and studies by chemical modification have been complicated by low-level contamination with native or unreacted hormone or subunits (1). To avoid these problems, we have employed a synthetic peptide strategy to identify the regions of both subunits that interact with the glycoprotein hormone receptors.

We previously reported two regions of the common glycoprotein hormone α subunit that are essential in both TSH (11) and LH/hCG (12) hormone–receptor interaction; these were residues 26–46 and 81–92, denoted α26–46 and α81–92, respectively. The former showed highest activity, and the synthetic peptide representing this sequence inhibited binding of ^{125}I-labeled bovine TSH (^{125}I-bTSH) to human thyroid membranes with an EC_{50} of 11 μM and binding of ^{125}I-labeled hCG (^{125}I-hCG) to ovarian membranes with an EC_{50} of 7.2 μM. Because the region had activity for both LH/hCG and TSH, it was believed to represent a binding site common to all glycoprotein hormones and, as such, is of great importance in structure–function analysis of the family.

In this study, we investigated further this important binding site by synthesizing four nested sets of peptide analogs representing various portions of the α26–46 sequence. The data identify the specific residues among the 21 examined that are involved in hormone–receptor interaction. Further, the data are comparable with previous data for similar peptides tested in hCG radioreceptor assay (13), indicating that the α subunit plays an analogous role in binding of both hormones to their respective and specific receptors.

MATERIALS AND METHODS

Peptide Synthesis. Four distinct nested sets of peptides were synthesized by using standard solid-phase synthetic chemistry (14). Sets 1, 2, and 4 were synthesized by using *tert*-butoxycarbonyl (tBOC)-protected amino acids on an automated peptide synthesizer (Applied Biosystems, ABI 430A) or by using 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids on an ABI 431A synthesizer. The peptides were cleaved from the resin with liquid hydrogen fluoride or 95% trifluoroacetic acid as indicated by the synthetic chemistry. Peptide set 3 was synthesized by using the "tea bag" method of manual peptide synthesis (15, 16) and Fmoc chemistry (13). All protected amino acids and resins used for manual synthesis were purchased from Advanced ChemTech, and those used for automated synthesis were purchased in preloaded cartridges from Applied Biosystems.

Each peptide was purified to homogeneity by reversed-phase HPLC in 0.1% trifluoroacetic acid on a Vydac C_{18} column developed with a gradient to 80% acetonitrile in 0.1% trifluoroacetic acid. Amino acid composition of all peptides was confirmed by subjecting each to acid hydrolysis and precolumn derivatization with phenylisothiocyanate then analysis by HPLC using the Waters Pico-Tag column as

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Abbreviations: TSH, thyroid-stimulating hormone (thyrotropin); LH, luteinizing hormone (lutropin); CG, chorionic gonadotropin; h-, human; b-, bovine; α26–46, etc., residues 26–46 of the glycoprotein hormone α subunit; ANOVA, analysis of variance.

described by Heinrikson and Meredith (17). Some peptides were also subjected to gas-phase microsequencing using an Applied Biosystems 470A automated sequenator when discrepancies were suspected after composition analysis.

Thyroid Membrane Preparation. Porcine thyroid glands were purchased frozen from Pel Freez Biologicals. Crude membrane preparations were made as previously described (18) and stored at −70°C in 8.5% sucrose until use. In the current studies the 10,000 × *g* fraction was employed.

TSH Radioreceptor Assays. Highly purified bTSH (a gift of John Pierce, University of California, Los Angeles) was radioiodinated ($Na^{125}I$, Amersham) by a lactoperoxidase technique to a specific activity of 40–60 μCi/μg (1 Ci = 37 GBq) as previously described (19) then purified by Sephadex G-100 chromatography.

The TSH radioreceptor assay has been described previously (11, 18). The incubation mixture consisted of 10 mg equivalents of crude thyroid membrane preparation, 0.125 ng of ^{125}I-bTSH, 0.2% Triton X-100, and the noted concentrations of synthetic peptides or unlabeled bTSH in 40 mM Tris·HCl, pH 7.4, with 0.1% bovine serum albumin at a final volume of 500 μl. After 2 hr of incubation at 25°C, bound label was separated from free by addition of 0.5 ml of buffer and 1 ml of 30% (wt/vol) polyethylene glycol 6000 (J. T. Baker) in 1 M NaCl and centrifugation. The radioactivity of the resulting pellet was measured in a γ counter. Nonspecific binding was determined by the addition of crude bTSH (Sigma) at 100 milliunits/ml.

Statistical Analysis. Each peptide was tested in three to five separate TSH radioreceptor assays in which a dose–response curve was generated and the dose required to inhibit specific binding of the tracer by 50% (EC_{50}) was determined. Within each set of synthetic peptides, the mean EC_{50} of a given peptide was compared to the peptide containing its respective native sequence by using analysis of variance (ANOVA). A P value ≤ 0.05 was considered statistically significant. For the truncation series (series 1, 2, and 4) to test the effect of removing a given residue, the activity of each peptide was compared with the activities of the peptide one residue longer and one residue shorter by ANOVA. A P value < 0.05 was interpreted as evidence of the importance of the residue in question.

RESULTS

Peptide Set 1. Peptide set 1 consisted of 16 distinct peptides that were progressively shorter than the entire α26–46 sequence by one residue at the amino terminus, so that peptides α27–46, α28–46, α29–46, etc. were made. The synthetic strategy of this set is shown in Fig. 1. Thus, reduction in activity of peptide 36 (which contains the sequence 36–46) as compared with peptide 35 (which contains the sequence 35–46) indicates the importance of residue Arg^{35} in receptor interaction, whereas equivalent activity of peptide 30 as compared with peptide 29 suggests that residue Met^{29} is not critical in the activity of the sequence.

Fig. 2 shows the activity of the set 1 peptides. As demonstrated, significant reductions in activity were noted with removal of residues Cys^{32}, Phe^{33}, and Arg^{35}. Essentially 100% of native activity was retained until removal of residue Cys^{31}. Removal of Cys^{31} also reduced the activity, but the change was less pronounced than with the above-named residues and was not statistically significant. After removal of Arg^{35} only low levels of activity remained (EC_{50} > 500 μM and ≤ 2% of native α26–46; because of the appearance of precipitation at higher peptide concentrations the activity could not be accurately quantified further). This suggested that the region bounded by Cys^{31} and Arg^{35} contained the majority of the activity. However, the low activity remaining in peptides 36–41 also suggested that residues after Leu^{41} may also contribute somewhat to binding activity.

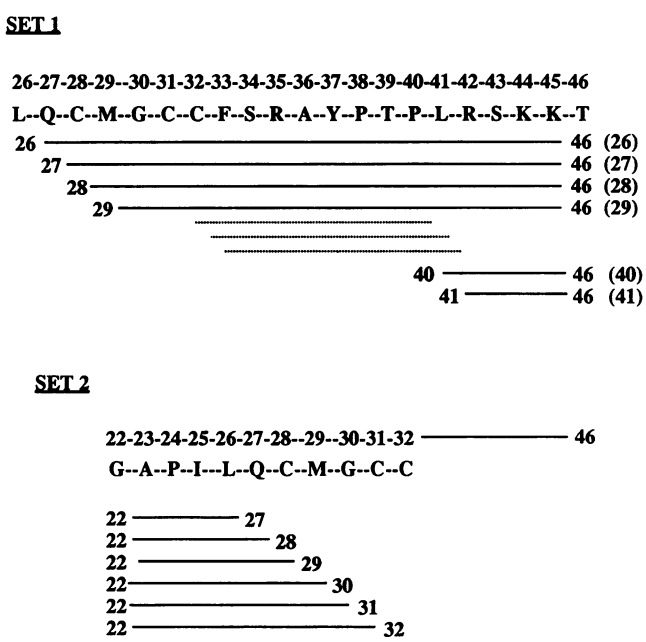

FIG. 1. Strategy for synthesizing set 1 and set 2 peptides. The native α22–46 sequence is shown at the top. In set 1, 16 separate synthetic peptides were synthesized, beginning with α26–46. Each subsequent peptide was one residue shorter than the previous peptide, such that a nested set was generated displaying systematic truncation of the sequence from the amino terminus. The peptides were numbered according to the number of the first residue in the human α subunit (2). Numbers are noted in parentheses at right. In set 2, 6 peptides were generated, beginning with α22–27 and each subsequent peptide was lengthened by one residue until the series was ended with α22–32. None of these peptides demonstrated activity in the TSH radioreceptor assay (data not shown).

Peptide Set 2. Peptide set 2 also examined the amino-terminal portion of α26–46. This set consisted of six distinct peptides that began at residue Gly^{22} and ended at residues Gln^{27} through Cys^{32} in series, as outlined in Fig. 1. None of these peptides significantly inhibited ^{125}I-bTSH binding to

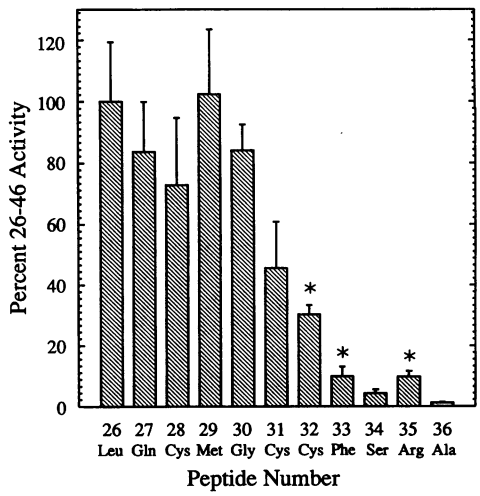

FIG. 2. Activities of set 1 peptides. Results are expressed as mean ± SEM of three to five assays as compared with the activity of the native α26–46 sequence. Peptides are numbered according to the position of the first residue of the peptide in the human α subunit as noted in Fig. 1. *, $P < 0.05$ for given peptide versus its next shortest neighbor by ANOVA, indicating significant reduction in activity with removal of the amino-terminal residue.

receptor (EC_{50} > 1000 μM; data not shown), indicating that these residues were not critical to the binding activity of the sequence. Thus, the results agreed with the findings from set 1 in that residues after Cys^{33} were involved in the receptor binding site.

Peptide Set 3. Set 3 further examined the region of activity noted above. This set consisted of seven analogs of the α29–46 sequence in which the native residue was systematically replaced by alanine, as illustrated in Fig. 3. The peptides were generated simultaneously by using the "tea bag" method of peptide synthesis (15, 16), and the native α29–46 was resynthesized for use as a control. Activity was expressed as percent of the native α29–46 peptide. Fig. 4 shows that substitution for residues Arg^{35}, Phe^{33}, Cys^{32}, Cys^{31}, and Gly^{30} resulted in a statistically significant loss of binding inhibition, suggesting that those residues were most important to the activity. Some loss of activity occurred with the substitution for Ser^{34}, although the reduction was lower than that seen with substitution for the above-named residues and was not statistically significant.

Fig. 5 compares the results of data generated from sets 1 and 3. The data from the two experiments generally agreed quite well in that residues Cys^{32}, Phe^{33}, and Arg^{35} were noted to be most critical, and also in that residues Met^{29} and Ser^{34} were seen to be less important. Some discrepancy did exist, however, for residues Cys^{31} and Gly^{30}, both of which showed higher activity in the substitution peptides than in the truncation series.

Peptide Set 4. Peptides of set 4 were designed to examine the carboxyl-terminal region of α26–46. The set consisted of seven peptides all beginning with Met^{29} but each progressively shorter at the carboxyl terminus by one residue as compared with its neighbor, as illustrated in Fig. 3. Fig. 6 shows that removal of Lys^{45}, Lys^{44}, and Arg^{42} resulted in

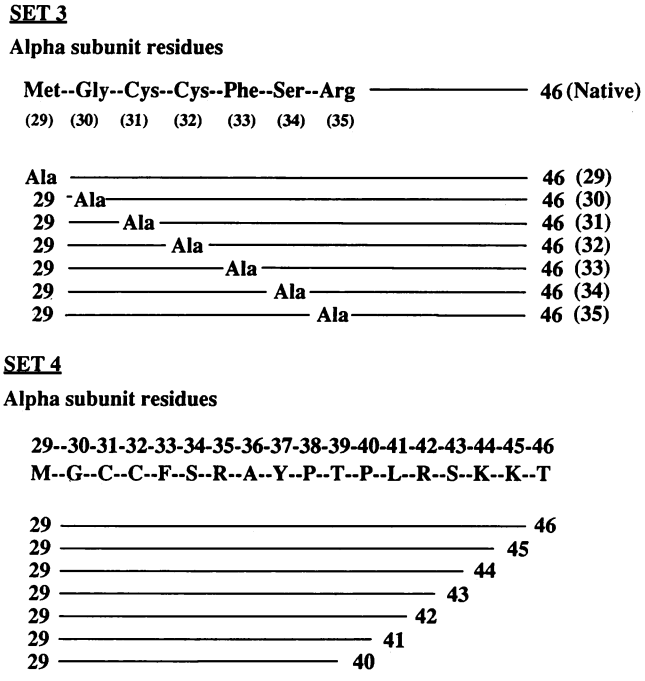

FIG. 3. Strategies for synthesizing set 3 and set 4 peptides. In set 3, seven peptides containing the α29–46 sequence were made in which the native residues at positions 29 through 35 were systematically replaced by alanine. The peptides were numbered according to the residue replaced. The peptide α29–46, containing the native sequence, was also synthesized for use as control. In the set 4 peptides, the carboxyl-terminal region of the α29–46 sequence was systematically truncated by one residue for residues 40 through 46, generating seven different peptides.

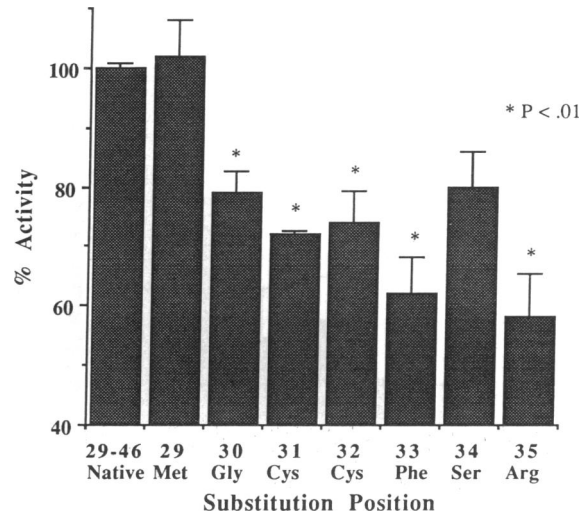

FIG. 4. Activities of set 3 peptides. The synthetic scheme for the peptides is shown in Fig. 3. The activity of each peptide was compared to that of the peptide containing the native α29–46 sequence. Numbers and amino acid symbols below the bars indicate the residue for which alanine was substituted. Data shown are mean ± SEM of results from three to five separate assays. *, $P < 0.01$ versus native sequence by ANOVA.

significant losses of activity, whereas removal of residues Thr^{46}, Ser^{43}, and Leu^{41} had no effect. The data therefore support the presence of a second binding site within the α26–46 sequence in addition to the region between residues Gly^{30} and Arg^{35} noted above. As shown, some activity remained in the peptides α29–40 and α29–41. This could be explained by the presence of the α30–35 sequence within each of the set 4 peptides, which as noted above possesses binding activity in itself.

DISCUSSION

Considerable evidence suggests that both the common α subunit as well as the hormone-specific β subunit interact

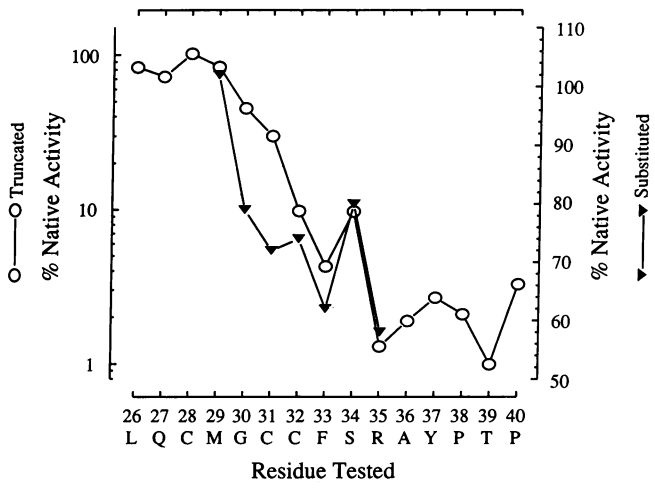

FIG. 5. Comparison of activities of set 1 (truncated) and set 3 (substituted) peptides. The x-axis represents the residue of interest of human α subunit tested by the two experiments. Results are expressed as percent activity of the native sequence for each experiment. Because the loss of activity in the truncated series is additive with removal of each active residue and they therefore demonstrate a much wider range of potency, the y scale for set 3 peptides is logarithmic, whereas that for the substituted peptides (in which only the effect of each individual residue is observed) is linear.

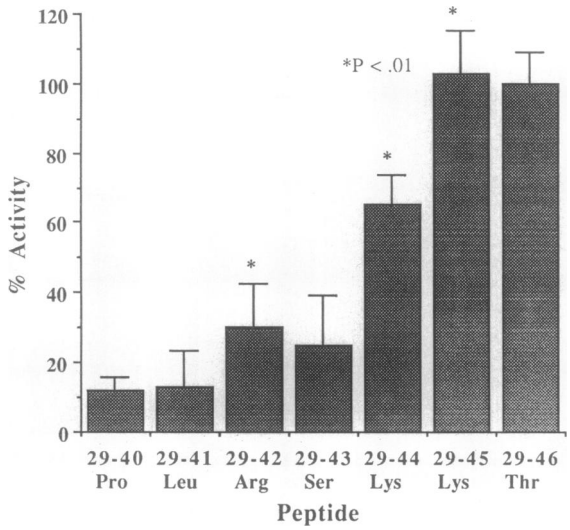

FIG. 6. Activities of set 4 peptides. The synthetic scheme is shown in Fig. 3. Results represent mean ± SEM of three to five separate assays and are expressed as percent activity of the native α29–46 peptide. *, $P < 0.01$ for given peptide versus its next shortest neighbor, indicating significant change in activity with removal of the carboxyl-terminal residue.

with the glycoprotein hormone receptor (1, 2, 20). Previously, we determined that a region of the α subunit between residues 26 and 46 was responsible for binding not only of TSH to the thyroidal TSH receptor (11) but also of hCG and LH to the ovarian LH receptor (12). The current studies were designed to delineate further which specific residues within this 21-amino acid span were responsible for this activity.

The data show that two regions of the α26–46 sequence are important to the binding activity observed. The first is bounded by Gly^{30} and Arg^{35}, and also includes Cys^{31}, Cys^{32}, and Phe^{33} (Fig. 5). Gly^{30} showed higher activity in peptide set 3 than in set 1, whereas Cys^{31} showed activity in both experiments but the difference reached statistical significance only in set 3. Thus, the importance of these residues is less certain because of the discrepancy between the two experiments. In contrast to the above amino acids, Ser^{34} lies within this region but it appears less important than the residues surrounding it. The second receptor binding region is bounded by Arg^{42} and Lys^{45} and includes Lys^{44} as an important component and Ser^{43} as a less important constituent. The data also indicate that the α30–35 region interacts at higher affinity than the α41–45 region because the activity of the later sequence could be detected only when the former was also present; however, α30–35 was presumably responsible for the activity of peptides α29–40 and α29–41 (EC_{50} = 244 ± 13 μM and 243 ± 74 μM, respectively) even in the absence of the α42–45 sequence.

The technique of systematic truncation of synthetic peptides to determine binding regions has been used previously to localize the α-bungarotoxin binding site of the acetylcholine receptor (21) and to determine the regions of hemoglobin that bind haptoglobin (22). In these studies, one must be concerned about reductions in activity, with truncation representing only a nonspecific effect of reducing the length of the molecule beyond some critical level. We addressed these issues by performing alanine substitution of the residues of interest in addition to truncating the sequences. As shown above the two experiments agreed quite well. Indeed, the substitution studies seemed to be more sensitive in that activity was seen with two residues by alanine substitution that had little or no activity by truncation. This finding argues strongly against length requirements as the cause of the loss of activity, at least in these experiments with this protein.

In our previous studies we observed activity with two synthetic α-subunit peptides that both include the α26–46 region, α21–35 and α31–45. Thus, the activity present in those two peptides is quite reasonably reconciled with the current data. We also previously observed that the activity of the peptide α26–46 was considerably higher than that of either α21–35 or α31–45 alone [EC_{50} = 39.9 μM and 31.2 μM for α21–35 and α31–45, respectively, versus 11.4 μM for α26–46 (11)]. This is also well explained by the current data in that the peptide α26–46 contains both of the binding regions in their entirety, whereas α21–35 contains only the Gly^{30}–Arg^{35} sequence and α31–45 contains only the Arg^{42}–Lys^{45} sequence and a truncated form of the more amino-terminal site.

Although little prior data concerning this region exists for TSH, several lines of evidence suggest involvement of the α31–35 region in hormone–receptor interaction for other glycoprotein hormones (1, 13, 23, 24). The importance of Arg^{35} was also suggested by studies showing reduced receptor binding of hCG after derivatization of that residue (1). These studies support our findings of the importance of the α30–35 region. In addition, the absence of activity in the region α36–41 is in agreement with Bidart et al. (8), who found that an antibody recognizing the residues α36–41 bound only free α subunit and not intact heterodimer. Bidart et al. suggested that the α36–41 region may therefore be in contact with the β subunit and form part of the subunit interface. The absence of receptor interaction that we found for these residues in our studies would be compatible with this hypothesis.

Several of the residues found to be important in receptor interaction in these studies are positively charged at neutral pH (Arg^{35}, Arg^{42}, Lys^{44}, and Lys^{45}). This finding is not surprising in view of previous isoelectric focusing studies of TSH and the TSH receptor (25). The isoelectric point of TSH has been reported to be around 9.0 (26) and the pI of the TSH receptor has been reported to be about 5.0 (27, 28). Thus, it should be expected that positively charged residues in the hormone would be critical in interaction with the predominantly negatively charged receptor. Indeed, the initial attraction between hormone and receptor is very likely to involve long-range forces such as electrostatic interactions (25). After initial association other weaker electrostatic forces such as hydrogen bonding (involving Cys^{31}, Cys^{32}, and Ser^{34}), van der Waals forces, and hydrophobic interactions (e.g., of Phe^{33}) may become involved.

In bovine α subunit, Lys^{49} corresponds to Lys^{45} of human α subunit (2). This residue can be chemically cross-linked to bovine β subunit by a carbodiimide (29–32). This finding has been taken to imply that the residue is near or involves the region of subunit contact. Our results do not support interaction of this residue with the β subunit, as it would seem unlikely that the residue could interact with both the receptor and the β subunit. However, our data are not inconsistent with the hypothesis that Lys^{45} (or Lys^{49} of bLHα) is *near* the subunit contact surface. Indeed, if the region between α36–41 is involved in subunit interaction, as mentioned above, and residues on either side of that sequence are involved in receptor binding, it follows that the two regions (subunit contact surface and receptor binding surface) may lie in close proximity to each other. This arrangement would also allow the receptor binding regions from both subunits to approach each other, thereby forming a continuous receptor binding surface on the native dimer.

The results of these studies are very similar to those of studies in which the interaction of the α26–46 region with the hCG/LH receptor was examined (13). In these studies, residues Gly^{30}, Cys^{32}, Phe^{33}, Arg^{35}, Arg^{42}, and Lys^{44} were found to be important in the interaction of the region with the hCG/LH receptor. However, some differences were also

apparent. We found that Cys^{31} and Lys^{45} were important for TSH interaction with its receptor, whereas they were not found to be so for hCG/LH. In addition, Reed *et al.* (13) found that Ser^{43} was important in the binding of hCGα with its receptor, whereas we found no activity of this residue in our TSH binding assays. Therefore, while the interaction of the α subunit with the two receptor species (i.e., TSH versus hCG/LH) is quite similar, our data would suggest that it is not identical.

In summary, we have used a comprehensive synthetic approach in the study of the structure–activity relationships of the receptor binding region of the human glycoprotein hormone α subunit. Several specific residues, which were confined to two distinct regions of the α26–46 sequence, were determined to be critical in the expression of full activity. The studies demonstrate the utility of the synthetic peptide approach in structure–function analysis.

This work was supported by grants from the National Institutes of Health (HD 9140), the Mellon Foundation, and the Mayo Foundation.

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