Substitution of the Seat-belt Region of the Thyroid-stimulating Hormone (TSH) β-Subunit with the Corresponding Regions of Choriogonadotropin or Folliclotropic Activity to Chimeric TSH*

(Received for publication, September 10, 1996, and in revised form, February 24, 1997)

Mathis Grossmann‡, Mariusz W. Szkudlinski, Rosemary Wong§, James A. Dias¶, Tae H. Ji‖, and Bruce D. Weintraub

From the Laboratory of Molecular Endocrinology, Department of Medicine, University of Maryland School of Medicine and the Institute of Human Virology, Medical Biotechnology Center, Baltimore, Maryland 21201, the Wadsworth Center, New York State Department of Health, Albany, New York 12201, and the Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071

The region between the 10th and 12th cysteine (Cys88, Cys105) in human thyroid-stimulating hormone β-subunit (hTSH(β)) of the glycoprotein hormone β-subunits corresponds to the disulfide-linked seat-belt region. It wraps around the common α-subunit and has been implicated in regulating specificity between human choriogonadotropin (hCG) and human follicle-stimulating hormone (hFSH), but determinants of hTSH specificity are unknown. To characterize the role of this region for hTSH, we constructed hTSH chimeras in which the entire seat-belt region Cys88-Cys105 or individual intercysteine segments Cys88-Cys95 and Cys95-Cys105 were replaced with the corresponding sequences of hCG and hFSH or alanine cassettes. Alanine cassette mutagenesis of hTSH showed that the Cys95-Cys105 segment of the seat-belt was more important for TSH receptor binding and signal transduction than the Cys88-Cys95 determinant loop region. Replacing the entire seat-belt of hTSHβ with the hCG sequence conferred full hCG receptor binding and activation to the hTSH chimera, whereas TSH receptor binding and activation were abolished. Conversely, introduction of the hTSHβ seat-belt sequence into hCGβ generated an hCG chimera that bound to and activated the TSH receptor but not the CG/lutropin (LH) receptor. In contrast, an hTSH chimera bearing hFSH seat-belt residues did not possess any follitropic activity, and its thyrotropic activity was only slightly reduced. This may in part be due to the fact that the net charge of the seat-belt is similar in hTSH and hFSH but different from hCG. However, exchanging other regions of charge heterogeneity between hTSHβ and hFSHβ did not confer follitropic activity to hTSH. Thus, exchanging the seat-belt region between hTSH and hCG switches hormonal specificity in a mutually exclusive fashion. In contrast, the seat-belt appears not to discriminate between the TSH and the FSH receptors, indicating for the first time that domains outside the seat-belt region contribute to glycoprotein hormone specificity.

Thyrotropin (thyroid-stimulating hormone (TSH))³ choriogonadotropin (CG), follitropin (follice-stimulating hormone (FSH)), and lutropin (luteinizing hormone (LH)) are structurally related heterodimers that together form the glycoprotein hormone family (1). These hormones belong to the superfamily of cystine-knot growth factors (2, 3) and activate specific G-protein-coupled receptors notable for large extracellular domains containing multiple leucine-rich motifs (4). The primary structure of the α-subunit, which is encoded by a single gene, is identical in these hormones. The distinct β-subunits, despite conservation of all 12 cysteine residues and similar overall folding, are sufficiently different to confer specificity to each hormone (1–3).

The molecular mechanisms whereby glycoprotein hormones activate their receptors are largely unknown, but multiple contact points between ligand and receptor, perhaps in a stepwise fashion, appear necessary to induce conformational changes favoring receptor G-protein coupling and subsequent second messenger generation (5–9). Recently, we have described several α-subunit domains important for hTSH activity (10–13), but there is little information on how the hTSH β-subunit contributes to receptor activation.

Previous studies have shown that the region between the 10th and 12th cysteine of the β-subunit is important not only for subunit association, receptor binding, as well as activation (14–16), but also for specific receptor recognition (17–20) of hCG and hFSH. In the crystal structure of hCG (2, 3), this region corresponds to the “seat-belt” region (Cys88-Cys105) in hTSH(β), so-called because it wraps around the α-subunit and orients it in the heterodimer while remaining covalently bonded to the β-subunit through disulfide linkages between Cys4-Cys90 and Cys88-Cys105. This seat-belt consists of two intercysteine segments, a surface-exposed hydrophilic segment between the 10th and 11th cysteine (Cys88-Cys95 in hTSH(β)) and a carboxyl-terminal segment between the 11th and 12th cysteine (Cys95-Cys105 in hTSH(β)), and is in close proximity to α-subunit domains important for the structural integrity and

* Preliminary portions of these results were presented at the 10th International Congress of Endocrinology, San Francisco, CA (1996). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence and requests for reprints should be addressed: Laboratory of Molecular Endocrinology, Institute of Human Virology, Medical Biotechnology Center, 725 W. Lombard St., N457, Baltimore, MD 21201. Tel.: 410-706-0993; Fax: 410-706-4574; E-mail: grossman@umbi.umd.edu.

§ Wadsworth Center, New York State Department of Health, Albany, New York 12201, and the Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071

¶ The abbreviations used are: TSH, thyroid-stimulating hormone; hTSH, human TSH; hTSHβ, human TSH β-subunit; CG, choriogonadotropin; FSH, follitropin; LH, lutropin; rh, recombinant human; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; wt, wild-type.
activity of the glycoprotein hormones (2, 3).

In contrast to the work on the gonadotropins, the role of the seat-belt for hTSH is not known. A single study using a set of overlapping synthetic peptides spanning the entire hTSH subunit (21) showed that none of the peptides encompassing the seat-belt region, hTSHβ 81–85 or hTSHβ 91–105, inhibited TSH receptor binding, but a peptide containing the carboxyl terminus (8101–112) possessed the highest TSH receptor binding activity. However, the role of the seat-belt region in the context of the intact hTSH heterodimer has not been investigated. Interestingly, recent studies on a naturally occurring hTSHβ mutation from patients with secondary hypothyroidism have shown the importance of Cys8105 (corresponding to Cys110 in hCG) for hTSH activity (22).

In the present study, using a chimeric mutagenesis approach, we demonstrate the importance of the seat-belt for hTSH action as well as specificity. Moreover, our findings reveal previously unrecognized differences in the regulation of specificity among the glycoprotein hormones.

EXPERIMENTAL PROCEDURES

Materials—The following materials were generous gifts. CHO cells stably transfected with the rHTSH receptor (clone JPO9) was from Dr. G. Vassart (Brussels, Belgium) (23); the gonadotropin-responsive murine Leydig cell line MA-10 was from Dr. M. Ascoli, (Iowa City, IA) (24); and cAMP antibody was from Dr. J. L. Vaitukaitis, National Institutes of Health (Bethesda, MD). Embryonic kidney 293 cells (FSH-R/293 cells) and Y-1 cells expressing the human FSH receptor have been described previously (20, 25). Cell culture media and reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD); [125I]-cAMP (specific activity, 40–60 μCi/μg) and [125I]-hCG (specific activity, 50–70 μCi/μg) were from Hazleton (Vienna, VA), and polymerase chain reaction (PCR) reagents were from Boehringer Mannheim and New England Biolabs (Beverly, MA).

Site-directed Mutagenesis—The chimeric hTSH were constructed with the PCR-based megaprimer method of site-directed mutagenesis (26), as described (11, 13). Individual intercysteine segments C10-C11 (hTSHβ Cys89-Cys96) or C11-C12 (hTSHβ Cys95-Cys105) of the hTSH β-minigene were replaced with nucleotides coding for the respective sequence of hCG and hFSH or Ala cassettes. To replace the entire seat-belt (hTSHβ Cys89-Cys105), chimeras with individually mutated intercysteine segments were used as templates for subsequent PCR reactions. The hTSHβ seat-belt was introduced into the hCG β-subunit in a single PCR reaction using a primer coding for the entire hTSH β-seat-belt. Further hTSH/hFSH chimeras were constructed in which the carboxyl-terminal residues hTSHβ 105–112 or amino acids hTSHβ 44–52 were replaced with the sequence of hFSH. In addition, Asp94 of the determinant loop was replaced with Lys (TSHβ o Glu94). After subcloning into the expression vectors, the entire PCR products of all constructs were sequenced to verify the mutations and to rule out any undesired polymerase errors. Construction of the quadruple α-subunit mutant bearing Lys residues at positions α13, 14, 16, and 20 (α4K) was described previously (13).

Transient Expression—CHO-K1 cells maintained as described (10) were transiently cotransfected with the various constructs using a transient transfection protocol based on a liposome formulation (LipofectAMINE reagent, Life Technologies, Inc.) (10). After culture in CHO serum-free medium (CHO-SFM, Life Technologies, Inc.) for 48 h, conditioned media including control medium from mock transfections were harvested, concentrated with Centriprep 10 concentrators (Amicon, Beverly, MA), and stored at −70 °C to prevent neuraminidase digestion.

Immunooassays—Wild type and mutant hTSH analogs were quantified with a panel of four different hTSH immunooassays, which were described in detail previously (12). hCG immunoreactivities were measured with two different specific third-generation immunooassays without crossreactivity to other glycoprotein hormones (Nichols Institute, San Juan Capistrano, CA; ICN, Costa Mesa, CA), and hFSH immunoreactivity was measured with an hFSH-specific third-generation immunooassay (Nichols Institute).

Hormone Binding Assays—The TSH receptor-binding activity of wild type and hTSH mutants was determined by their ability to displace 125I-hTSH from a solubilized porcine thyroid membrane receptor preparation (Kronus, Dana Point, CA), as described previously (10). Binding to the CG/LH receptor was studied in MA-10 cells following a previously employed protocol (13, 24), and FSH receptor binding was analyzed using a rat testis membrane radioreceptor assay as described in detail previously (20).

Hormone Activity Assays—The ability of the various chimeras to induce cAMP production was studied at the TSH receptor using JPO9 cells (23), at the CG/LH receptor using MA-10 cells (24), and at the FSH receptor using FSH-R/293 cells (25). Briefly, confluent cells in 96-well tissue culture plates were incubated in a modified Krebs Ringer buffer for 2 h at 37 °C, 5% CO2 with serial dilutions of wild type and mutant hTSH, as well as control medium from mock transfections. The amount of cAMP released into the medium was assayed by radioimmunooassay (10). Progesterone production at the CG/LH or FSH receptor was determined using a commercially available progesterone radioimmunoassay kit (ICN) after incubation of the chimeric constructs with MA-10 cells or Y-1 cells, respectively, as detailed previously (20, 24).

RESULTS

Construction of Chimeric Glycoprotein Hormone Mutants—Replacing individual intercysteine segments C10-C11 of the hTSH β-subunit (the determinant loop, hTSHβ Cys89-Cys96) or C11-C12 (the carboxyl-terminal segment, hTSHβ Cys95-Cys105) (Fig. 1) with Ala cassettes or with the corresponding sequences of hCG and hFSH generated hTSHβ constructs designated hTSHβ o40-46, hTSHβ o32, hTSHβ o52, and hTSHβ o26 and exchange of the entire hTSH β seat-belt chimeras hTSHβ 44-52 were obtained by introduction of the hTSHβ seat-belt residues into the hCG β-subunit (Fig. 2). In addition, hTSHβ Asp94, which is conserved in all known β-subunits and essential for hCG activity

![FIG. 1. Domains of hTSH important for activity. The schematic drawing of hTSH is based on a molecular homology model of hTSH (13) and built on a template of an hCG model derived from crystallographic coordinates (2). The α-subunit is shown in gray, and the β-subunit is black. The seat-belt region between the 10th (Cys94) and 12th (Cys105) Cys of hTSH corresponds to the dotted line (●●●●), and the carboxyl-terminal segment (Cys89-Cys105) in hTSHβ corresponds to the dashed line (— — — —). Since the carboxyl terminus beyond hCGβ 111 was not traceable in the original electron density map, hTSHβ is only drawn to the corresponding residue 106. Domains of the α-subunit of known importance for hTSH activity are boxed. Because of hydrogen fluoride treatment of hCG prior to crystallization, the oligosaccharides, including that linked to αAsn83, are not shown. The αAsn83 carbohydrate is predicted to project into the proposed receptor binding domain, which also includes the α40–46 helix and the α-carboxyl terminus α88–92 (2, 3). In contrast, the α11–20 domain is not located in proximity to the β seat-belt (see ‘Discussion’).]
**hTSH-β Seat-belt Chimeras**

Shown are the native amino acid sequences of the seat-belt region between the 10th and 11th Cys for hTSH, hCG, and hFSH subunits in the 1-letter code. For comparison, the Cys numbering is maintained according to its position in the respective subunit. Also depicted are the mutant seat-belt hTSH constructs and the hCG chimera prepared by site-directed mutagenesis, with the mutated segments underlined. In the Ala cassette constructs, either the determinant loop (between the 10th and 11th Cys, 89TSH104) or the carboxyl-terminal segment (between the 11th and 12th Cys, 94TSH109) were replaced with Ala residues. In the hTSH/hCG and hTSH/hFSH chimeras, intercysteine segments or the entire seat-belt of the hTSH β-subunit were replaced with the respective gonadotropin residues. In contrast, in 94TSH109, the hTSH β-seat belt was introduced into the hCG β-subunit. (27), was replaced with Lys (hTSHβLys94) or with Glu (hTSHβGlu94). Finally, sequences of the hTSH β-subunit outside the seat-belt were replaced with the corresponding sequence of hFSH to create 4FSH105 and 105FSH112. These regions were chosen because they display the greatest charge heterogeneity among these β-subunits, based on the proposed role of variable charges for glycoprotein hormone specificity (17) and see below. Receptor binding and biological properties of these analogs, described in detail below, are summarized in Table I. A comparison of the receptor specificity of glycoprotein hormone seat-belt chimeras from this and other studies (18–20) is given in Table II.

**Secretion of Chimeric Mutants**—All chimeric hTSH heterodimers were secreted from the transfected CHO cells, and hFSH subunits in the 1-letter code. For comparison, the Cys numbering is maintained according to its position in the respective subunit. Also depicted are the mutant seat-belt hTSH constructs and the hCG chimera prepared by site-directed mutagenesis, with the mutated segments underlined. In the Ala cassette constructs, either the determinant loop (between the 10th and 11th Cys, 89TSH104) or the carboxyl-terminal segment (between the 11th and 12th Cys, 94TSH109) were replaced with Ala residues. In the hTSH/hCG and hTSH/hFSH chimeras, intercysteine segments or the entire seat-belt of the hTSH β-subunit were replaced with the respective gonadotropin residues. In contrast, in 94TSH109, the hTSH β-seat belt was introduced into the hCG β-subunit. (27), was replaced with Lys (hTSHβLys94) or with Glu (hTSHβGlu94). Finally, sequences of the hTSH β-subunit outside the seat-belt were replaced with the corresponding sequence of hFSH to create 4FSH105 and 105FSH112. These regions were chosen because they display the greatest charge heterogeneity among these β-subunits, based on the proposed role of variable charges for glycoprotein hormone specificity (17) and see below. Receptor binding and biological properties of these analogs, described in detail below, are summarized in Table I. A comparison of the receptor specificity of glycoprotein hormone seat-belt chimeras from this and other studies (18–20) is given in Table II.

**Role of Asp94 for hTSH Activity**—A single mutation of the conserved Asp94 to Lys (hTSHβLys94) completely abolished TSH receptor binding and activation, whereas preserving the negative charge at this position by mutating Asp94 to Glu (hTSHβGlu94) did not have a significant effect on TSH receptor binding or activation (Table I). This confirmed the importance of a negative charge in this particular position for glycoprotein hormone activity (27).

**hTSH/hCG Chimeras**—Replacement of the hTSH seat-belt segments with the respective sequences of hCG either substantially decreased (89CG104) or abolished measurable TSH receptor binding and activation (94CG104, 89CG104) of the chimeras (Fig. 4, A and B). hTSH/hCG chimeras 89CG104 and 94CG104 showed only very little CG/LH receptor binding and activation, both with cAMP stimulation as well as progesterone production. Thus, at the highest doses possible within the limitations of the transient transfection system, between 100–200 ng/ml, cAMP or progesterone production was only 10–18% that of hCG-wt (Fig. 5, A–C). To more conclusively test whether the Cys88–Cys95 determinant loop was important for differential hormonal activity, we constructed a hTSH mutant α4K/98CG94. In α4K/89CG94, residues α13, 14, 16, and 20 were substituted with Lys residues in addition to the replacement of Cys88–Cys95 with the respective hCG residues. We had previously shown that introduction of positive charges into this α11–20 domain led to substantial increases of glycoprotein hormone receptor binding affinity (13). We therefore expected that increasing the binding affinity of 89CG94 (Fig. 5A) should accentuate its gonadotropin properties. Maximal cAMP and progesterone production with this α4K/89CG94 combination chimera increased to 35% and 50% of hCG-wt levels, respectively (Fig. 5, B and C). At the same time, the thyrotropic activity of α4K/89CG94 remained unchanged (data not shown).

Remarkably, replacement of the entire seat-belt of hTSHβ with the hCG sequence (89CG104) resulted in a chimera with hCG receptor binding comparable to hCG-wt, suggesting that the two individual intercysteine segments confer hCG specificity in a synergistic fashion (Fig. 5A). Further, the 89CG104 chimera was able to induce biological responses in MA-10 cells expressing the CG/LH receptor (Fig. 5, B and C). Whereas potency and efficacy of progesterone production as well as efficacy of cAMP induction were similar to hCG-wt, the potency of 89CG104 for cAMP production was 10-fold less than that of hCG-wt. These differences may stem in part from differences in the cAMP and progesterone assay conditions in MA-10 cells (see “Experimental Procedures”). Moreover, such generation of full hormonal responses at submaximal cAMP levels, termed “the cAMP superfluity concept,” has been well recognized in studies on structure-function relationships of glycoprotein hormones (8, 28). Analogous findings for recombinant analogs with substantially higher progesterone-inducing than cAMP-inducing ability have been described by others (29). Interestingly, hTSH/hCG specificity appeared mutually exclusive since the 89CG104 chimera did not possess significant thyrotropic activity (Fig. 4, A and B).

**hCG/hTSH Chimera**—Conversely, the reciprocal chimera 94TSH109, which bears the hTSHβ seat-belt in the context of the hCG-β subunit, bound to the TSH receptor and was able to activate cAMP production in JP09 cells, with an EC50 that was 26.7 ± 4.7-fold higher than that of hTSH-wt (Fig. 6, A and B).
At the same time, $[^{125}I]$/hTSH did not bind to the CG/LH receptor, nor did it stimulate cAMP or progesterone production in MA-10 cells at concentrations up to 1000 ng/ml (Table I).

**hTSH/fFSH Chimeras**—Analogous replacement of individual intercysteine segments of hTSH with the corresponding hFSH residues only slightly reduced TSH receptor binding or activation of these hTSH/hFSH chimeras (Fig. 7, A and B). Further, in contrast to $^{89}$CG/104, the $^{89}$FSH/104 construct bearing the entire hFSH seat-belt sequence was able to significantly bind to the TSH receptor and induce 50% of maximal hTSH-wt cAMP production in JP09 cells. Interestingly, none of the three hTSH/hFSH chimeras showed significant follitropic activity. Unlike hFSH-wt, the chimeras did not stimulate cAMP production at the hFSH receptor expressed in 293 cells (Fig. 8). Further, they did not show significant binding in a rat testis FSH radioreceptor assay and did not stimulate progesterone production in Y-1 cells expressing the hFSH receptor (Table I).

Since charge heterogeneity could play a role for hTSH/hFSH specificity (17), we replaced candidate regions hTSH$_b^{44–52}$ and the carboxyl-terminal residues 105–112 with hFSH sequences. Thus, hTSH$_b^{4K}$AL$S$QDVC$_{52}$ has a net charge of 0, and the corresponding hFSH$_b$ sequence ARPKIQKTC has a
charge of +3. hTSHβ¹⁰⁵CTKPQKSY¹¹² has a net charge of +2, and the corresponding hFSHβ sequence CSFGEMKE has a charge of −1. The hTSHβ 44–52 region corresponds to the carboxyl-terminal part of the long β2 loop identified by Keutmann et al. (30), which forms a wedge-shaped and partly surface-exposed extrusion in proximity to the determinant loop (2). However, none of the resulting hTSH/hFSH chimeras showed any follitropic activity in the three different assay systems (Table I). Interestingly, the thyrotropic activity of hTSH/hCG chimeras was performed in confluent MA-10 cells incubated in 96-well plates with serial dilutions of recombinant hormones in assay medium and ¹²⁵I-hCG (70,000–100,000 counts/well) for 20 h at room temperature. After repeated washing, cells were dissolved in 1 N NaOH and counted in a γ counter. B, cAMP production at the CG/LH receptor. cAMP induction by the hTSH/hCG chimeras was determined in MA-10 cells according to the protocol described in Fig. 4B and under “Experimental Procedures.” Basal cAMP levels were < 1.5 pmol/ml. C, progesterone production in MA-10 cells. Cells were incubated with hCG-wt or hTSH/hCG constructs for 6 h at 37 °C, 5% CO₂. Progesterone concentrations were determined in the supernatant using a commercially available progesterone radioimmunoassay kit (ICN). A representative experiment, repeated at least twice, is shown.

**DISCUSSION**

Previous studies on glycoprotein hormone specificity had focused on analogs that bound either to the CG/LH or the FSH receptor. These studies had suggested that domains within the seat-belt region of the β-subunit are involved in directing gonadotropin specificity (18–20). It is unknown, however, how hTSH specificity is achieved and whether the seat-belt is critical for interaction with the TSH receptor. We directly compared the effects of systematically replacing the hTSHβ seat-belt and its individual intercysteine segments with the corresponding regions of two different hormones, hCG and hFSH in parallel. Conversely, the hTSHβ seat-belt residues were introduced into hCG. This strategy allowed us to characterize the role of the seat-belt for hTSH activity as well as specificity and to reveal divergent principles of specificity regulation among the members of the glycoprotein hormone family (see Table II). Ala cassette mutations showed that the primary sequence of

![Graph A](image1.png)

**FIG. 5.** Luteotropic activity of the hTSH/hCG seat-belt chimeras. CG/LH receptor binding. A, receptor binding affinity of the hTSH/hCG chimeras was performed in confluent MA-10 cells incubated in 96-well plates with serial dilutions of recombinant hormones in assay medium and ¹²⁵I-hCG (70,000–100,000 counts/well) for 20 h at room temperature. After repeated washing, cells were dissolved in 1 N NaOH and counted in a γ counter. B, cAMP production at the CG/LH receptor. cAMP induction by the hTSH/hCG chimeras was determined in MA-10 cells according to the protocol described in Fig. 4B and under “Experimental Procedures.” Basal cAMP levels were < 1.5 pmol/ml. C, progesterone production in MA-10 cells. Cells were incubated with hCG-wt or hTSH/hCG constructs for 6 h at 37 °C, 5% CO₂. Progesterone concentrations were determined in the supernatant using a commercially available progesterone radioimmunoassay kit (ICN). A representative experiment, repeated at least twice, is shown.
the seat-belt is essential for hTSH receptor binding and activation. Of central importance was the negatively charged Asp94 of the determinant loop since a single mutation of this residue to Lys, but not to Glu, abolished hTSH receptor binding and activity. The critical role of the negative charge of Asp94, which is conserved in all known glycoprotein hormone β-subunits and forms a non-bonded interaction with Thr54 (2, 3), was first identified in hCG by Chen et al. (27), suggesting that this residue is universally important for the members of the glycoprotein hormone family.

Our chimeric studies demonstrated that the seat-belt region of the hTSH β-subunit, if placed into the context of the hCG β-subunit, confers thyrotropic activity although the seat-belt alone was not sufficient for full thyrotropic activity. This suggests that additional hTSH β domains beside the seat-belt may contribute to hTSH specificity or that the hCG β-subunit contains segments that restrict interaction with the TSH receptor. In this respect, it had been shown that removal of the C-terminal extension peptide of hCG (31) as well as of the N-linked carbohydrate side chain at Asn52 increased the weak inherent thyrotropic activity of hCG (11). This thyrotropic activity of native hCG however, unlike the chimera described here, requires 1000-fold higher concentrations than TSH itself in most systems (11, 31). In contrast to the results with the hCG/hTSH chimera, the hCGβ seat-belt, in the context of the hTSH β-subunit, was sufficient for full CG/LH receptor binding and secretory response. This reciprocal exchange of hCG/hTSH receptor specificity was mutually exclusive as both chimeras possessed no significant residual activity at their native receptor.

Remarkably, introduction of the hFSH seat-belt into the hTSH β-subunit did not result in FSH receptor binding or follitropic activity, and the hTSH/hFSH chimera retained most...
of its thyrotropic activity. In contrast to this finding, hCG could be converted to hFSH by placing the hFSH seat-belt into the hCG β-subunit (18, 19), and hFSH adopted partial CG/LH receptor binding after exchange of its determinant loop with the corresponding hCG sequence (20) (see Table II). Hence, the role of the seat-belt in conferring specificity appears to depend on the particular subunit into which it is introduced. These findings are best reconciled by considering the concept of “negative specificity,” which proposes that specificity of glycoprotein hormones evolved independently from signal transduction by the introduction of domains that block inappropriate ligand-receptor interactions (9, 19). In this respect, it is interesting to note that the net charge of the determinant loop, the N-terminal part of the seat-belt region, is similar in hTSH (−2) and hFSH (−3) but different from that in hCG (+1). Thus, it is conceivable that a net positive charge of the determinant loop, in conjunction with the carboxyl-terminal segment of the seat-belt, interferes with hormone binding to the TSH as well as FSH receptor; whereas a net negative charge, again in conjunction with carboxyl-terminal residues reduces interaction with the CG/LH receptor. From an evolutionary standpoint, it is justifiable to assume that diversification and ligand selectivity did not evolve by development of new mechanisms of receptor activation but rather by the emergence of inhibitory domains that impose steric hindrances, thus allowing only the intended ligand to interact with the common activation domain. This concept of negative specificity is not without precedent among cysteine-knot growth factors. Thus, binding specificity among members of the neurotrophin family is achieved by the cooperation of distinct active and inhibitory binding determinants that restrict ligand-receptor interactions, enabling the creation of analogs with multiple specificities (32).

Our findings extend the original observation of Moore et al. (17), who proposed that the variable charge of this loop may act as a determinant of hormone specificity. However, our data show that the carboxyl-terminal segment of the seat-belt is of similar importance for specificity, and charge differences of the determinant loop per se are, therefore, not sufficient for the switch of hormonal activity. Indeed, conversion of hTSH to a full hCG analog required concomitant replacement of the determinant loop as well as the carboxyl-terminal segment of the seat-belt, which displays a high degree of sequence but not charge heterogeneity among the glycoprotein hormones. Interestingly, the luteotropic activity of the hTSH/hCG determinant loop chimera could be increased by concomitant introduction of a cluster of Lys residues into the spatially unrelated α11–20 domain, previously shown to increase receptor binding of hTSH as well as hCG (13).

In an attempt to identify domains determining hTSH/hFSH specificity outside the seat-belt, we focused on regions hTSHβ 44–52, which correspond to the carboxyl-terminal part of the long β2 loop identified by Keutmann et al. (30), and the β carboxyl terminus 105–112. These domains were chosen because they display the greatest degree of charge heterogeneity between their β-subunits. The decrease of thyrotropic activity of the 105FSH112 chimera confirmed the importance of the hTSHβ carboxyl terminus, which was identified with a synthetic peptide approach (21), for hTSH activity. However, introduction of hFSH residues into these regions of the hTSH β-subunit did not confer FSH receptor binding or follitropic activity to any of these chimeras. This was in agreement with the findings of Campbell et al., who showed that the long β2 loop was not important for hCG versus hFSH specificity (18). Thus, charged residues appear to play a lesser role in determining hTSH/hFSH specificity. It is possible that hTSH/hFSH specificity is not located within distinct segments of the β-subunit but is mediated by a combination of topically related domains although the present study was not designed to systematically test this possibility.

Our study cannot define the molecular mechanisms whereby the seat-belt determines glycoprotein hormone specificity as this will require complete elucidation of the structure of hormone-receptor complexes. In this respect, the seat-belt could either directly contact the receptor or influence the conformation of functionally important but unrelated portions of the hormone. An indirect effect of the seat-belt on the conformation of the α-subunit would be consistent with antibody binding studies showing that the conformation of the α-subunit could change depending upon with which β-subunit it associated (33), as well as with a recent model of glycoprotein hormone-receptor interaction predicting that the seat-belt does not directly contact the receptor (6). It could also explain the lack of
**TABLE I**

Receptor binding and biological activity of chimeric analogs at the TSH, CG, and FSH receptor

|                | TSH-RRA   | TSH-cAMP | CG-RRA | CG-cAMP | CG-Prog. | FSH-RRA | FSH-cAMP | FSH-Prog. |
|----------------|-----------|----------|--------|---------|----------|---------|----------|-----------|
| hTSH-wt        | 1.0       | 1.0      | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 89Ala24        | 0.22      | 0.24     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 96Ala104       | 0.08      | 0.13     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| D95K           | <0.01*    | <0.01*   | 1.1    | 0.96    |          |         |         |           |
| D95E           |           |          |        |         |          |         |         |           |
| 89FSH100       | 0.13      | 0.11     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| hCG-wt         | <0.01*    | <0.01*   | 1.0    | 1.0     | 1.0      | <0.01*  | <0.01*  | <0.01*    |
| 89CG24         | 0.21      | 0.20     | 0.15   | 0.05    | 0.1      |         |         |           |
| 89CG104        | <0.01*    | <0.01*   | 0.3    | 0.2     | 0.3      |         |         |           |
| 94CG104        | <0.01*    | <0.01*   | 0.1    | <0.01*  | 0.1      | <0.01*  | <0.01*  | <0.01*    |
| 89CG104        | <0.01*    | <0.01*   | 0.93   | 0.4     | 0.97     | <0.01*  | <0.01*  | <0.01*    |
| hFSH-wt        | <0.01*    | <0.01*   | <0.01* | <0.01*  | <0.01*  | 1.0     | 1.0      | 1.0       |
| 89FSH24        | 0.77      | 0.75     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 89FSH104       | 0.62      | 0.72     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 94FSH104       | 0.54      | 0.33     | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 105FSH122      | 0.28      |          | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |
| 44FSH123       | 1.1       |          | <0.01* | <0.01*  | <0.01*  | <0.01*  | <0.01*  | <0.01*    |

**TABLE II**

Glycoprotein hormone seat-belt chimeras and their receptor specificity

Results are from this paper unless specified otherwise. Receptor specificity was determined either by receptor binding and/or activity assays (see text for details). For most chimeras, binding affinity and activation correlated closely. hTSH bearing the entire hCG seat-belt was 10-fold more potent for progesterone compared with cAMP stimulation (see text). The region between the 10th and the 11th Cys (C10-C11) corresponds to the carboxyl-terminal segment of the seat-belt. +++, specificity equivalent to native hormone (80–100%); +, 20–80%; +, 5–20%; −, <5% of native hormone. The known cross-reactivity of natural glycoprotein hormones, such as thyrotropic activity of hCG, by comparison, occurs with much higher amounts and requires a >1000-fold molar excess relative to the native hormone in the assays employed (11, 31). R, receptor.

| Hormone  | Seat-belt sequence | Receptor specificity |
|----------|--------------------|----------------------|
|          | C10-C11            | C11-C12              |
| hTSH     | hCG                | +                    |
|          | hCG                | −                    |
| hCG      | hCG                | −                    |
| hFSH     | hFSH               | ++                   |
| hFSH     | hFSH               | ++                   |
| hTSH     | hTSH               | +                    |
| hFSH     | hFSH               | +                    |
| hFSH     | hFSH               | +                    |
| hCG      | hCG                | +                    |

TSH receptor binding of hTSHβ peptides spanning the seat-belt region (21), as well as observations that mutations of identical α-subunit residues have hormone-dependent effects on activity (10–13, 25, 34). In this respect, we have shown that identical mutations in the a39–44 domain, which includes a positively charged α-helix at a40–46, truncation of the α-carboxyl terminus, and deletion of the carbohydrate consensus sequence at aAsn39, all affected hTSH subunit association or activity differently than in the analogous hCG and hFSH mutants (10–12). Intriguingly, these α-subunit domains are in close proximity to the seat-belt in the crystal structure of hCG and an hTSH homology model (5, 13). It has thus been proposed that they may form a composite receptor-binding domain (2, 3). In contrast, a peripheral α-subunit receptor binding domain located at the tip of the α1 loop, α11–20, appears to be important for all glycoprotein hormones (13). It is tempting to speculate that the lack of specificity of the α11–20 domain is related to its distance from the seat-belt region. On the other hand, the α-subunit domains in close proximity to the β-subunit seat-belt may be spatially oriented by the seat-belt to contact the appropriate receptor in a hormone-dependent fashion. In this respect, a direct cooperation between the complementary charged residues Lys836 of the α-subunit and Asp2277 of the CG/LH receptor has recently been demonstrated (35).

Thus our findings suggest that, during the evolutionary divergence of the glycoprotein hormones from a common ancestor gene (36), determinants of ligand specificity have evolved independently and in different ways. The seat-belt region appears to be critical to direct glycoprotein hormone binding to either the CG/LH receptor or to the TSH and FSH receptor. Determinants mediating discrimination between the TSH and FSH receptor remain to be elucidated.
REFERENCES

1. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50**, 465–495
2. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **368**, 455–461
3. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure (Lond.)* **2**, 545–558
4. Segaloff, D. L., and Ascoli, M. (1993) *Endocrinol. Rev.* **14**, 324–347
5. Szkudlinski, M. W., Grossmann, M., and Weintraub, B. D. (1996) *Trends Endocrinol. Metab.* **7**, 11–20
6. Moyle, W. R., Campbell, R. K., Rao, S. N. V., Ayad, N. G., Bernard, M. P., Han, Y., and Wang, Y. (1995) *J. Biol. Chem.* **270**, 20020–20031
7. Baenziger, J. U. (1994) in *Glycoprotein Hormones* (Lustbader, J. W., Puett, D., and Ruddon, R. W., eds) pp. 167–174, Springer-Verlag New York Inc., NY
8. Bielinska, M., and Boime, I. (1995) in *Glycoproteins* (Montreuil, J., Vliegenhart, J. F. G., and Schachter, H., eds) pp. 565–587, Elsevier Science Publishers B.V., Amsterdam
9. Combarnous, Y. (1992) *Endocrinology* **13**, 670–691
10. Grossmann, M., Szkudlinski, M. W., Zeng, H., Kraiem, Z., Ji, I., Tropea, J. E., Ji, T. H., and Weintraub, B. D. (1995) *Mol. Endocrinol.* **9**, 948–958
11. Grossmann, M., Szkudlinski, M. W., Tropea, J. E., Bishop, L. A., Thotakura, N. R., Schofield, P. R., and Weintraub, B. D. (1995) *J. Biol. Chem.* **270**, 29378–29385
12. Grossmann, M., Szkudlinski, M. W., Dias, J. A., Xia, H., Wong, R., Puett, D., and Weintraub, B. D. (1996) *Mol. Endocrinol.* **10**, 769–779
13. Santacoloma, T. A., and Reichert, L. E., Jr. (1990) *J. Biol. Chem.* **265**, 5037–5042
14. Chen, F., and Puett, D. (1991) *J. Biol. Chem.* **266**, 6904–6908
15. Moore, W. T., Burchfield, B. D., and Ward, D. N. (1980) in *Chorionic Gonadotropin* (Segal, S. A., ed) pp. 89–126, Plenum Press, New York
16. Campbell, R. K., Dean-Emig, D. M., and Moyle, W. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 760–764
17. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368**, 251–255
18. Dias, J. A., Zhang, Y., and Liu, X. (1994) *J. Biol. Chem.* **269**, 25289–25294
19. Morris, J. C., McCormick, D. J., and Ryan, R. J. (1990) *J. Biol. Chem.* **265**, 1881–1884
20. Medeiros-Neto, G., Herodotou, D. T., Rajan, S., Komnareddi, S., de Lacerda, L., Sandrini, R., Boguszewski, C. S., Hollenberg, A. N., Radovick, S., and Wandsford, F. E. (1996) *J. Clin. Invest.* **97**, 1250–1256
21. Costagliola, S., Swillens, S., Nicoli, P., Dumont, J. E., Vassart, G., and Ludgate, M. (1992) *Endocrinology* **108**, 88–95
22. Yoo, J., Zeng, H., Ji, I., Murdoch, W. J., and Ji, T. H. (1993) *J. Biol. Chem.* **268**, 13034–13042
23. Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* **8**, 404–407
24. Chen, F., Wang, Y., and Puett, D. (1991) *J. Biol. Chem.* **266**, 19357–19361
25. Jones, M., and Hu, Y. (1990) in *Glycoprotein Hormones* (Chin, W. W., and Boime, I., eds) pp. 395–402, Serono Symposia U. S. A., Norwell, MA
26. Matznik, M. M., Keene, J. L., and Boime, I. (1989) *J. Biol. Chem.* **264**, 2409–2414
27. Keutmann, H. T., Charlesworth, M. C., Mason, K. A., Ostrea, A., Johnson, L., and Ryan, R. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2038–2042
28. Yoshimura, M., and Hershman, J. M. (1995) *Thyroid* **5**, 425–434
29. Ibanez, C. F. (1992) *J. Neurosci.* **25**, 1349–1361
30. Reuben, M. R., Linggen, J., Sairam, J., and Bhargavi, G. N. (1990) *Biochim. Cell Biol.* **68**, 889–893
31. Liu, C., Roth, K. E., Lindau Shepard, B. A., Shaffer, J. B., and Dias, J. A. (1993) *J. Biol. Chem.* **268**, 21613–21617
32. Ji, I., Zeng, H., and Ji, T. H. (1993) *J. Biol. Chem.* **268**, 22971–22974
33. Fiddes, J. C., and Talmadge, K. (1984) *Recent Prog. Horm. Res.* **40**, 43–78